Is the gut the key to obesity?

IS THE GUT THE KEY TO OBESITY ?

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The involvement of the intestine in obesity,
type 2 diabetes mellitus, and fatty liver disease in man

PROEFSCHRIFT

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There were times - I'm sure you knew - when I bit off more than I could chew

But through it all, when there was doubt, I ate it up and spit it out

I faced it, I stood tall and did it my way

I've loved, I've laughed, I've cried

I've had my fill, my share of losing

But as tears subside, one day, I will find it all amusing

vrij naar Frank Sinatra

Contents

Chapter 1

General introduction: the role of the intestine in obesity and its comorbidity

1.1 PREVALENCE OF OBESITY AND OBESITY-RELATED CO-MORBIDITY

According to the World Health Organization, over 65% of the world population lives in countries where more people die of overweight and obesity than of underweight. The classification of overweight and obesity is based upon body mass index (BMI); a heuristic measure of body weight based on a person's weight and height. This index was introduced in the 19th century by the Belgian statistician Adolphe Quetelet. Caucasians are considered to be overweight if they have a BMI of 25kg/m^2 and over, obese if they have a BMI of over 30kg/m^2, and morbidly or severely obese if they have a BMI of over 40kg/m^2 (Figure 1a). The prejudice about overweight and obesity is that they mainly form a problem in high-income countries, but their prevalence is dramatically on the rise in low- and middle-income countries, particularly in urban settings. In the Netherlands; the percentage of overweight adults was 47.2% in 2010, and the highest incidence of obesity is reported in the province of Limburg (13.3%, Figure 1b). Worldwide, the amount of obese subjects has more than doubled since 1980; more than 1.5 billion adults [1] and at least 43 million children under the age of 5 years are currently overweight [2]. The latest projections indicate that these numbers will rise to approximately 2.3 billion overweight adults and over 700 million obese adults by 2015 [3].

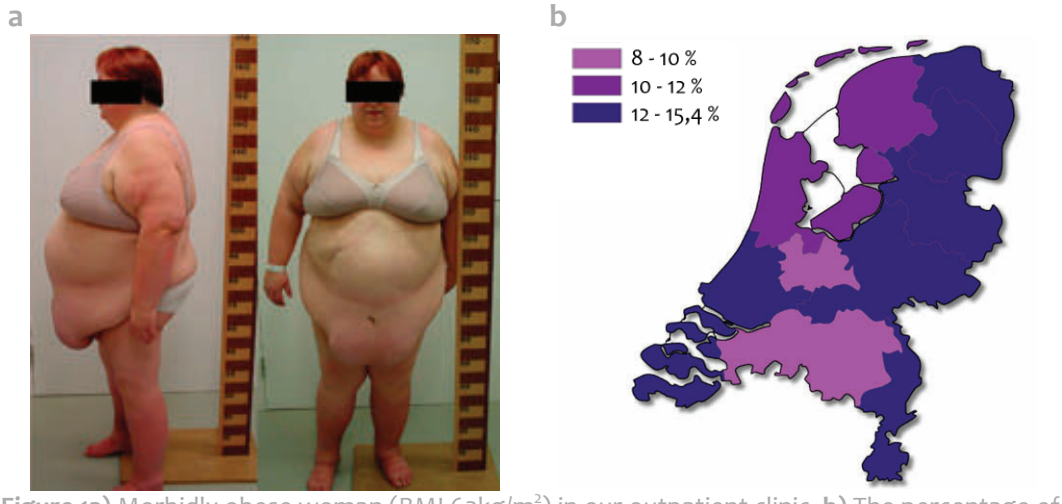

Figure 1a) Morbidly obese woman (BMI 63kg/m^2) in our outpatient clinic, **b)** The percentage of obese adults (BMI>30kg/m^2) per province in 2008-2009 (data provided by the central bureau of statistics).

This epidemic forms a growing burden on the health care system, welfare, economics, and leads to major health consequences. The prevalence of obesity-related comorbidity, such as type 2 diabetes (T2DM), Non-alcoholic Fatty Liver Disease (NAFLD), Obstructive Sleep Apnoea Syndrome (OSAS), and cardiovascular disease also continues to rise [4,5]. The spectrum of disturbed glucose- and insulin metabolism, dyslipidemia, hypertension, and abdominal fat accumulation, also referred to as the metabolic syndrome [6,7], was already estimated to affect one in five adults in 2005 [3]. This disturbed glucose- and insulin metabolism can lead to T2DM, and the number of patients suffering from T2DM is expected to double within the next decade [3]. Globally, the prevalence of diabetes is expected to rise to around 366 million patients in 2030, of whom 85% has T2DM [8]. In the Netherlands, 740.000 people were diagnosed with diabetes in 2007, a number expected to rise to around 1.3 million people in 2025 [9]. More than 40% of the diabetes burden is attributable to obesity [3], already leading to diabetes-associated healthcare costs of $98 billion in the USA alone over a decade ago [10]. In the Netherlands, next to the associated medical costs, patients with diabetes cost 5.5 billion Euros annually in loss of working hours alone.

T2DM results from a dynamic interplay of low-grade inflammation, metabolic derangements (mitochondrial dysfunction, oxidative and endoplasmic reticulum stress), neuronal and endocrine factors (e.g. a disturbed fatty acid metabolism) [11]. Fundamental to the development of T2DM is a combination of impaired insulin secretion from pancreatic β-cells, decreased glucose uptake in peripheral tissues, and increased hepatic glucose production [12]. The main consequences of these derangements are increased fasting and postprandial plasma glucose levels and peripheral insulin resistance (IR). This results in symptoms such as polyuria (frequent urination), polydipsia (increased thirst), and polyphagia (increased hunger). Currently, T2DM is treated by oral medication and insulin therapy in more severe cases, but complications occur nonetheless. For example, severe diabetes can lead to micro- and macro-vascular dysfunction (e.g. cardiovascular disease), ophthalmic abnormalities (such as diabetic retinopathy, retinal detachment, cataracts, and glaucoma, which can lead to blindness), nephropathy, and to peripheral neuropathy, erectile dysfunction, and foot problems (e.g. infections and deformities, leading to amputations) [10,13].

Another important comorbidity of obesity is NAFLD; currently the most common liver disease worldwide [14], estimated to occur in more than 25% of the Western population [15], and widely considered to be the hepatic manifestation of the metabolic syndrome. With the inexorable rise in obese patients, the impact and prevalence of NAFLD also continue to expand [15-17]. NAFLD is a multi-factorial liver disease; genetic, metabolic, inflammatory and environmental factors contribute to its pathogenesis [18]. Importantly, the exact triggering factors for progression from hepatic steatosis to steatohepatitis remain to be defined, although insulin resistance and free fatty acid metabolism are considered to play a role [19]. NAFLD can progress from hepatic fat accumulation or steatosis alone, to more severe stages characterized by hepatic inflammation, such as Non-Alcoholic Steatohepatitis (NASH). In turn, NASH is associated with the development of fibrosis and cirrhosis [20-22], which may progress towards liver failure and hepatocellular carcinoma [20,21]. Only in a relatively advanced stage such as in case of cirrhosis or liver failure, patients may experience symptoms such as fatigue, weight loss, and general weakness. Treatment options for NASH are under investigation. In severely obese subjects, weight reducing surgery has been shown to improve NAFLD and NASH [23], but in advanced stages such as severe liver failure, liver transplantation may become the only option offering chance of survival [14].

1.2 TREATMENT OF SEVERE OBESITY AND IMPACT ON CO-MORBIDITY

In general, a stable body weight is maintained by guarding a balance between daily food intake and energy expenditure. This balance is determined by appetite, satiation, metabolism, and partially regulated by mechanical and chemical stimuli produced by the intestine in response to food [24]. Overweight develops when this equilibrium between energy intake and energy expenditure is disrupted. In order to restore the balance, weight reduction can be achieved either by non-invasive or invasive methods. If non-invasive strategies such as dietary adjustments, physical exercise, life-style intervention, pharmacological therapy, behavioral, and/or psychotherapy are ineffective, patients become eligible for more invasive therapies.

Weight reducing surgery, also referred to as bariatric or metabolic surgery, is currently considered the most effective treatment option for severely obese patients with either a BMI of 40kg/m^2, or a BMI ≥35kg/m^2 accompanied by comorbidity. Ever since bariatric surgery was first performed in the 1950s, both quality and quantity of bariatric procedures have increased. In spite of a shift towards more complex and effective procedures, mortality rates have remained relatively low [25-27]. The number of eligible patients in the Netherlands is estimated to expand from 222,000 in 2007 to 336,000 in 2012, whereas the number of procedures conducted annually will rise from 3500 to 11,000 [28]. In the United States, only a few percent of the eligible severely obese candidates are treated by means of bariatric surgery [29].

Bariatric surgical procedures are roughly divided into gastric restrictive surgery on the one hand, and so-called malabsorptive procedures with a restrictive component on the other hand (Figure 2). Which procedure is preferably performed varies by continent; in Australia 95% of bariatric procedures are restrictive (gastric banding, Figure 2a), in Europe approximately half, while in South America almost only malabsorptive procedures are performed (mainly gastric bypass surgery, Figure 2b) [30].

The Laparoscopic Adjustable Gastric Banding (LAGB, Figure 2a) is a restrictive procedure, diminishing food intake and reducing the volume of the stomach [31,32]. The most frequently performed procedure combining restriction and malabsorption is the Roux-and-Y Gastric Bypass (RYGB, Figure 2b); the stomach size is reduced and a proximal part of the small intestine is bypassed. Subsequently, digestive juices pass through the bypassed part of the intestine, while this part remains excluded from nutrition. Digestive juices and nutrition join in the so-called common limb (Figure 2b). Gastric bypass procedures are very effective, but their malabsorptive character increases the risk of deficiencies of mainly iron, vitamin B12 and D, and calcium [33]. To prevent these and other complications, lifelong follow-up is essential [34].

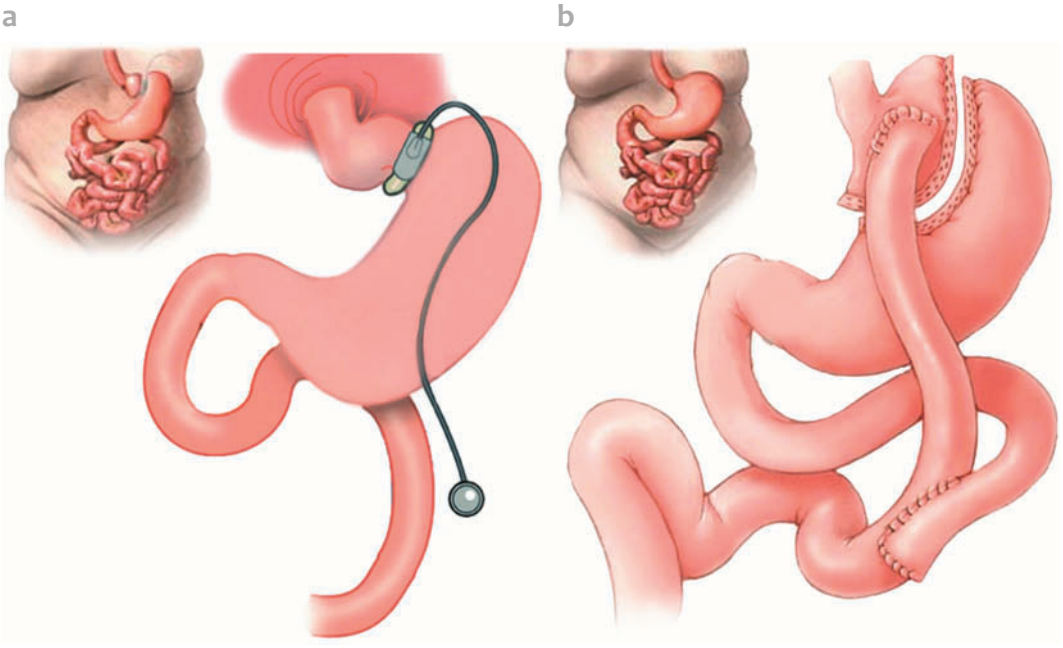

Figure 2. The most frequently performed bariatric procedures for severe obesity.
a) During adjustable gastric banding surgery, a hollow tube in the shape of a ring is placed around the upper part of the stomach, below the lower oesophageal sphincter. The amount of nutrition that passes through the band is regulated from outside the body, by varying the injected volume of saline in the band; the food channel is thereby tightened or loosened.
b) After Roux-en-Y gastric bypass surgery, the stomach and small bowel are re-arranged into a Y-configuration, enabling outflow from the small upper stomach pouch, via a so-called 'Roux limb', to a lower part of the small intestine. The food then flows directly from the upper part of the stomach to the small intestine, whereas the lower part of the stomach and the proximal small intestine are excluded. The digestive juices reunite with food after 80 to 150cm in the so-called common limb.

Bariatric surgery induces weight reduction [26,35-37] and decreases overall mortality [26]. Moreover, it has a positive effect on obesity associated co-morbidity such as T2DM, hypertension, hyperlipidemia [38,39], and both NAFLD and NASH [40]. These effects are remarkable; diabetes is improved and approximately 80% of diabetic patients even display complete resolution of their diabetes [38].

Surgical experts even suggest T2DM to be an operable intestinal disease [41,42]. However, the rapidity and the efficacy of these beneficial effects both depend on the type of procedure [43,44] and the status of pancreatic β-cells at the time of surgery [45]. In the short term, days to weeks postoperatively, and well before significant weight loss occurs [46-48], bypassing the proximal intestine (for example by RYGB) has a stronger effect on T2DM than the gastric banding procedure [49]. Interestingly, similar effects on glucose homeostasis are obtained by less invasive techniques excluding the proximal small intestine, such as the Endobarrier Liner [50].

The potential mechanisms underlying these effects have not been fully elucidated yet. Exclusion of the upper intestine from contact with ingested nutrients was suggested to be a determining factor in improving diabetes in experiments with rats [51,52]. However, these results were contradicted by experiments in other rat models, showing no improvement of diet-induced T2DM after a duodenojejunal bypass [53]. Recent experiments in non-obese diabetic rats have shown that the gastric bypass-induced improvement of glucose metabolism occurs simultaneously with regeneration of pancreatic beta-cells [54]. In 2005, it was discovered that the small intestine releases glucose of gluconeogenic origin after food absorption [55]. This endogenous de novo glucose production functions as a hepatoportal glucose sensor, with a quantitative effect on food intake [55]. This intestinal gluconeogenesis has been suggested to play a role in T2DM [56], and in the ameliorated glucose homeostasis after bypassing the proximal small intestine [44]. Last but not least, another factor that may contribute to the positive effects of gastric bypass surgery is the enhanced production of peptides regulating glucose metabolism (such as glucagon-like peptide 1, GLP-1) in response relatively undigested food passing through a more distal part of the small intestine [57]. As opposed to non-diabetics, subjects with T2DM show almost no GLP-1 increase following a meal [58,59], whereas this normalizes after malabsorptive bariatric surgery [60]. Collectively, these fast and positive effects on obesity-induced T2DM after partial exclusion of the proximal intestine strongly suggest that the intestine is involved in the pathophysiology of T2DM.

1.3 THE POTENTIAL ROLE OF THE INTESTINE IN OBESITY; INTESTINAL MICROBIOTA, PERMEABILITY AND PROLIFERATION

INTESTINAL MORPHOLOGY AND FUNCTION

Given the misbalance between energy intake and energy expenditure that characterizes obesity, the intestine absorbing our nutrition forms an obvious research topic. Nutrient absorption is realized by the intestinal epithelium, a folded structure built up of villi alternated by the crypts of Lieberkühn, with a surface area of 300-400m^2, similar to that of a tennis court [61]. As depicted in Figure 3, this surface area is built up of a tightly packed epithelial monolayer, formed by enterocytes or absorbing cells, alternated by goblet cells, all of which are interconnected by tight junction proteins (TJs) that seal the barrier. The luminal side of this epithelial monolayer consists of a brush border coated with mucus; a viscous, gel-like substance produced by goblet cells [62].

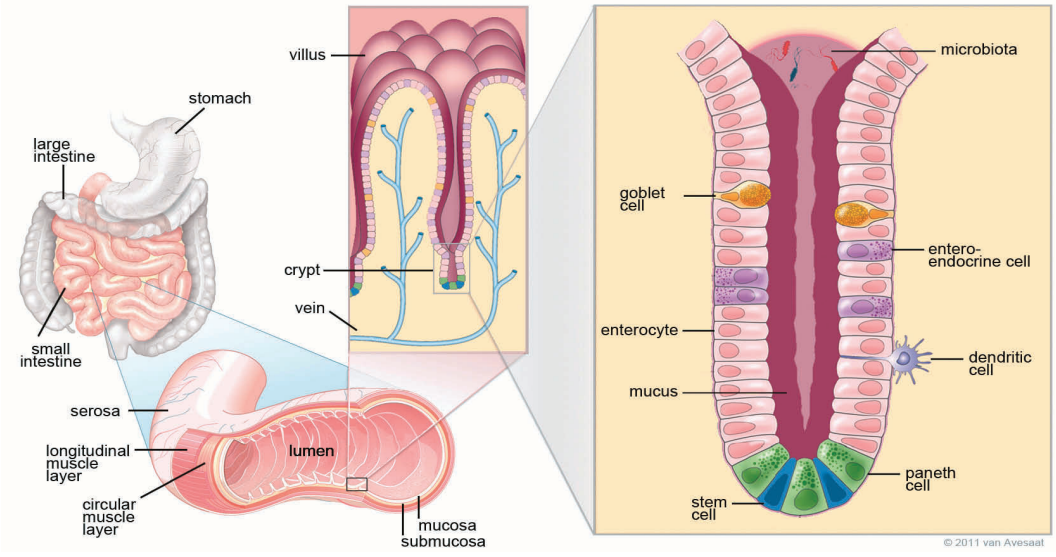

Figure 3. Simplified anatomy of the small intestine. The small intestinal wall forms the interface between the external milieu (the intestinal lumen and its microbial content) and the internal milieu (the veins, symbolizing the systemic circulation). The luminal side of the small intestine consists of villi and crypts. From the external to the internal milieu this figure illustrates the microbiota in the lumen, the mucus layer produced by goblet cells, and the monolayer of absorptive cells or enterocytes, goblet cells, entero-endocrine cells, and dendritic cells. At the bottom of the villus in the crypts reside the Paneth cells and stem cells.

The generally accepted primary function of the intestine is to absorb fluids and dietary nutrients essential for growth and development, meanwhile protecting the body from potentially harmful luminal components, such as bacteria, antigens, and toxic products [63]. Mucus limits both bacterial adhesion and infiltration [64], and is therefore considered pivotal in maintaining intestinal homeostasis [65]. Interestingly, both the number of goblet cells and the composition of the mucus can be influenced by bacterial colonization [66]. The microbiological community colonizing the human intestine, the gut microbiota, consists of over 1014 bacteria, and more than a thousand species, whose microbiome outnumbers the human genome by more than a hundredfold [67,68]. Imaginably, interactions at the intestinal epithelial barrier between this microbiological community and the host are highly complex and heterogeneous [69].

Microbiota inhabiting the intestine have been suggested to play a role in both the development of obesity and IR [70]. This role was first illustrated by experiments showing that germ-free mice are protected from diet-induced obesity [71]. Colonization of germ-free mice with microbiota of obese mice was shown to result in a larger fat deposition than colonization with microbiota of lean mice [70]. Moreover, microbiota composition was reported to influence inflammation, glucose tolerance, lipid metabolism, body weight gain [72], and fat mass development [73]. Various shifts in intestinal microbiota composition have been reported in animal and human obesity. Most studies report a shift in favor of the presence of Firmicutes over Bacteroidetes [70,74-77], whereas a shift towards Bacteroidetes [78], and a similar composition in lean and obese subjects [79] is also described. Furthermore, changes in microbiota composition are observed after weight loss, either induced by alterations in diet [76,80,81] or after malabsorptive bariatric surgery [82], and administration of pro- or antibiotics can improve glucose metabolism [83,84]. These interactions between microbiota, diet, body weight and T2DM are currently under intense investigation.

MICROBIOTA, MICROBIAL PRODUCTS AND INFLAMMATION

One of the proposed mechanisms by which intestinal microbiota may influence obesity is by altering expression levels of TJ proteins [72]. TJs seal the intestinal barrier by connecting cells that form the epithelial monolayer, thereby regulating intestinal permeability [85]. Both in obesity and after a high fat diet, increased intestinal permeability coincided with an altered distribution and diminished expression of TJ proteins in rodents [86-88]. A higher intestinal permeability was also related to the extent of bacterial translocation in genetically obese mice [72,86]. After translocation, lipopolysaccharide (LPS), a potent pro-inflammatory component of the cell wall of Gram-negative bacteria, can induce inflammation by activating the innate immune system [89-91]. Under normal conditions, an inflammatory response is avoided and homeostasis is maintained by means of interplay between commensal microbiota and the intestinal barrier [92].

In obesity [72] and during a high fat diet [88,92,93], a higher intestinal permeability favors LPS translocation from the intestinal lumen into the bloodstream. This is in line with reported positive relations between both high-fat and high-caloric intake and plasma LPS levels [94,95]. Moreover, obesity-associated increased intestinal permeability and higher LPS levels in the portal vein, also referred to as metabolic endotoxemia [72], are related to hepatic and systemic inflammation [86,87,95]. LPS induced inflammation in turn has been described in the context of obesity and IR/T2DM [96-101], NAFLD [102,103], and NASH [104].

THE INTERACTION BETWEEN MICROBIOTA AND INTESTINAL CELLS IN OBESITY AND T2DM

Paneth cells are important determinants of the intestinal microbiota composition, and thereby contribute to preserving gut homeostasis [85,105]. Paneth cells are named after the Austrian physician Joseph Paneth (1857–1890), and reside in the crypts of Lieberkühn (Figure 3). Paneth cells secrete granules containing defensins, lysozyme, tumor necrosis factor-alpha, and phospholipase A2, which play a role in the host's defense against enteric pathogens such as bacteria [85,105,106]. Paneth-cell-derived peptides can form pores in bacterial cell membranes, thereby disrupting membrane function and causing cell lysis.

These peptides or defensins are secreted both constitutively, and in response to LPS and intestinal microbiota [107]. Interestingly, in mice lacking Paneth cell antimicrobials a shift in microbiota composition has been observed [106], similar to the reported microbial shift in obesity [70,76,108]. Moreover, via their impact on microbiota, Paneth cells are also described to modulate translocation of bacterial products over the intestinal barrier [105,109], and intestinal permeability [110,111]. In addition, Paneth cells can regulate angiogenesis [112] and sustain a relatively sterile surrounding for the stem cell (Figure 3) [113].

Stem cells are in turn also pivotal in maintaining the intestinal barrier. They give rise to four cell types; the enterocytes with their apical microvilli, goblet cells that form the protective luminal mucus layer, neuro-endocrine cells releasing hormones, and the Paneth cells [114]. The single cell layer forming the intestinal barrier is renewed every 4-5 days, which is the highest turnover of all tissues in adult mammals [115]. Stem cells therefore provide around 300 newly generated cells per crypt daily [116], which migrate up onto an adjacent villus in a coherent columnar manner [116,117]. Epithelial proliferation and turnover, survival, and barrier function are influenced by commensal intestinal microbiota [92,106,118-122]. For example, commensal intestinal microbiota are required to prevent intestinal injury, and induce compensatory proliferation [92]. This is confirmed by experiments illustrating a slower intestinal epithelial turnover and different morphologic properties, e.g. atrophic crypts and longer villi, in germ free animals [123]. Interestingly, longer villi have also been described in rats with T2DM, together with generalized small intestinal hyperplasia, mucosal hypertrophy, and enhanced carbohydrate digestion [124].

1.4 THE PATHOPHYSIOLOGY OF NON-ALCOHOLIC FATTY LIVER DISEASE

In spite of the high prevalence of NAFLD, its etiology and the mechanism(s) responsible for progression towards NASH remain to be fully elucidated [125]. Many different factors of genetic, metabolic, inflammatory, and environmental origin are described to be involved [18]. Similar to histopathology of alcoholic steatohepatitis (ASH), NASH ranges from steatosis to hepatic inflammation (Figure 4), and can progress into fibrosis and cirrhosis. In turn, the presence of fibrosis and cirrhosis is associated with an increased risk of hepatocellular carcinoma [126].

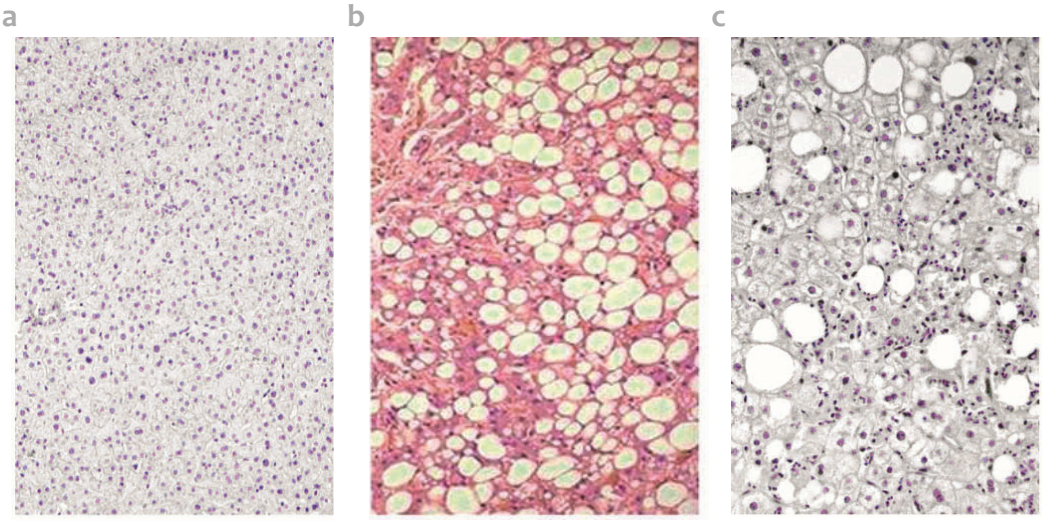

Figure 4. Histology of a healthy liver, a steatotic liver and non alcoholic steatohepatitis.
a) normal histology of a healthy liver. **b)** a steatotic liver (NAFLD), characterized by fat containing vacuoles or vesicular steatosis accumulated in hepatocytes. **c)** non-alcoholic steatohepatitis (NASH) as illustrated by the presence of steatosis, parenchymal inflammation, and a rearranged architecture. These biopsies were stained with hematoxylin-eosin (200x magnification, material was obtained during the writing of this thesis).

The hepatic inflammation characteristic of NASH is may be induced by the hepatotoxic component LPS [86,127], illustrated by studies reporting increased LPS levels in NASH [103,104,127,128]. Moreover, both small intestinal bacterial overgrowth [129-131] and increased intestinal permeability [86,103,104,129] have been observed in NASH.

It is therefore hypothesized that an enhanced intestinal permeability leads to increased LPS translocation and subsequent hepatic inflammation. Interestingly, translocation of LPS and hepatic inflammation have been associated with intestinal inflammation, emphasizing the role of the intestine in the development of NASH [128]. In line with this, administration of lactulose (an indigestible disaccharide stimulating growth and/or activity of beneficial bacteria), reduced LPS levels in the portal vein and diminished hepatic inflammation in rats with NASH [132]. This implies that the microbiota composition can modulate hepatic inflammation in NASH. Portal hypertension may be another contributing factor, since this is often seen in liver failure and known to lead to mucosal edema and mast cell infiltration in the small intestine [133,134]. In summary, even though the etiology of human NAFLD is not yet completely disentangled, several studies indicate that the intestine and translocation of bacterial compounds play a role.

Research in human NASH is hampered because the current gold standard to diagnose NAFLD is based upon histological evaluation of a liver biopsy [16,135,136], as illustrated in Figure 4. This scoring is essential to assess disease severity, but the procedure of obtaining a liver biopsy is invasive and associated with patient discomfort, morbidity, and high costs [137]. Therefore, risk assessment in clinical practice is often based on physical examination combined with blood tests and radiological tools [16]. Next to obesity, hypertension, and increased plasma levels of glucose, insulin, and triglycerides are also associated with the presence of NASH [138-141]. However, risk assessment based on these risk factors leads to an underestimation of NASH prevalence, even if screening is combined with radiological techniques [142,143]. In summary, it remains crucial to clarify the pathogenesis of human NAFLD and its progression to NASH, in order to prevent this disease, diagnose it in an early stage, and develop suitable treatment options.

1.5 AIMS OF THIS THESIS

Based on the literature reviewed in this chapter, there seems to be an interaction between intestinal microbiota, the integrity of the intestinal barrier, low-grade inflammation, and the pathophysiology of obesity and its comorbidity. This thesis therefore focuses on the role of the intestine in obesity, T2DM, and NASH in the human setting.

First, **Chapter 2** provides background information on the assessment of intestinal barrier integrity and function. Insight into currently used methods to assess intestinal integrity is indispensable to understand and investigate the potential role of intestinal barrier integrity in the pathophysiology of obesity. Next, we investigated the microbiota composition in lean and obese individuals, and the potential relation with intestinal permeability and inflammation **(Chapter 3).**

Intestinal microbiota composition is known to be regulated by Paneth cells. The reported obesity-associated shifts in microbiota composition in mice are similar to the shift observed in mice lacking Paneth-cell-derived antimicrobial peptides. Hence, the peptide production by Paneth cell in obese subjects was quantified in obese and lean subjects **(Chapter 4).**

Whereas the first part of this thesis is focused on factors related to the intestine in obesity, the second part of this thesis concerns potential differences between obese subjects with and without comorbidity. Several studies strongly suggest involvement of the intestine in obesity-induced T2DM. The small intestine of rats with T2DM is heavier and larger. Moreover, bypassing a part of the small intestine has an indisputable ameliorating effect on T2DM. This led us to examine markers for small intestinal mass and turnover in human T2DM **(Chapter 5).**

Next, based upon findings of increased LPS levels in the portal circulation of animal models with NASH, the potential relation between LPS exposure and NASH was studied **(Chapter 6).** The gold standard to diagnose NASH and to assess its severity is by means of histological examination of a liver biopsy.

In Chapter 7, we assessed the potency of analysis of exhaled breath as a non-invasive method to predict the presence of NASH. Moreover, the predictive value of this breath test was compared to the currently used plasma parameters to assess the presence of NASH. Chapter 8 concludes this thesis by discussing the findings in the context of the current literature, and providing potential implications for clinical practice and future research.

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Chapter 2

Assessment of human intestinal barrier function

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ABSTRACT

Over the past decades evidence has been accumulating that intestinal barrier integrity loss plays a key role in the development and perpetuation of a variety of disease, including inflammatory bowel disease (IBD) and celiac disease. Moreover, intestinal barrier integrity loss is a key player in the onset of sepsis and multiple organ failure (MOF) in situations of intestinal hypoperfusion including trauma and major surgery. Insight into gut barrier integrity and function loss is important to improve our knowledge on disease etiology and pathophysiology and contributes to early detection and/or secondary prevention of disease.

A variety of tests have been developed to assess intestinal epithelial cell damage, intestinal tight junction status and consequences of intestinal barrier integrity loss, i.e. increased intestinal permeability. This review discusses currently available methods for evaluating loss of human intestinal barrier integrity and function.

2.1 INTRODUCTION

The gastrointestinal tract is the most extended surface acting as a barrier between external environment and internal milieu. The host integrity is maintained by effective monitoring of the mucosal surface and sealing the host interior against potentially harmful compounds such as bacteria, toxins and antigens. This function of the gastrointestinal tract is referred to as intestinal barrier function. The intestinal epithelial barrier function consists of multiple defense mechanisms which can basically be subdivided into a physical and an immunological barrier [1-3].

The physical intestinal barrier is composed of a lining of epithelial cells, connected by tight junctions (Figure 1a). These adhesion structures serve as a fence sealing the paracellular pathway, thereby preventing exposure of the internal milieu to potentially harmful intraluminal microbiota and microbial products [4]. Tight junctions are anchored in the cell via the filamentous actin (F-actin) cytoskeleton [5,6]. Zonula occludens proteins (ZO-1, ZO-2 and ZO-3) are important intracellular tight junction proteins, linking the cell cytoskeleton to the transmembrane tight junction proteins: claudins, occludins and junctional adhesion molecules (JAM). Whereas occludin and JAM have a regulatory role, claudins are transmembrane proteins mainly responsible for the intestinal barrier function [7].

The physical barrier is reinforced by the presence of a mucus layer, produced and secreted by goblet cells [8]. The immune barrier is formed by specialized epithelial cells, the Paneth cells, located in the crypts of the small intestine, which can actively sense bacterial presence and prevent colonization of the crypts by releasing antimicrobial proteins including lysozyme and defensins [9,10]. Furthermore, lamina propria immune cells actively participate as immune sensors of microbial pathogens and commensal organisms. Bacterial recognition is dependent on transmembrane and intracellular pattern recognition receptors, including the structurally homologous toll-like receptor (TLR) and NOD-like receptor (NLR) family. Ligation to these bacterial receptors stimulates central signaling cascades (NF-kB, AKT/phosphatidylinositol-3'-kinase and mitogen-activated protein kinase pathways), resulting in an immunological response [11-14].

Disturbed intestinal barrier function is considered a key factor in the development and/or progression of intestinal inflammation, and is therefore thought to play a role in both the pathogenesis and the perpetuation of various intestinal diseases including

inflammatory bowel disease (IBD) and celiac disease [2,3]. Impaired intestinal barrier function has also been assumed to play a role in the development of sepsis and multiple organ failure (MOF) in patients with decreased gut perfusion following major surgery, trauma or shock [15,16]. Recently the occurrence of splanchnic hypoperfusion during major surgery was reported to result in intestinal ischemia and intestinal barrier integrity loss [5], which could in turn facilitate translocation of bacterial products from the intestinal lumen to the circulation. This phenomenon has been suggested to trigger an excessive inflammatory response, leading to sepsis and MOF in these patients [4,17]. In conclusion, intestinal barrier function loss is associated with a range of diseases; insight in gut barrier integrity and function loss is therefore imperative for clinical practice and important for improving our knowledge on disease etiology and pathophysiology. In this review, the currently available methods aiming to assess either human intestinal barrier integrity or intestinal barrier function will be discussed. In addition, applicability of these tests in different clinical and research situations is described.

2.2 ASSESSMENT OF THE EPITHELIAL BARRIER INTEGRITY

The intestinal barrier function is maintained by a lining of enterocytes and tight junctions, sealing the paracellular space between adjacent enterocytes. Intestinal barrier integrity loss can be assessed by evaluation of intestinal epithelial cell damage or tight junction loss.

INTESTINAL EPITHELIAL CELL DAMAGE: FATTY ACID BINDING PROTEINS

Fatty acid binding proteins (FABP) are small (14-15 kDa) cytosolic water-soluble proteins, present in mature enterocytes of the small and large intestine. Their function is the transport of fatty acids from the apical membrane of the enterocyte to the endoplasmic reticulum where biosynthesis of complex lipids occurs [18]. Three types of FABP are present in the gut; Intestinal FABP (I-FABP), Liver FABP (L-FABP) and Ileal Bile Acid Binding Protein (I-BABP). The distribution of these FABP was studied by Pelsers et al. and Derikx et al. who reported that I-FABP is in particular expressed in jejunum and to a lesser extent in the colon, whereas I-BABP is

exclusively present in the ileum [18-20]. In addition, I-FABP and I-BABP are exclusively present in the gut [18,21,22], whereas L-FABP is also present in the liver and kidney [18]. Since FABP are small, water-soluble cytosolic proteins they are easily released into the circulation upon enterocyte membrane integrity loss and are rapidly renally cleared (half-life of 11 minutes) [23]. Therefore FABP can be measured sensitively in both plasma and urine using an enzyme-linked immunosorbent assay (ELISA).

Basal levels of FABP have been reported to reflect the physiological turnover rate of enterocytes [24]. Several studies showed the usefulness of FABP as markers for intestinal epithelial cell damage. Elevated circulating or urinary FABP levels are reported in patients with intestinal ischemia [25], systemic inflammatory response syndrome and necrotizing enterocolitis [26-28]. High levels of FABP were also detected in patients with intestinal ischemia during major (vascular) surgery and in patients with mesenteric infarction [25,29,30]. Hence, in situations of acute intestinal damage, plasma and urine FABP levels are useful for the assessment of intestinal epithelial damage. In conclusion, measurement of plasma and urinary FABP levels is useful for the early detection of intestinal epithelial cell damage. Since FABP are differentially expressed along the intestinal tract, measurement of specific FABP could be a promising tool to provide information on disease localization.

INTESTINAL EPITHELIAL CELL DAMAGE: GLUTATHIONE S-TRANSFERASES

The Glutathione S-transferases (GST) are involved in cell protection, antioxidation and detoxification of a range of toxic and foreign compounds within the cell by conjugating them to glutathione. The GST family consists of four subgroups displaying tissue variation; αGST, μGST, πGST and θGST. Whilst μGST, πGST and θGST are present in cells of various organs, αGST is predominantly present in liver, kidney and intestine and has been proposed as a potential marker for, amongst others, intestinal epithelial cell damage [31,32].

Several studies reported that mesenteric ischemia could reliably be predicted by plasma αGST levels in patients with abdominal pain, suspected to be due to acute mesenteric ischemia [33-35]. McMonagle et al. found that circulating αGST were significantly elevated in patients who displayed signs of intestinal pathology after cardiac surgery with cross clamping of the aorta and consequent intestinal ischemia [32].

It has to be kept in mind that increased plasma or urine levels of αGST can indicate intestinal damage as well as liver and kidney damage, because αGST is expressed in epithelial cells of all these organs. Therefore, this test might be useful for assessment of intestinal damage when isolated intestinal damage is suspected.

PARACELLULAR BARRIER INTEGRITY LOSS: TIGHT JUNCTION STATUS

Intestinal epithelial cells are tightly connected by a surrounding system of tight junction strands. Claudins are transmembrane proteins which are mainly held responsible for the intestinal barrier function [7]. These claudins are abundantly present between adjacent healthy intestinal epithelial cells [7,36]. Zeissig et al. showed a disturbance of the barrier function which was accompanied by downregulation of several claudins, e.g. claudin-1, 3, 5, 7 and 8 in intestinal biopsies of patients with Crohn's disease. Claudin-2, a pore-forming claudin, was upregulated in these patients [1].

Non-invasive assessment of claudins could provide information on paracellular gut barrier integrity. Claudin-3 seems to be a suitable candidate marker for early non-invasive detection of intestinal tight junction integrity loss due to its small size, abundant endogenous intestinal expression and paracellular localization [36]. Recent studies showed a strong relationship between intestinal tight junction loss and urinary claudin-3 levels in both a rat hemorrhagic shock model and in a clinical setting in patients with IBD, necrotising enterocolitis and in patients undergoing major surgery, thereby suggesting that measurement of urinary claudin-3 can indeed be used as non-invasive marker for intestinal tight junction loss [5,37,38].

In conclusion, measurement of urine claudin-3 levels offers the opportunity to study paracellular intestinal barrier damage. Detection of tight junction loss offers new opportunities for early diagnosis and follow-up of patients with intestinal diseases and for elucidation of the pathophysiology of gut-related diseases in man. A clear research or clinical question with careful interpretation of results is however imperative, since tight junction distribution is not limited to the intestine. The usefulness of other tight junction proteins for non-invasive evaluation of intestinal paracellular integrity remains to be established.

2.3 FUNCTIONAL ASSESSMENT OF INTESTINAL BARRIER LOSS

Methods for the functional assessment of intestinal barrier loss have been studied extensively. Currently available methods are either based on actively measuring either paracellular intestinal leakage of orally administered test substances, or passively measuring the consequences of intestinal barrier function loss, i.e. translocation of luminal content to the circulation.

'Active' assessment of barrier function loss is based on the hypothesis that orally administered large molecular probes cannot cross the paracellular intestinal pathway unless the intestinal barrier function is compromised. In case of barrier function loss such probes cross the intestinal barrier, appear into the circulation and can be detected in urine after renal excretion. 'Passive' assessment of barrier function loss is based on the hypothesis that intestinal luminal compounds, such as endotoxins and bacterial fermentation products, translocate to the circulation in cases of barrier function loss. Plasma levels of these bacterial components or products are therefore hypothesized to reflect barrier function integrity.

ACTIVE MEASUREMENT OF INTESTINAL BARRIER FUNCTION LOSS

In the early 1970s, Menzies introduced oligosaccharides as test probes for the functional assessment of intestinal barrier failure [2,39]. It was hypothesized that large oligosaccharides such as lactulose would not traverse the intestinal membrane in the healthy situation. However, as a result of intestinal barrier integrity loss, these probes cross the intestinal barrier to the circulation and are detectable in urine after being excreted renally. Using this method, increased intestinal permeability was detected in patients with celiac disease [39]. Although test results were promising, the test was prone to various premucosal and postmucosal confounders such as gastric dilution and gastric emptying, bacterial degradation, intestinal transit and renal function. This led to the development of differential sugar absorption tests, where both a di- or oligosaccharide and a monosaccharide, serving as a large- and a small- molecular probe respectively, are administered orally simultaneously, after which their recovery is measured in urine (Figure 1b) [40]. The smaller molecular probe is thought to traverse the intestinal barrier freely, independent of barrier function loss, and is affected in the same way as the large molecular probe by the pre- and postmucosal confounders.

The ratio of the urinary concentration of both compounds would therefore more accurately reflect the paracellular passage across the intestinal barrier than isolated measurement of urinary oligosaccharides [2,3].

Currently, the most frequently used sugar probes to assess intestinal permeability are lactulose as oligosaccharide and mannitol or L-rhamnose as monosaccharide. Other macromolecular probes are differently sized polyethylene glycols (PEG: 4000, 1500, 400), and radioactively labeled macromolecules such as chromium labeled EDTA (51Cr-EDTA). These tests will be discussed respectively in the following section.

DIFFERENTIAL SUGAR ABSORPTION TESTS

The differential sugar absorption tests are based on the oral administration of two sugars that differentially cross the intestinal barrier to the circulation upon barrier integrity loss, after which they are rapidly cleared into urine. The ratio of oligosaccharides and monosaccharides in urine, collected over five to six hours after oral intake, is considered to reflect small intestinal barrier function loss most accurately. Laboratory analysis is usually performed using High Pressure Liquid Chromatography (HPLC) or Liquid Chromatography in combination with Mass Spectrometry (LC/MS) [2]. Oligosaccharides (for example lactulose, cellubiose) and monosaccharides (mannitol, L-rhamnose) have been used with similar results. Since some of the saccharides, as lactulose, can cause increased intestinal motility, the administered dose should be kept as low as possible [41]. It is important to bear in mind that the classical DST are only useful for assessing small intestinal permeability, since lactulose is degraded by bacteria in the large intestine [2,3]. To evaluate whole intestinal permeability, non-degradable probes as sucralose, which remain unaffected by bacteria in the colon, are added to classical DST, resulting in the so-called triple sugar test. The lactulose excretion over 24h (likely to represent only small intestinal permeability), subtracted from 24h sucralose excretion, is considered to give an isolated measure of colonic permeability [42]. Other studies focused on measurement of gastroduodenal permeability, have used sucrose as test substance. Sucrose is rapidly degraded by sucrase, an enzyme secreted in large amounts by mature enterocytes in the duodenum. Therefore, enhanced plasma or urinary levels of sucrose are thought to reflect only permeability of the stomach and proximal duodenum [43,44].

In conclusion, DST are useful to assess small intestinal permeability and additional information on gastroduodenal or colonic permeability can be obtained by adding sucrose or sucralose, respectively, as test probes.

DIFFERENTIAL SUGAR ABSORPTION TESTS IN DISEASE

DST have been valuable for evaluation of both etiology and disease activity in various intestinal diseases. Increased permeability for saccharides has been reported in patients with Crohn's disease [45-47], celiac disease [48,49] and food intolerance [3]. However, the test has never gained a place in everyday practice for diagnosis and follow up of such patients groups, mainly because the test is impractical in use and detection methods are complex and not widely available [50]. Apart from intestinal diseases, DST have also been used to assess intestinal permeability in critically ill patients, since the intestinal barrier function has been hypothesized to play a central role in the development of sepsis. Indeed, many studies report increased permeability in patients who are critically ill or who undergo major surgery [51,52]. Therefore DST might be useful for early detection of patients at risk of developing severe complications, although the administration of probes and the necessity of urine collection for several hours is impractical and moreover, these patients often have limited urine production. Furthermore, some studies have showed that permeability measurements using DST in intensive care patients with MOF has pitfalls. Firstly, decreased motility and altered clearance of the different sugars as a result of renal dysfunction is a complicating factor in these patients. Secondly, the use of mannitol appeared to be unsuitable in patients receiving red blood cell transfusion, since mannitol is used in the storage solution of bank blood [53].

POLYETHYLENE GLYCOLS

Ethylene glycol polymers (PEG) with a molecular weight of 400-4000Da have also been used to assess intestinal barrier function. It is hypothesized that, as saccharides in the DST, large molecular PEG will only cross the intestinal mucosa to the circulation in the case of barrier integrity loss, as measured after renal excretion using Gas Chromatography (GC) or HPLC [2]. Increased urinary levels of large molecular PEG therefore reflect increased intestinal permeability. Since PEG is biochemically inert and not degraded by bacteria, 24h urinary levels could provide information on whole intestinal permeability.

POLYETHYLENE GLYCOL MEASUREMENT IN DISEASE

Variously sized PEG probes were used to investigate bowel permeability in a broad range of intestinal diseases. PEGs have the advantage of being inert and can therefore be used to measure both small and large intestinal permeability. They have been used successfully to assess permeability changes in patients with irritable bowel syndrome [54], pancreatitis [55-57], liver cirrhosis [58], and intestinal ischemia reperfusion injury [59].

They have also been used in patients with Crohn's disease, although both decreased and increased intestinal permeability was found in this patient group. In addition, some studies also reported high inter- and intra-individual variations in test results, even in controls [50,60,61]. Hence, future studies on the permeation pathways of PEG are necessary to improve interpretation of results.

CHROMIUM LABELED EDTA

Chromium labaled EDTA (51Cr-EDTA) has similar physiological properties to oligosaccharides with the advantage of being easily detectable. Furthermore, 51Cr-EDTA is not degraded by bacteria in the colon, which makes it a useful marker for both small and large intestinal permeability. Some studies have reported increased colorectal permeability for 51Cr-EDTA in patients with IBD [62]. A disadvantage of 51Cr-EDTA is its radioactivity. It should, therefore, be avoided for research purposes in children and for screening in healthy subjects.

PASSIVE MEASUREMENT OF INTESTINAL BARRIER FUNCTION

In the healthy situation the intestinal barrier prevents translocation of intraluminal compounds whilst in situations of impaired intestinal barrier function, bacteria and bacterial products can find their way to the circulation. The presence of such compounds in plasma could therefore provide information on intestinal barrier integrity and function. In the next section, tests for the measurement of bacterial compounds or bacterial fermentation products are summarized. An advantage of these tests is that they can be performed without the need to administer test substances and time consuming urine collection.

MEASUREMENT OF CIRCULATING ENDOTOXIN

Limulus Amebocyte Lysate Assay (LAL assay): Endotoxin is a lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria. It is capable of inducing multiple effects in man, varying from fever and leucocytosis to thrombocytopenia and coagulopathies [63]. The LAL assay allows quantitative determination of plasma endotoxin levels. This assay is based on the fact that endotoxin causes intravascular coagulation in the horseshoe crab (Limulus polyphemus), via the enzymatic conversion of a clottable protein derived from the circulating blood cells (amebocytes) of the crab. The lysate from the amebocytes is also sensitive to the presence of endotoxin in vitro.

Several assays have been developed to detect endotoxin using LAL, such as the gel clot LAL assay and the more recent chromogenic LAL assay [63]. In LAL assays, the presence of plasma endotoxin neutralizing factors, detergents, urea and variation in pH influences the test results strongly and can yield false positive or false negative results. In order to minimize the effect of plasma endotoxin neutralizing factors, Ditter et al. reported a modified chromogenic LAL assay. The principle of this assay is that each sample contains a specific amount of factors interfering with the LAL-endotoxin reaction. Therefore, an endotoxin reference curve is established in each sample by spiking it with certain concentrations of endotoxin. The deviation from the standard curve then represents the endogenous unknown endotoxin content. Using this method, each sample has an internal standard, correcting for plasma endotoxin neutralizing factors [64].

Still, the specificity of the LAL assay remains a point of concern since cell wall products of fungi, Gram-positive bacteria and polynucleotides have been reported to account for a (false) positive test. Furthermore, due to its high sensitivity, the LAL assay is prone to false positive results caused by exogenous endotoxin contamination [63,65]. In spite of these complicating factors, several studies have successfully used the LAL assay to show endotoxemia, mostly in patients with sepsis [66,67], which might indicate bacterial translocation from the gut lumen to the circulation as a consequence of intestinal barrier function failure.

2.4 INDIRECT MEASUREMENT OF TRANSLOCATION OF BACTERIAL PRODUCTS

MEASUREMENT OF CIRCULATING ENDOTOXIN CORE ANTIBODIES

The endotoxin core antibody (EndoCAb) assay measures the concentration of immunoglobulins (IgG, IgM and IgA) against the inner core of endotoxin. This inner core consists of a hydrophobic part, lipid A, which is attached to a core oligosaccharide. Lipid A is highly conserved across the whole range of Gram-negative microbiota. Moreover, it is this part that is considered most responsible for endotoxin toxicity [68]. In 1989, Barclay et al. described the potential value of EndoCAb for diagnostic use in patients with a Gram-negative sepsis [69]. They hypothesized that anti-endotoxin antibodies were consumed by the superabundance of endotoxin in such patients (Figure 1c). In a later stage, IgM EndoCAb levels increase as endotoxin stimulates the synthesis of antibodies to endotoxin [69].

Several studies showed decreased EndoCAb levels postoperatively, accounting for the degree of exposure to endotoxin [70,71]. In addition, successive studies have shown that preoperative low circulating levels of anti-endotoxin antibodies are related to poor outcome in patients after major cardiac [72], and abdominal aortic aneurysm surgery [73,74]. Stable EndoCAb levels have high individual variation and the determining factors for an individual's stable EndoCAb level have not been fully understood, potentially hampering the interpretation of circulating EndoCAb levels [75]. In summary, EndoCAb assays detect anti-endotoxin immunoglobulins; consumption of these circulating immunoglobulins following translocation of gut-derived endotoxins can therefore be used to acquire indirect information on the intestinal epithelial barrier function.

MEASUREMENT OF PLASMA D-LACTATE LEVELS

D-lactate is a fermentation product produced by many bacteria present in the human gastrointestinal tract, and was proposed in the 1980s as a marker for diagnosis of bacterial infections [76]. Low circulating levels of D-lactate are found in healthy individuals, but in case of intestinal barrier function loss, these levels will rise as a consequence of increased translocation across the intestinal mucosa. Therefore, plasma D-lactate levels could serve as a measure of impaired barrier function (Figure 1d). Various studies proposed a relationship between plasma D-lactate and intestinal

permeability. Sun et al. evaluated plasma D-lactate as a marker for increased intestinal permeability in a rat model of intestinal ischemia reperfusion and acute necrotising pancreatitis [77]. Plasma endotoxin levels, measured using the LAL assay, correlated significantly with plasma D-lactate levels at an early stage of intestinal injury. Few human studies have been performed to evaluate the potential role of plasma D-lactate as a marker for impaired barrier function. In one study, performed in patients undergoing open aortic surgery, a rapid increase of plasma D-lactate levels was observed, which correlated with a histologically proven ischemic colitis [78]. D-lactate measurement has especially been valuable in assessment of ischemic colonic injury and seems to be a reliable marker for colonic barrier function loss in animal models. Results should however be interpreted cautiously in case of bacterial overgrowth since the augmented presence of bacteria could result in increased fermentation of undigested carbohydrates to D-lactate [79]. The usefulness of plasma D-lactate as marker for colonic barrier function in man is a subject for future research.

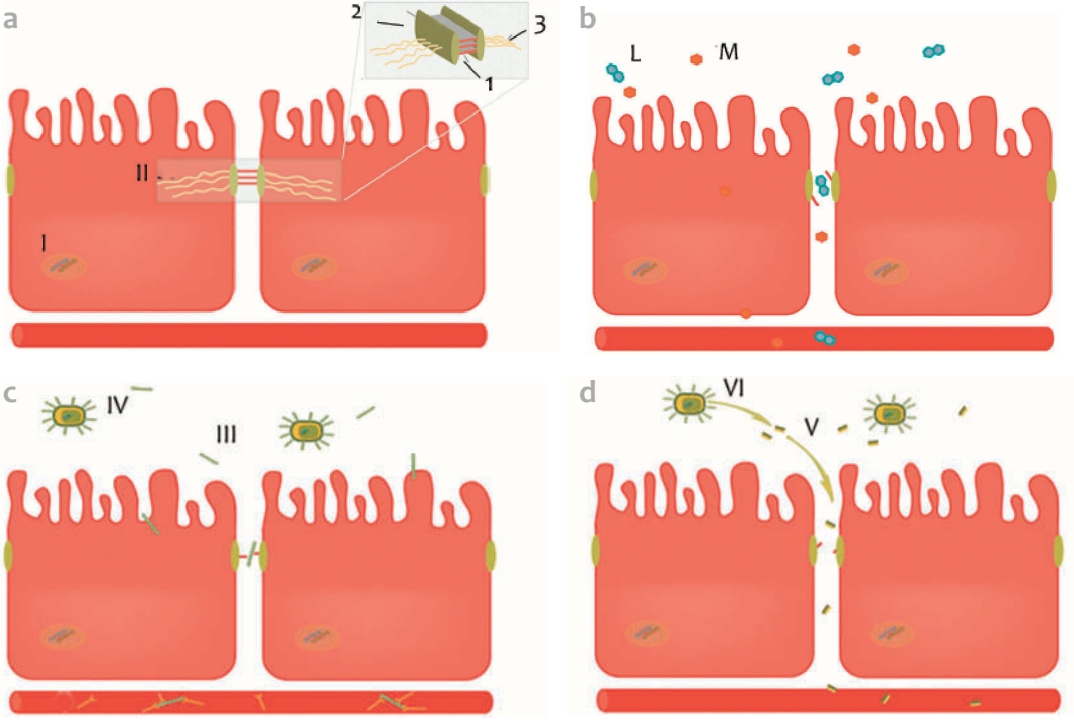

Figure 1a) the intestinal epithelial barrier is composed of a lining of enterocytes (I) tightly connected by tight junctions (II) to prevent the translocation of intraluminal compounds to

the circulation. Claudins (1), important transmembrane tight junction proteins responsible for sealing the paracellular space, are tightly connected to intracellular protein ZO-1 (2), which is anchored to the cell cytoskeleton (3). Figure **b)** differential sugar absorption test: Lactulose (L), a disaccharide, is only able to traverse the paracellular pathway in case of compromised intestinal barrier function. Mannitol (M) is a monosaccharide which can cross the intestinal barrier both via the trans- and paracellular pathway, thereby serving as an internal control to correct for confounders as gastric emptying, mucosal perfusion and renal function. **c)** endotoxin Core Antibody (EndoCAb) is consumed when endotoxin (III), derived from intraluminal Gram-negative bacteria (IV), translocates from the intestinal lumen to the circulation via the defective intestinal barrier. **d)** D-Lactate (V) is a fermenting product from intestinal bacteria (VI). In case of barrier function loss, D-Lactate can be detected in plasma.

2.5 CONCLUSION

The intestinal epithelial lining should provide an efficient barrier that prevents entry of pathogens and antigens. Over the past decades it has become evident that dysfunction of the intestinal barrier has a significant impact on the health of an individual. The mucosal barrier may become compromised as a consequence of various (intestinal) disorders, and has also been suggested to play a role in the pathogenesis and perpetuation of disease. Although intestinal barrier function tests have been improved over the past decades and new tests have emerged, evaluation of intestinal barrier integrity and barrier function loss remains a challenge to both clinicians and scientists.

In this review, currently available tests that address different aspects of the intestinal epithelial barrier have been evaluated (Table 1). Markers for physical barrier loss as well as methods to assess functional barrier loss have been discussed. A combination of the various tests might provide clinicians and scientists with a more advanced insight in gut wall integrity status. Due to the evident importance of the intestinal barrier in development and perpetuation of disease, further studies should be aimed at validating current available tests for clinical application and development of new tests for accurate assessment of intestinal integrity and barrier function loss. Furthermore, the relationship between physical intestinal barrier damage and functional failure of the barrier function remains subject for future research.

TEST	MEASURED IN	INDICATIVE FOR	TISSUE SPECIFICITY
I-FABP	Blood or urine Single sample	Intestinal epithelial integrity	Yes *
I-BABP	Blood or urine Single sample	Intestinal epithelial integrity	Yes **
L-FABP	Blood or urine Single sample	Intestinal epithelial integrity	No
GST-α	Blood Single sample	Intestinal epithelial integrity	No
Claudin 3	Urine Single sample	Paracellular integrity	No
Dual Sugar Test	Urine 5h collection	Functional intestinal barrier function	Yes
PEG	Urine 6 h collection	Functional intestinal barrier function	Yes
51Cr-EDTA	Urine 24h collection	Functional intestinal barrier function	Yes
LAL-assay	Blood Single sample	Intestinal barrier function	N/A
EndoCAb	Blood Single sample	Intestinal barrier function	N/A
D-Lactate	Blood Single sample	Intestinal barrier function	Yes

***** predominantly proximal small gut, ****** predominantly ileum
I-FABP: intestinal fatty acid binding protein; I-BABP: ileal bile acid binding protein; GST: glutathione S-transferase-α; PEG: polyethylene glycol; 51Cr-EDTA: chromium labelled EDTA; LAL: limulus amebocyte lysate; EndoCAb: endotoxin core antibody.

Table 1. Methods for the assessment of intestinal barrier integrity status and intestinal barrier function loss.

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Chapter 3

Obesity-specific intestinal microbiota composition is related to local and systemic inflammation in man

Submitted as: Obesity-specific intestinal microbiota composition is associated with local and systemic inflammation but not related to intestinal permeability in man. Froukje Verdam, Susana Fuentes, Charlotte de Jonge, Erwin Zoetendal, Jan Willem Greve, Wim Buurman, Willem de Vos, Sander Rensen.

ABSTRACT

The intestinal microbiota play an important role in the development of obesity. Microbiota have been shown to enhance intestinal permeability in obese mice, thereby stimulating the low-grade inflammation characteristic of obesity. In view of this, we investigated the relation between intestinal permeability, microbiota composition, and inflammation in obese and non-obese subjects.

Gastroduodenal (sucrose), small intestinal (lactulose/rhamnose), and colonic (sucralose/erythritol) permeability were probed in 28 subjects (BMI 18.6-60.3kg/m^2) by a multi-saccharide test. Faecal microbiota composition was analyzed by a phylogenetic profiling microarray. Faecal calprotectin was used to evaluate intestinal inflammation while systemic inflammation was assessed using plasma C-reactive protein.

Whereas gastroduodenal permeability was doubled in obese subjects (4.1±0.7 vs. 1.9±0.3 µmol, p=0.003), small and large intestinal permeability were similar between obese and non-obese subjects. Obese subjects had a lower Bacteroidetes/Firmicutes ratio (0,274±0,044 vs. 0,069±0,019, p=0.0002). Moreover, the Bacteroidetes/Firmicutes ratio strongly correlated with BMI (r_s=-0.59, p=0.0009). Detailed analyses of the microbiota composition showed that the population could be divided into two clusters with predominantly obese (15/19) or non-obese (9/9) members. Microbiota composition was not related to intestinal permeability. Interestingly, however, faecal calprotectin was only detectable in subjects within the obese microbiota cluster (n=8/19, p=0.02). In addition, plasma C-reactive protein levels were also elevated in the obese microbiota cluster, and correlated with the Bacteroidetes/Firmicutes ratio (r_s=-0.41, p=0.03).

Intestinal microbiota alterations in obese subjects are associated with both local and systemic inflammation, but not with intestinal permeability, indicating a direct effect of the gut microbiota on inflammation in human obesity.

3.1 INTRODUCTION

The intestinal microbiota are increasingly acknowledged to be involved in the development of obesity and the metabolic syndrome [1]. For instance, germ-free mice are protected from diet-induced obesity [2]. Furthermore, transplanting lean germ-free mice with intestinal microbiota from obese mice results in a larger fat deposition than transplantation of intestinal microbiota from lean donor mice [3]. Phylogenetic profiling and metagenomics of the GI tract microbiota indicated that compared to lean controls, both genetically and diet-induced obese animals have a different intestinal microbiota composition, which is characterized by a reduction in the abundance of Bacteroidetes paralleled by an increase in Firmicutes [4,5].

Comparable profound differences in intestinal microbiota composition have been reported in man [6,7,8], although a lower Firmicutes/Bacteroidetes ratio in obesity [9] and similar microbiota composition in lean and obese subjects [10] have also been described. The mechanisms by which intestinal microbiota affect obesity and metabolic disorders are the focus of intense research. Microbiota have been shown to influence intestinal permeability in obese mice, thereby promoting translocation of bacterial products and stimulating the low-grade inflammation characteristic of obesity and insulin resistance [11,12]. Furthermore, obesity-prone rats were found to exhibit intestinal inflammation combined with an altered microbiota composition [13]. Finally, several studies suggest that intestinal microbiota influence energy extraction from nutrition and subsequent fat storage in adipose tissue [2,3,14].

In view of these data, we investigated the intestinal microbiota composition in obese and non-obese subjects, and correlated these data to parameters of intestinal permeability and local and systemic inflammation. We present the first evidence that the obese gut microbiota may be related to both increased intestinal and systemic inflammation in man.

3.2 MATERIALS AND METHODS

SUBJECTS

From May to September 2010, 28 subjects with a Body Mass Index (BMI) ranging from 18.6 to 60.3kg/m^2 (mean 35.0kg/m^2) were recruited through advertising at the Atrium Medical Centre Parkstad in Heerlen, the Netherlands. Subjects were grouped according to BMI as non-obese (BMI<30kg/m^2, n=13) or obese (BMI>30kg/m^2, n=15). Population characteristics are presented in Table 1.

	NON-OBESE	OBESE	P-VALUE
No. of patients	13	15	ns
Age (years)	28.2 ± 3.3	35.3 ± 2.8	<0.04
Sex (F : M)	8 : 5	12 : 3	ns
BMI (kg/m^2)	23.4 ± 0.8	44.2 ± 2.3	<0.01
HbA1c (%)	5.4 ± 0.1	6.1 ± 0.3	<0.02
Cholesterol (mmol/L)	4.8 ± 0.4	4.6 ± 0.2	ns
HDL (mmol/L)	1.5 ± 0.1	1.1 ± 0.1	<0.02
LDL (mmol/L)	2.8 ± 0.3	2.7 ± 0.3	ns
TG (mmol/L)	1.4 ± 0.3	1.8 ± 0.3	ns
AST (IU/L)	17 ± 2	19 ± 2	ns
ALT (IU/L)	21 ± 2	29 ± 3	ns
CRP (mg/L)	1.5 ± 0.2	12.4 ± 2.5	<0.01

Table 1. Population characteristics.

Exclusion criteria were acute and chronic inflammatory diseases (e.g. Crohn's disease, colitis, hepatitis, type 1 diabetes, auto-immune diseases, asthma, and chronic obstructive pulmonary disease). Subjects were also excluded if they used anti-inflammatory drugs, reported alcohol consumption over 8 units/week, or received antibiotic treatment in the last six months. The study was approved by the Medical Ethics Committee of the Atrium Medical Centre, and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Informed consent in writing was obtained from each subject.

BLOOD SAMPLING AND ANALYSIS

Venous blood samples were obtained in the outpatient clinic, collected into pre-chilled EDTA tubes (BD Vacutainer, Becton Dickinson Diagnostics, Erembodegem-Aalst, Belgium), and kept on ice. Parameters reflecting inflammation (high sensitivity C-Reactive Protein: CRP) and obesity co-morbidity (HbA_{1c}, plasma glucose, insulin, cholesterol, HDL, LDL, free fatty acids (FFA), and liver transaminases (AST and ALT)) were assessed at the Department of Clinical Chemistry according to the protocol of the Atrium Medical Centre.

FAECAL MICROBIOTA AND FAECAL CALPROTECTIN ANALYSIS

Subjects collected feces during 24 hours prior to the intestinal permeability test, and kept this refrigerated until the morning of the test, when samples were stored in aliquots at -20°C. DNA was isolated as previously described [15] and used for the assessment of intestinal microbiota composition. This was performed using the Human Intestinal Tract Chip (HITChip), a phylogenetic profiling DNA microarray containing over 4,800 probes based on 16S rRNA gene sequences of over 1,100 intestinal bacterial phylotypes. This microarray identifies both variation and relative quantity of the human intestinal tract communities [16]. Hybridizations were performed in duplicate with samples labeled with Cy3 and Cy5 dyes, respectively. Slides were scanned and the data was extracted from the microarray images using the Agilent Feature Extraction software, version 10.7.3.1 (http://www.agilent.com). Array normalization was performed as previously described [16,17] using a set of R-based scripts (http://r-project.org) in combination with a custom designed relational database which runs under the MySQL database management system (http://www.mysql.com). This was implemented on both dyes for each sample, and duplicates with a Pearson correlation over 0.98 were considered for further analysis. Ward's minimum variance method was used for the construction of hierarchical clusters of the total microbiota probe profiles, while the distance matrix between the samples was based on Euclidian distance. Furthermore, faecal calprotectin levels were analyzed by ELISA (Hycult Biotech, Uden, the Netherlands), according to previously described instructions by Van der Sluis Veer et al. to improve sensitivity [18]. The detection limit was 20µg/g feces.

ASSESSMENT OF INTESTINAL PERMEABILITY

Intestinal permeability was assessed as previously described [19]. In short, a multi saccharide mix was orally administered after at least eight hours of fasting and a double challenge with a non-steroid anti-inflammatory drug (400mg ibuprofen the evening prior to the test, and the following morning) to magnify potential differences in intestinal permeability [20]. The saccharide mix consisted of 1g sucrose (Van Gilse, Dinteloord, the Netherlands), 1g lactulose (Centrafarm, Etten-Leur, the Netherlands), 0.5g L-rhamnose (Danisco, Copenhagen, Denmark), 1g sucralose (Brenntag, Sittard, the Netherlands), and 1g erythritol (Danisco), dissolved in 150mL tap water. Urinary excretion of these saccharides reflects their translocation over different parts of the gastrointestinal tract; sucrose excretion after one hour reflects gastroduodenal permeability [21,22,23], the ratio of Lactulose/L-rhamnose (L/R) after five hours reflects small intestinal permeability, and large intestinal permeability is reflected by the ratio of sucralose/erythritol (S/E) after five hours [24,25]. After oral administration of the saccharide mix, total urine collection after one and five hours was recorded. At these time points, urine samples were taken and centrifuged at 4°C for 15 minutes at 2300g, and immediately stored in aliquots at -80°C until analysis. Urinary excretion of mono- and disaccharides was quantified by combined high pressure liquid chromatography and mass spectrometry (Model LTQ-XL, Thermo Electron, Breda, the Netherlands) as previously described [26].

STATISTICAL ANALYSIS

Data analysis for the microbiota was performed in R (version 2.12.2). Multivariate statistical software Canoco 4.5 for Windows [27] (Biometrix, Plant Research international, Wageningen) was used to perform redundancy analysis (RDA) on log transformed data, and statistical significance was evaluated using a Monte Carlo Permutation Procedure (MCPP). The log transformed sum of the hybridization signals for the 131 genus like phylogenetic groups targeted by the HITChip was used as species variables. Comparisons between groups at the genus-level (subsets of phylotypes with 90% or more 16S rRNA sequence similarity) were performed using the Wilcoxon signed-rank test corrected for multiple comparisons (q value), in which $q<0.05$ was considered statistically significant.

To evaluate differences of the Bacteroidetes/Firmicutes ratio, the Student t-test for normally distributed data was used. Additional statistical analyses were performed using Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Correlations were calculated using Spearman's rank correlation coefficient. Differences between groups were analyzed by the nonparametric Mann-Whitney test or the Chi-square test. A p-value of <0.05 was considered statistically significant (denoted with an * in the figures). Data are presented as mean±standard error of the mean.

3.3 RESULTS

ALTERED PERMEABILITY OF THE PROXIMAL GASTRO-INTESTINAL TRACT IN OBESE SUBJECTS

Since animal studies suggest that obesity is characterized by increased intestinal permeability [12,28], we first studied permeability of different segments of the gastro-intestinal tract in non-obese and obese subjects. Gastro-duodenal permeability as reflected by urinary sucrose excretion after one hour was twice as high in obese compared to non-obese subjects (4.1±0.7µmol vs. 1.9±0.3µmol, p<0.01, Figure 1a). In contrast, the lactulose/rhamnose (L/R) ratio signifying small intestinal permeability was comparable in both groups (0.06±0.02 vs. 0.05±0.01; p=0.9, Figure 1b). Permeability of the colon, as indicated by the urinary sucralose erythritol ratio (S/E), was also similar (0.03±0.01 vs. 0.04±0.01; p=0.65, Figure 1c). Thus, permeability of the proximal gastro-intestinal tract appears to be increased in the obese population, whereas permeability of the lower parts of the intestine is normal.

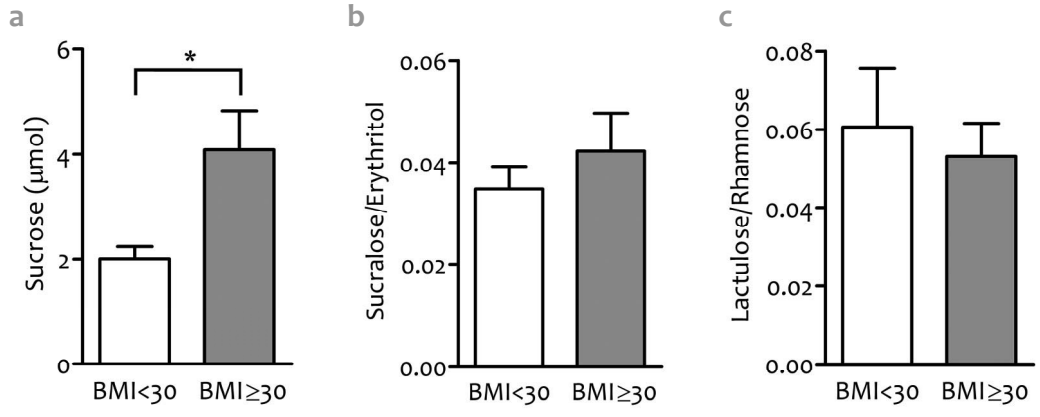

Figure 1. Permeability of the gastro-intestinal tract in non-obese and obese subjects.

a) Significantly higher gastroduodenal permeability in obese subjects (n=15) compared to non-obese subjects (n=13), reflected by urinary sucrose levels after one hour. **b)** The similar lactulose/rhamnose ratio in both groups indicates a comparable small intestinal permeability. **c)** The sucralose/erythritol ratio was not significantly different between the groups, reflecting a similar large intestinal permeability.

FIRMICUTES / BACTEROIDETES RATIO IS ASSOCIATED WITH BMI AND STRONGLY INCREASED IN OBESE SUBJECTS

The microbial profiles obtained from the faecal samples of all 28 subjects (13 non-obese subjects and 15 obese subjects) were hierarchically clustered based on the signal intensity of 3699 distinct HITChip oligonucleotide probes. The majority of samples from obese subjects clustered separately from those of non-obese subjects, although four non-obese subjects clustered with the obese subjects (Figure 2).

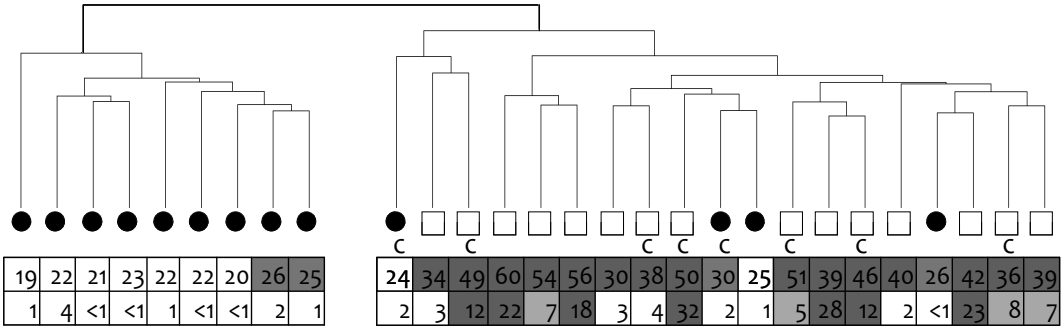

Figure 2. Microbiota alterations in obesity.
Hierarchical clustering of the HITChip profiles of the 28 faecal samples of obese (square) and non-obese (dot) subjects. Corresponding BMI and CRP values are shown below in white (BMI<25kg/m^2; CRP<5mg/L), light grey (25>BMI<30kg/m^2; 5>CRP<10mg/L) and dark grey (BMI>30kg/m^2; CRP>10mg/L). Subjects with detectable faecal calprotectin are denoted by a 'c'.

Comparison of the microbiota composition of non-obese and obese subjects based on the sum of hybridization signals for 131 genus-like phylogenetic groups (taxa with ≥90% sequence identity) revealed that for 17 groups, abundance was significantly different between the two study groups (Table 2).

LEVEL 1	LEVEL 2	RELATIVE ABUNDANCE (%)		
		non-obese	obese	q-value
Bacteroidetes	Allistipes	2.72	0.48	0.0001
	Bacteroides fragilis	0.84	0.25	0.0093
	Bacteroides intestinalis	0.72	0.21	0.0179
	Bacteroides ovatus	1.20	0.52	0.0243
	Bacteroides plebeius	1.66	0.42	0.0127
	Bacteroides splachnicus	1.52	0.30	0.0016
	Bacteroides stercoris	1.08	0.39	0.0356
	Bacteroides uniformis	0.91	0.26	0.0179
	Parabacteroides distasonis	2.11	0.60	0.0045
	Prevotella oralis	0.57	0.13	0.0120
	Prevotella ruminicola	0.50	0.16	0.0179
	Prevotella tannerae	1.24	0.53	0.0182
	Tannerella	0.83	0.33	0.0182
	Uncultured Bacteroidetes	0.19	0.01	0.0016
Firmicutes Clostridium cluster IV	Papillibacter cinnamivorans	0.30	0.77	0.0321
Firmicutes Clostridium cluster XIVa	Clostridium symbiosum	2.54	3.76	0.0179
Firmicutes	Dorea formicigenerans	4.11	6.27	0.0511

Table 2. Bacterial groups present at significantly different relative abundance (%) in the microbiota of non-obese and obese subjects. The level 2 phylogenetic groups with higher relative abundance in obese subjects are indicated in darker grey.

Most phyla did not significantly differ in their relative abundance between obese and non-obese subjects (in obese vs. non-obese: Actinobacteria 7.8±9.8% vs. 5.9±5.8%, Cyanobacteria 0.003±0.001% vs. 0.004±0.002%, Fusobacteria 0.01±0.003% vs. 0.01±0.002%, Proteobacteria 0.3±0.1% vs. 0.4±0.2%, Spirochaetes 0.005±0.001% vs. 0.005±0.001% and Verrucomicrobia 0.1±0.3% vs. 0.2±0.5%). However, major differences between groups were found with respect to the large phyla of the Bacteroidetes and the Firmicutes. The Bacteroidetes phylum accounted for 19.2±9.2% of the total hybridization signal in the non-obese, whereas this number was significantly lower in the obese (5.9±5.8% of the total signal; $q<0.002$, Figure 3a). Bacteroidetes showed a coherent drop in the obese, in line with their reduced abundance in the cecal microbiota of obese mice [4] and their increased abundance in correlation to weight loss in obese subjects [3].

Members of the Firmicutes phylum were most abundantly present in all samples, differing significantly between the non-obese group, where they accounted for 74.6±9.2% of the total hybridization signal, and the obese group, where they contributed up to 85.8±8.5% (q=0.002, Figure 3a). As a result of the shifts in Bacteroidetes and Firmicutes abundance, the Bacteroidetes to Firmicutes ratio was strongly decreased in obese samples compared to non-obese samples (p=0.0002, Figure 3b). These data are further supported by the strong negative correlation observed between Bacteroidetes/Firmicutes ratio and BMI (r_s=-0.59, p=0.0009, Figure 3c).

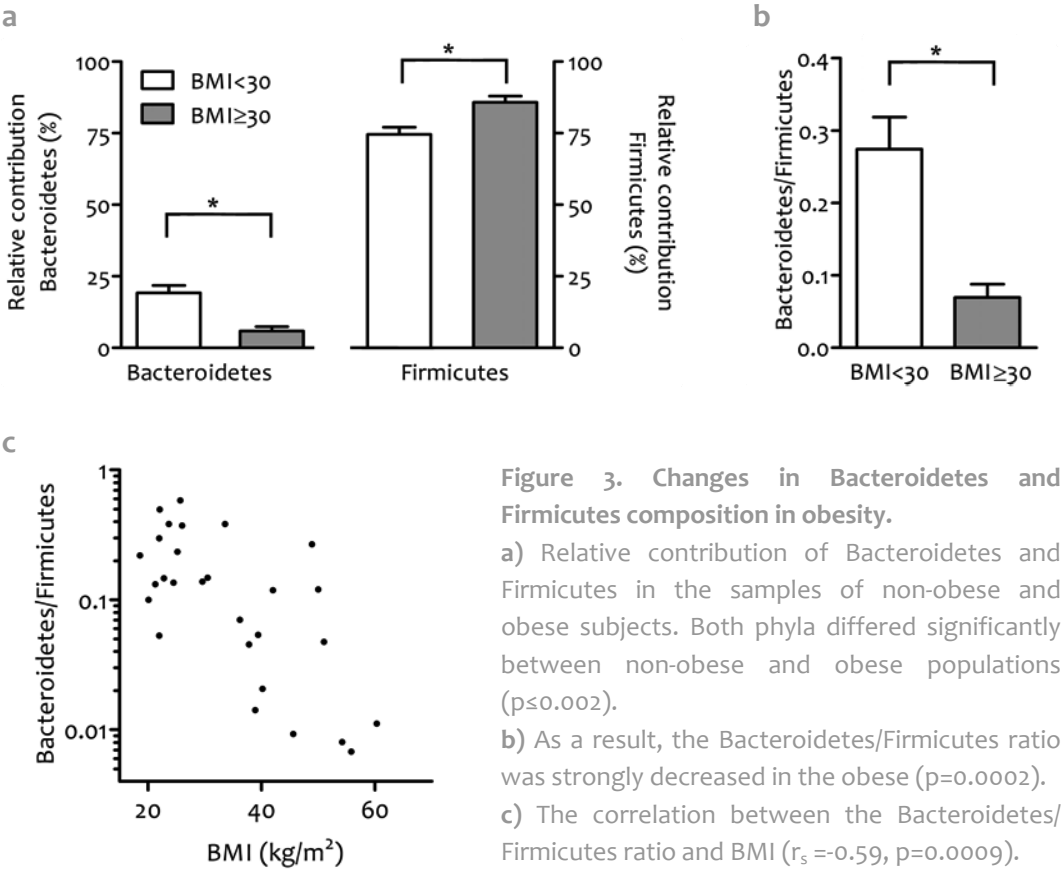

Figure 3. Changes in Bacteroidetes and Firmicutes composition in obesity.

a) Relative contribution of Bacteroidetes and Firmicutes in the samples of non-obese and obese subjects. Both phyla differed significantly between non-obese and obese populations (p≤0.002).

b) As a result, the Bacteroidetes/Firmicutes ratio was strongly decreased in the obese (p=0.0002).

c) The correlation between the Bacteroidetes/ Firmicutes ratio and BMI (r_s =-0.59, p=0.0009).

MICROBIOTA COMPOSITION IS ASSOCIATED WITH BMI AND FAECAL CALPROTECTIN

To determine which bacterial groups and/or subject characteristics were primarily associated to obesity-related differences in microbiota composition, multivariate analysis was performed taking into account BMI, age, CRP, HbA_{1c}, faecal calprotectin, lactulose/rhamnose ratio, sucralose/erythritol ratio and the Bacteroidetes/Firmicutes ratio. A total of 34.5% of the variation in the microbiota composition could be related to these subject characteristics.

Interestingly, only BMI (p=0.002), Bacteroidetes/Firmicutes ratio (p=0.01), and faecal calprotectin (p=0.004) contributed significantly to the microbiota variations as assessed by MCPP (Figure 4), implying that other factors were relatively unimportant in this respect. RDA further supported that obese subjects had a relatively higher abundance of bacteria belonging to the Firmicutes phylum, whereas Bacteroidetes were more dominant in the non-obese (Figure 4).

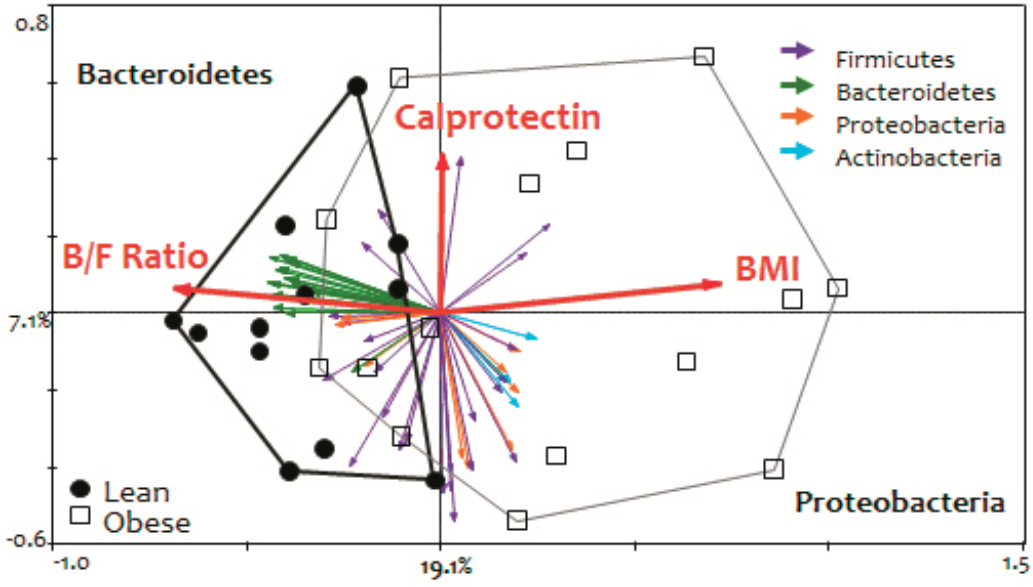

Figure 4. BMI, Bacteroidetes/Firmicutes ratio, and faecal calprotectin levels contribute to microbiota variations in obesity. The RDA plot of non-obese (dot, n=12) and obese (square, n=15) subjects is based on their microbiota composition. The plotted first and second ordination axes explain 19.1% and 7.1% of the variability in the dataset, respectively.

The variation in the abundance of 53 level 2 groups shown (represented by the coloured arrows) belonging to the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria

is explained to at least 15% by the ordination space (subject characteristics). The statistically significant characteristics (red arrows) are BMI (p=0.002), faecal calprotectin (Calprotectin) p=0.004, and Bacteroidetes/Firmicutes ratio (B/F) p=0.01.

Interestingly, the Firmicutes positively correlated to BMI included butyrate producing bacteria such as Roseburia intestinalis and Eubacterium rectale, whereas the only butyrate producing phylotype present in the non-obese cluster was Faecalibacterium prausnitzii. Overall, known butyrate producing bacteria accounted for 21.4±7.4% of the total hybridization signal of the samples. The total signal corresponding to butyrate producers was not significantly different between non-obese and obese subjects. However, species that were positively correlated to BMI, CRP, and faecal calprotectin (Table 3) were significantly more abundant in the obese subjects (9.8±4.4%) compared to the non-obese subjects (5.9±4.2%) (p=0.02). Proteobacteria, which were recently described to be increased in mice on a high fat diet [29], were also positively associated with BMI and more abundant in obese subjects (Figure 4).

Table 3 on the next page shows the bacterial groups that are significantly correlated to BMI, CRP, and/or faecal calprotectin and their relative abundance (%) in non-obese and obese subjects.

LEVEL 1	LEVEL 2	CORRELATION COEFFICIENT			ABUNDANCE (%)	
		BMI	**CRP**	**calp.**	**non-obese**	**obese**
BACTEROIDETES						
	Allistipes	-0.642**	-0.470*		2.733	0.446
	Bacteroides fragilis	-0.552**			0.832	0.239
	Bacteroides intestinalis	-0.539**	-0.433*		0.730	0.210
	Bacteroides plebeius	-0.508**	-0.413*		1.680	0.413
	Bacteroides splachnicus	-0.539**	-0.429*		1.525	0.275
	Bacteroides uniformis	-0.483**			0.921	0.264
	Bacteroides vulgatus	-0.499**	-0.449*		1.663	0.599
	ParaBacteroides distasonis	-0.505**	-0.443*		2.130	0.583
	Prevotella oralis	-0.389*			0.576	0.121
	Prevotella ruminicola	-0.404*			0.506	0.158
	Tannerella	-0.511**	-0.440*		0.815	0.308
FIRMICUTES						
Bacilli	*Aneurinibacillus*	0.375*	0.458*		0.004	0.010
	Lactococcus			0.395*	0.002	0.002
C. cluster IV	*Faecalibacterium prausnitzii*	-0.374*			9.324	6.245
	Papillibacter cinnamivorans	0.522**	0.579**		0.295	0.775
	Subdoligranulum variable		0.402*		3.669	5.377
C. cluster XIVa	*Clostridium colinum*	0.409*			0.506	0.780
	Clostridium nexile			0.437*	2.094	3.114
	Clostridium sphenoides		0.374*		2.898	3.819
	Dorea formicigenerans	0.487**			4.154	6.357
	Eubacterium rectale #			0.378*	3.556	5.175
	Roseburia intestinalis #	0.448*	0.479**		2.366	4.652
	Ruminococcus gnavus	0.396*			1.772	2.792
C. cluster XV	*Eubacterium limosum*	0.395*			0.001	0.003
C. cluster XVIII	*Coprobacillus catenaformis*	-0.424*			0.059	0.026
PROTEOBACTERIA						
	Alcaligenes faecalis	-0.416*			0.002	0.000
	Enterobacter aerogenes	0.585**			0.006	0.018
	Klebsiella pneumoniae	0.530**			0.004	0.008
	Vibrio	0.498**			0.001	0.003
	Yersinia	0.562**			0.001	0.002
ACTINOBACTERIA						
	Bifidobacterium	0.386*			4.627	6.621

Significant correlations are indicated by * at the 0.05 level and by ** at the 0.01 level (2-tailed). Grey shading indicates a negatively correlation to the different variables. # butyrate producing bacteria.

Table 3. Microbiota composition in non-obese and obese subjects.

THE OBESE MICROBIOTA CLUSTER IS ASSOCIATED WITH INTESTINAL AND SYSTEMIC INFLAMMATION INDEPENDENT OF INTESTINAL PERMEABILITY

Because the obese intestinal microbiota have been suggested to promote inflammation due to an increased intestinal permeability [11,12,13,28], we next focused on the potential relation between gut microbiota composition, inflammation, and gut permeability.

As opposed to animal studies, neither small intestinal nor colonic permeability, as reflected by the L/R ratio and the S/E ratio respectively, were related to the Bacteroidetes/Firmicutes ratio (r_s=-0.01, p=0.95; r_s=-0.14, p=0.50, respectively) or to CRP levels reflecting systemic inflammation (r_s=-0.03, p=0.89; r_s=0.05, p=0.80, respectively). Furthermore, intestinal permeability of subjects within the obese microbiota cluster was not different from that of subjects within the non-obese microbiota cluster (data not shown).

Strikingly though, the intestinal inflammation marker faecal calprotectin was only detectable in subjects within the obese microbiota cluster (n=8/19, 42% of subjects, vs. n=0/9 in the non-obese microbiota cluster, p=0.02; Figure 2, Figure 5a). These subjects showed a mean faecal calprotectin level of 279±70ng/ml, ranging from 80 to 570ng/ml. As expected, all subjects displaying intestinal inflammation were characterized by a low Bacteroidetes/Firmicutes ratio.

Of note, the Bacteroidetes/Firmicutes ratio also correlated with plasma CRP levels (r_s=0.41, p=0.03, data not shown), implying a relation between microbiota composition and systemic inflammation. In line with this, plasma CRP levels were significantly higher in subjects within the obese microbiota cluster (Figure 5b).

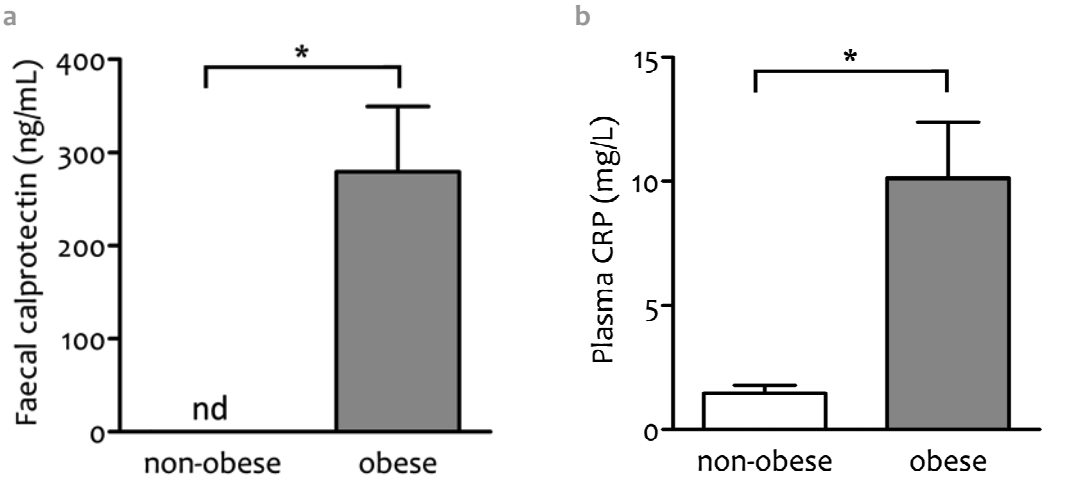

Figure 5: The obese microbiota cluster is associated with intestinal and systemic inflammation a) None of the subjects within the non-obese microbiota cluster have detectable faecal calprotectin, whereas 8 out of 19 subjects within the obese microbiota cluster (42%) show calprotectin in their feces (p=0.02). 'nd' is not detectable. **b)** Plasma CRP levels were significantly higher in subjects within the obese microbiota cluster (p=0.0005).

3.4 DISCUSSION

Gut microbiota are considered to play an important role in the development of obesity and obesity-associated chronic low grade inflammation by increasing gut permeability [11,12,28]. In the present study, we found significant obesity-associated differences in faecal microbiota composition in the absence of marked differences in intestinal permeability. Interestingly though, the microbiota composition changes in obesity were associated with intestinal and systemic inflammation.

Therefore, the data presented in the current paper suggest that obesity-associated modulation of inflammation by intestinal microbiota occurs independent of changes in gut permeability.

Based on animal data, several mechanisms by which bacterial factors can affect the intestinal barrier and promote inflammation in the context of obesity have been suggested.

First of all, translocation of pro-inflammatory bacterial components such as endotoxin, facilitated by increased intestinal tight junction permeability, has been shown in obese animals [11,12,28] and suggested in obese subjects with non-alcoholic steatohepatitis [30]. Furthermore, a high fat diet may enhance endotoxin absorption through chylomicron-facilitated transport [31,32]. In addition, microbiota may control endogenous GLP-2 production, thereby affecting both gut permeability and the consequent inflammatory phenotype associated with obesity [12]. Finally, it was recently shown that high-fat diet-induced translocation of bacteria over the intestinal wall occurs after phagocytosis by dendritic cells, and leads to adipose tissue and systemic inflammation [33]. Our data are in best agreement with the last mechanism since the microbiota alterations that we observed were not related to transcellular or paracellular gut permeability as probed by oligosaccharides, but nevertheless associated with both local intestinal and systemic inflammation.

Interestingly, intestinal inflammation as reflected by elevated faecal calprotectin levels has previously been reported in obese subjects [34]. More recently, Brignardello et al. found no association between the altered microbiota composition in obese subjects and faecal calprotectin levels or gut permeability [35]. However, subjects in that study had a lower BMI (mean 35.9kg/m^2) and a less sensitive calprotectin assay was used. This may have prevented the detection of the calprotectin levels that we observed, which are relatively low and not considered pathological [10,34,36,37]. Of note, the presence of subclinical chronic intestinal inflammation has previously been suggested to underlie the increased risk of colorectal cancer in human obesity.

In line with our results, intestinal inflammation has also been observed in obese rats and in mice on a high fat diet concomitant with microbiota alterations [13,38]. We detected intestinal inflammation only in subjects within the obese microbiota cluster, implying that these microbiota may have a local pro-inflammatory effect in the intestine. Along this line, it is well known that interactions of the microbiota with the intestinal epithelium can provoke an inflammatory response, for example by increasing cytokine expression [38,39,40,41,42,43,44].

In addition to the local intestinal inflammation, we found a positive relation between the obese microbiota and systemic inflammation. Interestingly, the chronic low grade

systemic inflammation characterizing obesity was recently shown to be reduced by administration of probiotics or antibiotics in mice [12]. Moreover, the microbiota dysbiosis in high fat diet-induced obese mice could be reversed by oral prebiotic administration, which was accompanied by improvement of intestinal barrier function and diminished systemic inflammation [45]. It would be of interest to investigate whether prebiotics or probiotics would have similar anti-inflammatory effects in human obesity.

The nature of the bacteria responsible for the inflammation remains unknown, though it is conceivable that they belong to the Firmicutes phylum, which were increased in obese individuals, in line with the seminal studies by Ley and colleagues [4,7]. A decreased Bacteroidetes/Firmicutes ratio in obese individuals has been confirmed by other groups as well [3,6,8], although others found no differences in microbiota composition [10] or even an opposite change in this ratio in obesity [9]. These conflicting data may be attributable to confounding factors such as diet [5,46,47], recent use of antibiotics [48], host physiology [49], and the presence of obesity associated co-morbidity such as insulin resistance [50]. Perhaps more importantly, the subjects in these studies were less obese. Our study of subjects with a BMI up to 60.3kg/m^2 and as low as 18.6kg/m^2 who did not receive recent antibiotic treatment, corroborates the findings of Ley et al. but also indicates that a decreased Bacteroidetes/Firmicutes ratio is particularly characteristic of severely obese individuals with a BMI>35kg/m^2. Indeed, the ratio of Bacteroidetes/Firmicutes in subjects with a BMI<35kg/m^2 was remarkably constant, suggesting a threshold effect. Further support for an important interplay between BMI and microbiota composition comes from the observation that the four non-obese subjects within the non-obese microbiota cluster had a relatively high BMI.

The mean age of the subjects in the two study groups differed to some extent. However, the microbiota are formed by a stable core of microbes in adulthood [16], and an effect of ageing on gut microbiota has only been described for later stages of life (>63 years) [51]. As the age of the subjects in our study ranged from 18.9 to 48.6 years, it can be assumed that the age difference between non-obese and obese individuals did not strongly affect microbiota composition. This was further confirmed by the multivariate analysis where age was not found to contribute to the observed variation in microbiota composition (p=0.74).

The increase in Firmicutes in obese subjects was primarily due to the abundance of Clostridium cluster XIVa, which contains many butyrate producing species. Interestingly, increased synthesis of SCFA such as butyrate by the obese microbiota has been suggested to contribute to increased energy harvesting in obesity [3,9]. Therefore, Clostridium cluster XIVa species may actively contribute to the development of obesity. On the other hand, F. prausnitzii, a butyrate producer from Clostridium cluster IV, was associated with the non-obese group. Butyrate and other SCFAs are known to inhibit inflammation by limiting immune cell migration, adhesion, and cytokine production [52]. In line with this, F. prausnitzii has been found to stimulate anti-inflammatory responses in mice [53], suggesting that this microbe belonging to the Firmicutes may protect non-obese subjects from inflammation. Indeed, F. prausnitzii abundance was previously shown to be negatively correlated with inflammatory markers like CRP and IL-6 in obese non-diabetic subjects [54]. Next to energy harvest and inflammation, microbiota also affect the energy consuming process of enterocyte turnover, which we recently found to be increased in obese subjects with diabetes type 2 [55]. Further studies investigating the specific effects of obesity-associated bacteria in the human context are warranted. The potential causes of the microbiota composition changes in obesity remain speculative, though evidence for the involvement of dietary factors and antimicrobial peptides secreted by Paneth cells has been reported [56,57].

In conclusion, for the first time we here provide evidence for a link between the intestinal microbiota and intestinal and systemic inflammation in obese subjects. Since there was no relation between the obese microbiota and intestinal permeability, our data suggest that microbiota-derived factors may directly promote inflammation in obesity.

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Chapter 4

Paneth cell antimicrobial protein levels are reduced in obesity and correlate with activation of the unfolded protein response

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ABSTRACT

The intestinal microbiota is increasingly acknowledged to play a crucial role in the development of obesity. A shift in intestinal microbiota composition favouring the presence of Firmicutes over Bacteroidetes has been observed in obese subjects. A similar shift has been reported in mice with deficiency of Paneth cell α-defensins. We aimed at investigating changes in Paneth cell antimicrobial levels in the gut of obese subjects. Next, we studied activation of the unfolded protein response (UPR) as a possible mechanism involved in altered Paneth cell function.

Paneth cell numbers were counted in jejunal sections of 15 severely obese (BMI>35kg/m^2) and 15 normal weight subjects. Expression of Paneth cell antimicrobials human α-defensin 5 (HD5) and lysozyme were investigated using immunohistochemistry, qPCR, and western blot. Activation of the UPR was assessed with western blot.

Severely obese subjects showed decreased protein levels of both HD5 and lysozyme, while Paneth cell numbers were unchanged. Lysozyme protein levels correlated inversely with BMI. Increased expression of HD5 and lysozyme transcripts in the intestine of obese subjects prompted us to investigate a possible translational block caused by UPR activation. Binding protein (BiP) and activating transcription factor 4 (ATF4) levels were increased, confirming activation of the UPR in the gut of obese subjects. Furthermore, protein levels of both proteins correlated with BMI. Involvement of the UPR in the lowered antimicrobial protein levels in obese subjects was strongly suggested by a negative correlation between BiP levels and lysozyme levels. Additionally, indications of ER stress were apparent in Paneth cells of obese subjects.

Our findings provide the first evidence for altered Paneth cell function in obesity, which may have important implications for the obesity-associated shift in microbiota composition. In addition, we show activation of the UPR in the intestine of obese subjects which may underlie the observed Paneth cell compromise.

4.1 INTRODUCTION

The intestine is colonised by a complex microbiota, which plays an important role in physiological and homeostatic functions. A close relationship between the intestinal microbiota and obesity has been revealed. First, it has been shown that germ-free animals are protected from diet-induced obesity [1,2]. Second, colonisation of the gut of lean germ-free mice with the intestinal microbiota of obese mice results in significant weight gain of these mice, while this was not observed when germ-free lean mice were inoculated with the intestinal flora of lean donor mice [3]. Underlying mechanisms explaining these observations were suggested to be increased bacteria-mediated energy harvest from the diet and the ability of bacteria to influence host lipid metabolism, thereby affecting energy expenditure and storage [1,2]. Finally, obesity-related changes in the composition of the microbiota have been described in both man and animals [3-5]. A large shift in microbiota favouring the presence of Firmicutes over Bacteroidetes was reported in obesity [3-5], which remarkably reverted to normal after weight loss [5]. Strikingly, a similar shift in microbiota composition was observed in mice deficient in the active form of the antimicrobial peptides α-defensins [6]. On the contrary, mice overexpressing α-defensins showed a higher percentage of Bacteroidetes and a lower percentage of Firmicutes [7,8]. In the gut, α-defensins are produced by Paneth cells. These cells residing in the crypts of the small intestine produce large amounts of antimicrobials including human α- defensin 5 (HD5) and lysozyme, which makes them key players in controlling the microbiota composition of both the small and large intestine [9-11].

In view of the major impact of Paneth cell antimicrobials on intestinal microbiota composition and the striking similarity between microbiota alterations in mice defective in active α-defensins and obesity, we hypothesized that compromised Paneth cell function underlies the microbial shift described in obese subjects. We report a decrease in protein expression of both HD5 and lysozyme in the jejunum of obese subjects, as compared to normal weight controls. Moreover, activation of the unfolded protein response (UPR) in the small intestine and indications of ER stress in Paneth cells are observed in obese subjects, which we propose to be a putative mechanism underlying Paneth cell compromise. Our study for the first time suggests a host factor to be involved in the obesity-associated intestinal microbial shift.

4.2 MATERIALS AND METHODS

ETHICS

The study was approved by the medical ethical committee of Maastricht University Medical Centre and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written consent of all patients was obtained.

STUDY POPULATION AND TISSUE COLLECTION

A sequentially included cohort of 15 severely obese subjects (BMI>35kg/m^2) undergoing gastric bypass surgery was studied retrospectively. Exclusion criteria for this study were acute or chronic inflammatory diseases, degenerative diseases, >10g alcohol consumption per day, or use of anti-inflammatory drugs.
During surgery, jejunal biopsies were obtained and divided into two pieces. Control jejunal biopsies were obtained from 15 consecutive patients undergoing pancreato-duodenectomy. During this procedure a variable length of jejunum is routinely resected in continuity with the head of the pancreas and duodenum.
One piece of tissue was immediately formalin-fixed and embedded in paraffin, another piece was snap-frozen in liquid nitrogen. Snap frozen samples were stored at -80°C until further processing for western blot or qPCR. Patient characteristics are summarized in Table 1.

CHARACTERISTIC	OBESE (N=15)	NORMAL WEIGHT (N=15)
Male:female	2:13	9:6*
Age (years)	43.3 ± 2.7	58.9 ± 3.3**
BMI (kg/m^2)	43.7 ± 1.6	24.6 ± 1.5**
*p<0.05, **p<0.01		

Table 1. Patient characteristics.

ANTIBODIES AND REAGENTS

The following primary antibodies were used: rabbit anti-HD5 (kindly provided by Dr. T. Ganz, Department of Medicine, David Geffen School of Medicine, LA), rabbit anti-human lysozyme (Dakocytomation, Glostrup, Denmark), mouse anti-human BiP/Grp78 (BD Biosciences, San Jose, CA), rabbit anti-human ATF4, rabbit anti-human GADD34 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-β-actin (Sigma, St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit, streptavidin-biotin HRP complex were from Dakocytomation and HRP-conjugated secondary antibody rat anti-mouse was from Jackson (West-Grove, PA). All other reagents were purchased from Sigma, unless mentioned otherwise.

QUANTITATIVE POLYMERASE CHAIN REACTION

cDNA was amplified using a three-step program (40 cycles of 10s at 95°C, 20s at 60°C and 20s at 70°C) performed with the MyiQ System (Bio-rad). Specificity of amplification was verified by melt curve analysis. The primers used for the qPCR assays are listed in Table 2.

GENE	FORWARD	REVERSE
Lysozyme	5'-GATAACATCGCTGATGCTGTAGCT-3'	5-'CATGCCACCCATGCTCTAATG-3'
HD5	5'-ACCTCAGGTTCTCAGGCAAGAG-3'	5'-GGGACTCACGGGTAGCACAA-3'
Cyclophilin A	5'-CTCGAATAAGTTTGACTTGTGTTT-3'	5'-CTAGGCATGGGAGGGAACA-3'
β2-microglobulin	5'-TCCATCCGACATTGAAGTTG-3'	5'-CGGCAGGCATACTCATCTT-3'

Table 2. Primers used for qPCR assays.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

4µm tissue sections were deparaffinised in xylene and rehydrated in graded ethanol to distilled water. For quantification of Paneth cell number, sections of all test and control subjects were stained with haematoxylin and eosin (H/E), and cells were counted in 40 crypts (200x) in representative microscopic fields (n=15/group). Immunohistochemistry was performed by primary blocking with endogenous

peroxidase with 0.3% H_2O_2 in methanol. After blocking non-specific antibody binding with 5% bovine serum albumin, sections were incubated with specific antibodies to human lysozyme (1µg/ml in 0.1% BSA/PBS) and HD5 (1µg/ml in 0.1% BSA/PBS) at room temperature for 60 min. Next, an appropriate biotin-conjugated secondary antibody (1.5µg/ml in 0.1% BSA/PBS) was applied. Binding of the primary antibody was visualized by the streptavidin-biotin HRP system and 3-amino-9-ethylcarbazole. Nuclei were stained with haematoxylin, all sections were mounted in aqueous mounting medium (Dakocytomation). To avoid inter-assay variability, immunohistochemistry was performed on all study subjects simultaneously.

WESTERN BLOTTING

For western blot, full thickness tissue samples were homogenised in lysis buffer (200mM NaCL, 10mM Tris buffer, 5mM EDTA, 10% glycerol and 1% NP-40) using a Biospec mini-beadbeater and glass beads (Bartlesville, OK). Samples were centrifuged at 18,000 g for 15 min at 4°C. Protein concentration of supernatants was determined using a BCA protein assay kit (Pierce Thermo Fisher Scientific Inc, Rockford, IL). Protein (10µg) was heated for 5 min in reducing SDS sample buffer, separated by SDS-PAGE on a 15% polyacrylamide gel and transferred onto polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA). After transfer of proteins, membranes were blocked with phosphate-buffered saline (PBS) supplemented with 5% non-fat dry milk. Next, antibody incubation was performed overnight at 4°C in PBS-0.05% Tween (PBST) supplemented with 3% non-fat dry milk, with anti-lysozyme (1µg/ml), anti-BiP (0.5µg/ml), anti-ATF4 (0.2µg/ml) or anti-GADD34 (0.8µg/ml) antibody. To confirm equal protein loading, membranes were reprobed with anti-β-actin (1µg/ml) antibodies. After washing with PBST, membranes were incubated with an appropriate HRP-conjugated secondary antibody (0.1µg/ml) for 90 minutes at room temperature. Signals were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Thermo Fisher Scientific Inc, Rockford, IL) on blue X-ray film (Fuji, SuperRX, Tokyo, Japan). Band intensity was semi-quantitatively analysed using Quantity One (Bio-rad, Hercules, CA).

QUANTITATIVE POLYMERASE CHAIN REACTION

RNA was extracted from full tickness jejunal tissue using TRI reagent according to the manufacturer's protocol. RNA samples were treated with DNAse (Promega) to ensure removal of contaminating genomic DNA. RNA concentration was determined by Nanodrop (Nanodrop, Wilmington, DE) and 750ng RNA was used as template for reverse-transcription in a cDNA synthesis reaction using iScript cDNA synthesis kit (Bio-rad). qPCR reactions were conducted in a volume of 20µl containing 10 ng of cDNA, 1x Absolute qPCR SYBR Green Fluorescein Mix (Westburg, Leusden, the Netherlands) and 150 nM of gene-specific forward and reverse primers. Sequences of primers used are provided in the Supplementary table. Gene expression levels of lysozyme, and HD5 were determined with iQ5 software (Bio-rad) using a Δ-Ct relative quantification model. The geometric mean of the expression levels of two reference genes, Cyclophilin A and β2-microglobulin was calculated and used as normalisation factor.

ELECTRON MICROSCOPY (EM)

For electron microscopic scanning, jejunal tissue (n=4 per group) was immersed in 1.5% glutaraldehyde fixative buffered in 0.067 M cacodylate at pH 7.4. Next, samples were washed in cacodylate buffer and transferred to a 1% OsO4 fixative solution buffered with PBS for subsequent immersion fixation for 1 hour at 4°C. After washing in PBS, dehydration was carried out rapidly in graded ethanol series, followed by embedding in Epon. Tissue sections were examined with a Philips CM 100 electron microscope at an accelerating voltage of 80KV.

STATISTICAL ANALYSIS

Statistical analysis was performed using Prism 5.02 for Windows (GraphPad Software Inc. San Diego, CA). A two-tailed Mann-Whitney U test was used to detect differences between groups. Data are represented as mean ± standard error of the mean (SEM). Spearman's correlation coefficient was determined to study associations between variables. Differences were considered statistically significant at $p<0.05$. Multivariate linear regression analysis was used to test for confounding in the data analysis.

4.3 RESULTS

REDUCED LEVELS OF PANETH CELL ANTIMICROBIALS IN OBESE SUBJECTS

To study potential Paneth cell alterations in obesity, we first investigated HD5 expression in the jejunum of obese and normal weight control subjects. Immunohistochemical staining for HD5 showed intense staining in Paneth cell granules of normal weight subjects, whereas staining for this antimicrobial protein was substantially reduced in the jejunum of obese individuals (Figure 1a). Next, to investigate whether the reduction in HD5 expression in obese subjects was a result of generalised Paneth cell compromise, we studied lysozyme levels, another highly expressed antimicrobial protein in Paneth cells. Similar to HD5, lysozyme was abundantly present in granules of Paneth cells of normal weight control subjects while staining was strongly diminished in obesity (Figure 1b).

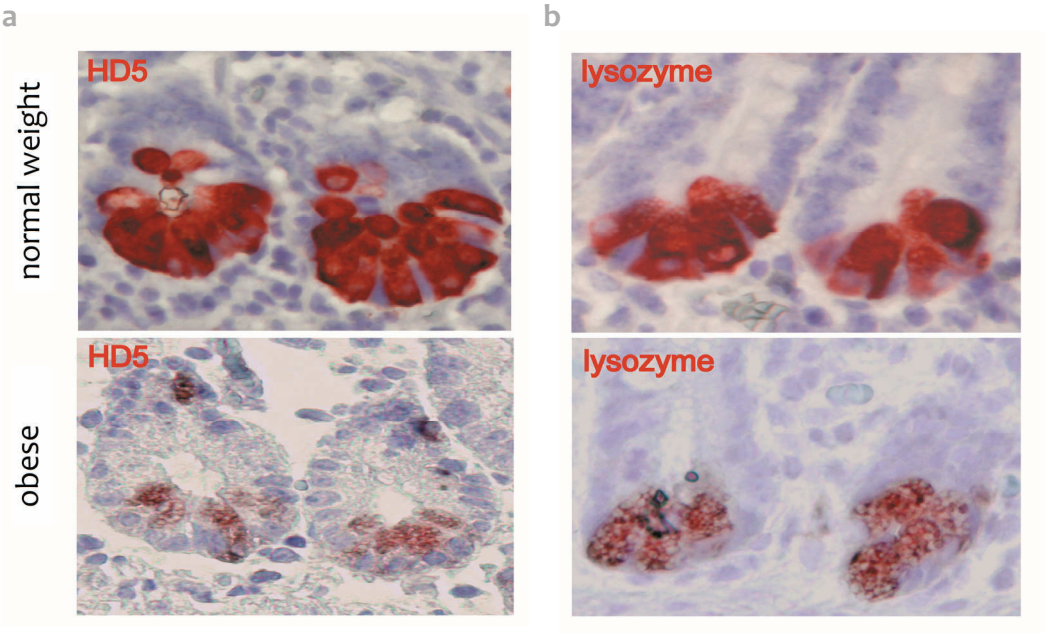

Figure 1. HD5 and lysozyme levels are decreased in Paneth cells of obese subjects.

a) Immunohistochemistry for HD5 in jejunum of normal weight (upper panel) versus obese (lower panel) subjects showed strongly reduced staining in Paneth cells of obese subjects. **b)** Immunohistochemistry for lysozyme in jejunum of normal weight (upper panel) versus obese subjects (lower panel) showed a markedly reduced expression in Paneth cells from obese subjects. This histology is representative for all tissue samples studied (magnification 200x).

Semi-quantitative analysis by western blot confirmed a reduced amount of lysozyme in the small intestine of obese patients compared to intestinal samples from normal weight subjects (Figure 1c; $p<0.05$). A relationship between obesity and antimicrobial protein expression in the gut of obese subjects was evidenced by an inverse correlation between BMI and lysozyme protein levels (Figure 1d; $r_s= -0.42$, $p<0.05$).

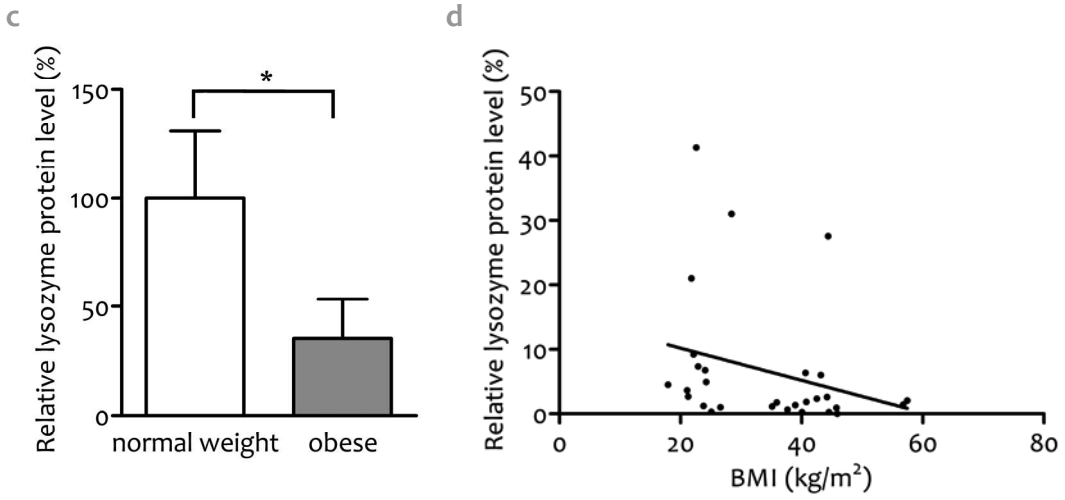

Figure 1c) Western blot analysis revealed a significant decrease in jejunal levels of lysozyme in obese subjects compared to normal weight subjects confirming staining results ($p<0.05$). β-Actin was used to confirm equal protein loading. **d)** Jejunal lysozyme content was inversely correlated with BMI ($r_s=-0.42$, $p<0.05$).

Quantification of Paneth cells revealed equal numbers in the jejunum of both study groups, excluding the possibility that the observed decrease in antimicrobial expression in Paneth cells was caused by reduced cell numbers (Figure 2).
Taken together, these data indicate that antimicrobial protein levels are reduced in Paneth cells of obese subjects.

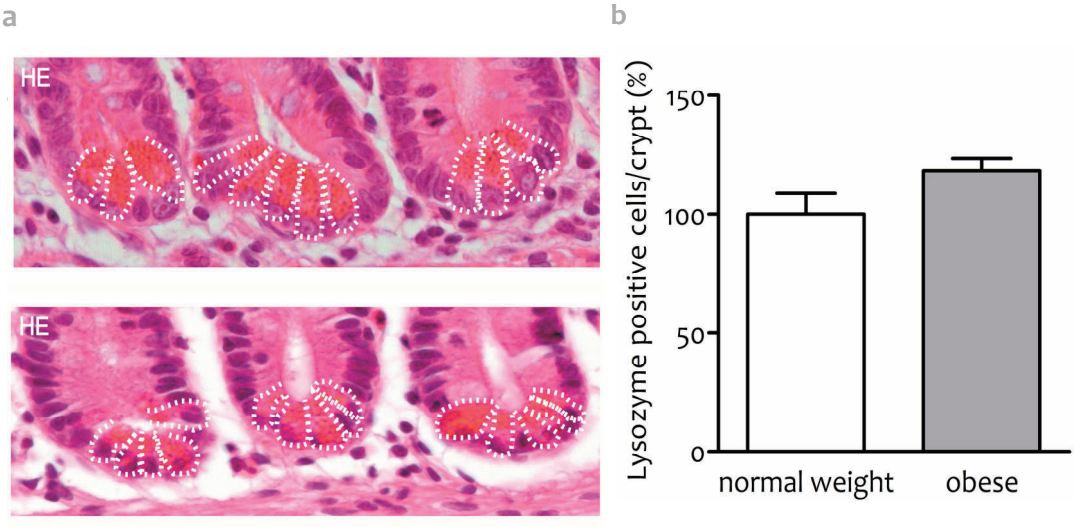

Figure 2. Equal Paneth cell numbers in the jejunum of obese and normal weight subjects.
a) H/E staining demonstrated equal numbers of Paneth cells in the jejunal crypts of normal weight (upper panel) and obese subjects (lower panel). The histology shown is representative for all tissue samples studied (magnification 200x). **b)** Quantification of the number of Paneth cells present in the jejunum of obese subjects showed no difference in Paneth cell count compared to control tissues.

ENHANCED EXPRESSION OF PANETH CELL ANTIMICROBIAL GENES IN OBESITY

To investigate whether the reduced antimicrobial protein levels in obese subjects were due to reduced gene expression, we quantified mRNA expression of HD5 and lysozyme. Surprisingly, qPCR data showed a 2.6 fold increased expression of HD5 (Figure 3a; $p<0.01$) and a 1.6 fold increased expression of lysozyme (Figure 3b; $p=0.15$) in jejunal tissue of obese subjects compared to normal weight controls.
Since the discrepancy seen in protein expression and mRNA expression of HD5 and lysozyme could point towards a translational arrest due to ER stress, we next assessed activation of the UPR.

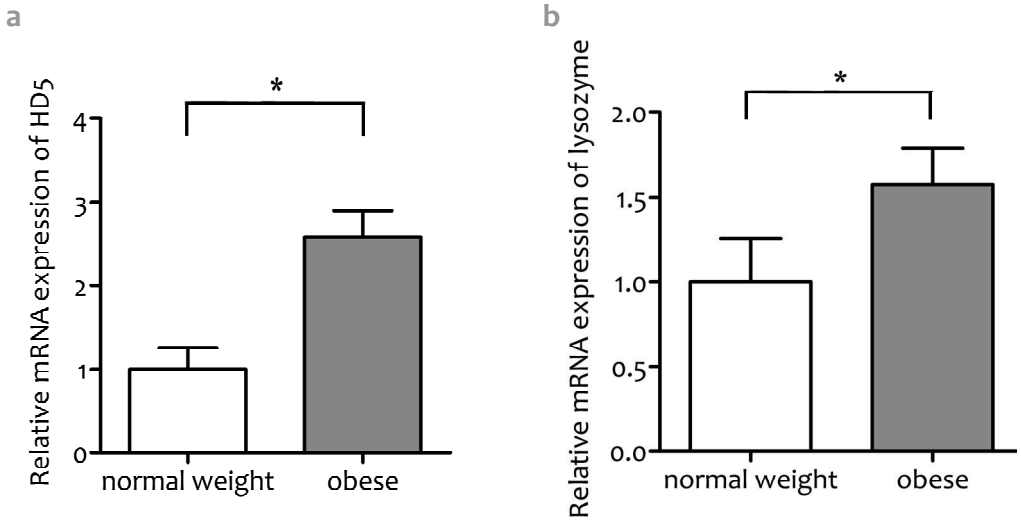

Figure 3. Increased HD5 and lysozyme gene expression in jejunum of obese subjects. **a)** qPCR analysis showed a 2.6 fold increase in HD5 mRNA ($p<0.01$)., and a 1.6 fold increase in gene expression of lysozyme ($p=0.15$, panel **b**).

ACTIVATION OF THE UNFOLDED PROTEIN RESPONSE AND INDICATIONS OF ER STRESS IN SMALL INTESTINE OF OBESE SUBJECTS

ER stress arises from circumstances resulting in accumulation of misfolded or unfolded proteins in the ER. Upon ER stress, the UPR is activated, which is aimed at restoring ER homeostasis. To investigate the putative involvement of ER stress in the reduced antimicrobial protein expression by Paneth cells in obesity, activation of the protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathway was assessed. This branch of the UPR is responsible for a translational arrest which reduces protein load to the ER and thus alleviates ER stress [12]. We first studied binding protein (BiP), an important player in the initiation and maintenance of the UPR [13]. BiP levels were significantly increased in jejunal samples of obese patients compared to normal weight subjects, signifying activation of the UPR (Figure 4a and b; $p<0.05$). A correlation between BMI and BiP levels was found and further indicated a relationship between obesity and the activation of the UPR in the gut (Figure 4d; $r_s=0.45$, $p<0.05$).

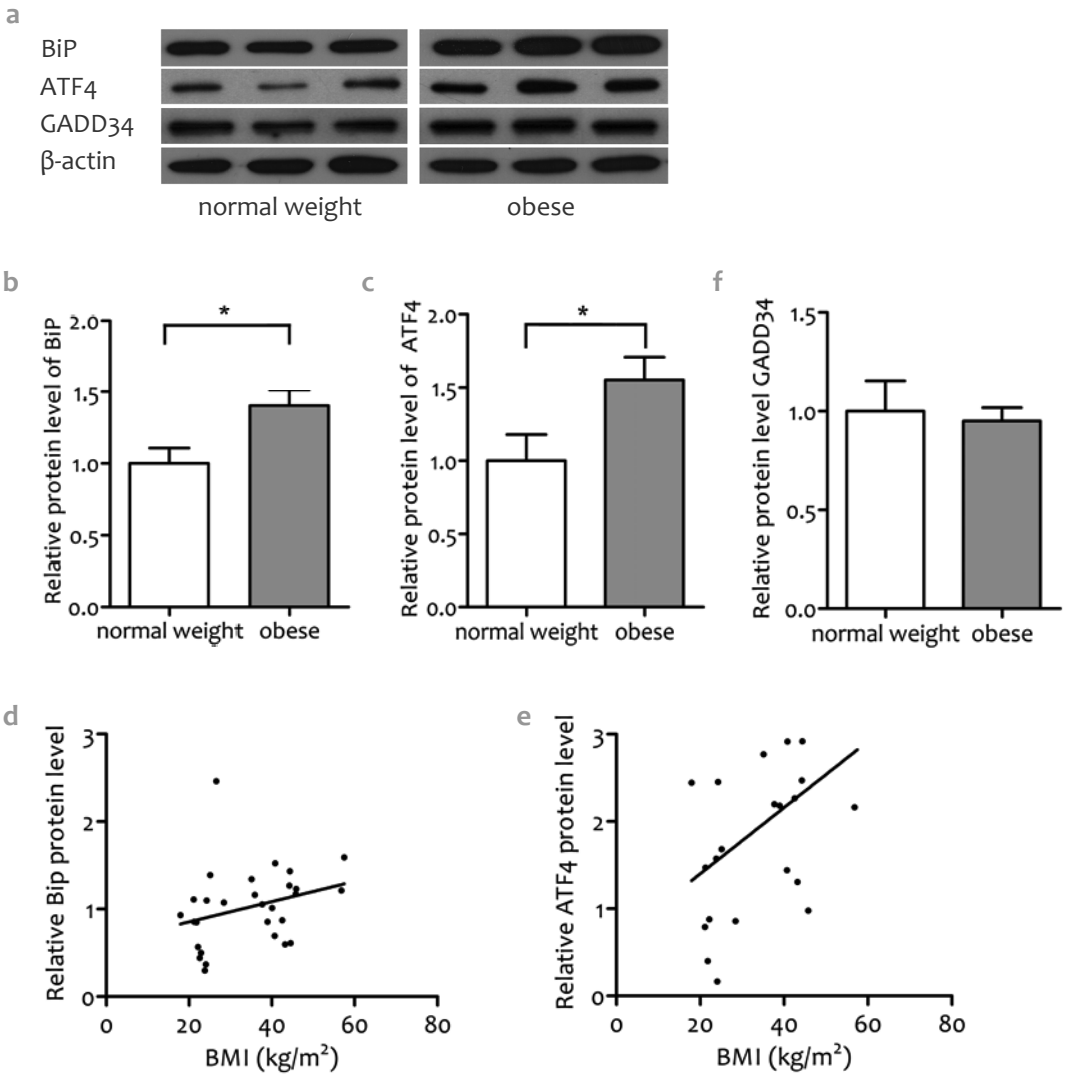

Figure 4. The UPR is activated in jejunum of obese subjects.
a) Western blot analysis showed increased band density for BiP and ATF4 in obese samples (representative bands of 3 subjects per group). Quantification of western blots demonstrated a significant ($p<0.05$) upregulation of both BiP **(b)** and ATF4 **(c).** Both BiP **(d)** and ATF4 **(e)** concentrations correlated with BMI ($r_s=0.45$, $p<0.05$ and $r_s=0.41$, $p<0.05$ respectively). No difference in GADD34 expression was observed **(f).**

In addition, protein levels of activating transcription factor 4 (ATF4), which induces transcription of downstream mediators in the PERK pathway, were also significantly increased in obese subjects (Figure 4a and c; $p<0.05$). ATF4 protein levels correlated with BMI as well, providing additional evidence for a relationship between obesity and UPR activation (Figure 4e; $r_s=0.41$, $p<0.05$). Next, we analyzed protein expression of growth arrest and DNA damage-inducible protein 34 (GADD34), a downstream target of ATF4 that provides a negative feedback loop to the PERK pathway to reinitiate protein translation. GADD34 levels were similar in both study groups (Figure 4a and f).

ER compromise was studied using EM to assess the contribution of Paneth cells to the results obtained by western blot on UPR activation. Indeed, Paneth cells of obese subjects showed an enlarged ER with vacuoles (Figure 5b), indicative of ER stress, whereas a normally structured ER was found in Paneth cells of normal weight subjects (Figure 5a).

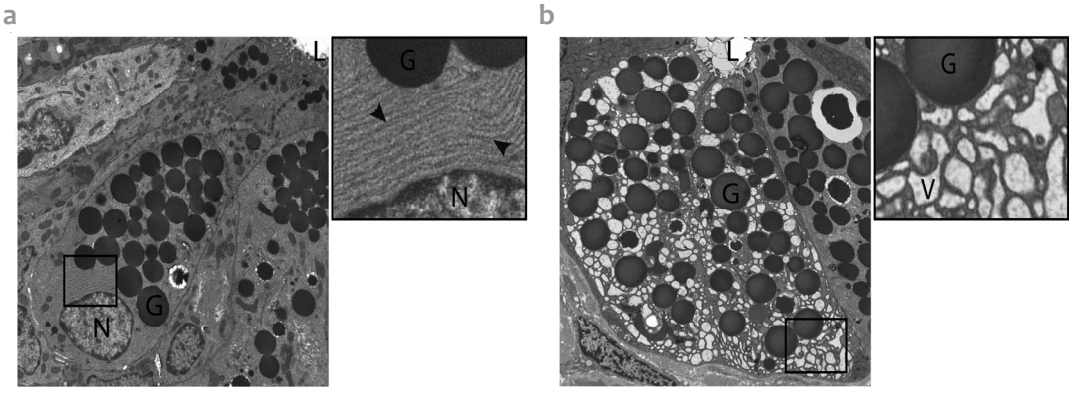

Figure 5. Expansion of the ER indicates ER stress in Paneth cells of obese subjects.
a) Representative EM images of Paneth cells in jejunum of normal weight subjects reveal a normally structured ER (dark arrowheads), while the ER in Paneth cells of obese subjects **(b)** displays vacuoles (V) and is expanded (open arrowheads) indicative of ER stress. L= lumen, N= nucleus, G= granule.

Importantly, involvement of the UPR in the lowered antimicrobial protein levels in obese subjects was strongly suggested by a negative correlation between BiP levels and lysozyme levels (Figure 6; $r_s=-0.39$, $p<0.05$).

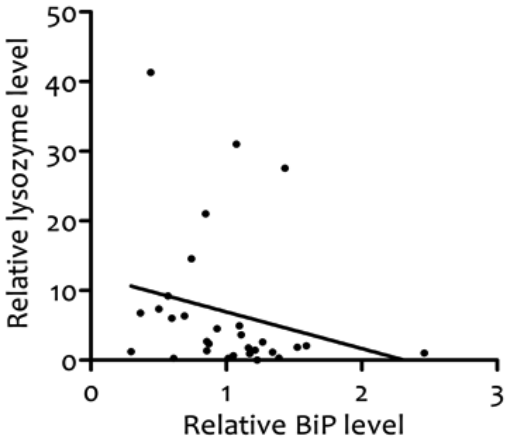

Figure 6. Activation of the UPR is associated with a reduction in lysozyme expression in the gut of obese subjects.

The differences in age and gender between the study groups do not confound the results on UPR activation and Paneth cell products, as assessed by multivariate analysis ($p>0.05$ for all variables tested).

All in all, these results suggest that activation of the UPR contributes to the diminished expression of antimicrobial proteins in the jejunum of obese individuals.

4.4 DISCUSSION

Over the past decade, the influence of Paneth cells on controlling intestinal microbiota composition and limiting bacterial translocation has become increasingly clear [6,10,14]. Intestinal microbiota on their part have been shown to play an important role in obesity. In this study, we provide the first evidence for altered Paneth cell properties in human obesity. We show decreased levels of the crucial Paneth cell antimicrobials HD5 and lysozyme in jejunum of obese subjects compared to normal weight subjects. In addition, we provide new insight into the mechanisms that could account for this phenomenon showing activation of the UPR in the gut of obese subjects for the first time.

Changes in α-defensin levels, as also described in Crohn's disease [8], have been shown to influence the composition of the intestinal microbiota in mice [6,8]. A reduction in α-defensin levels has been shown to result in a higher percentage of Firmicutes and a lower percentage of Bacteroidetes in the small intestine [6]. Moreover, Paneth cell-produced antimicrobial proteins were proven to act in the

colon [11]. Interestingly, a similar Firmicutes-Bacteroidetes shift has been observed in the colon of obese subjects [5]. The decreased HD5 expression in the gut of obese subjects that we observed could therefore very well explain the reported shift in bacterial composition. However, the composition of the intestinal microbiota in our study groups could not be investigated since the clinical study from which the tissue samples were derived did not include faecal samples and remains subject for future studies. Alternatively, it should be noted that changes in expression of antimicrobial peptides could occur secondary to alterations in the composition of the intestinal microbiota.

Since changes in gut microbiota have been implicated in obesity-associated increase in intestinal permeability and metabolic endotoxemia, we hypothesize that disturbed Paneth cell function may underlie these phenomena [15,16]. Furthermore, increased intestinal permeability in obesity has been suggested to lead to enterogenous endotoxemia contributing to non-alcoholic steatohepatitis [17-20].

In addition to the change in microbiota composition, a generalised Paneth cell malfunction might contribute to these obesity-related complications in other ways. For example, Paneth cells prevent bacterial translocation in the healthy intestine and act as a second line of defence in limiting bacterial translocation in situations of physical intestinal barrier loss [10,21]. An overall reduction in antimicrobial protein production by Paneth cells might therefore play an important role in facilitating bacterial translocation, as has been suggested to occur in obesity [15,22,23], thereby adding to the role of an increased intestinal permeability.

The diminished antimicrobial protein expression and increased mRNA levels of their corresponding genes could be explained by a Paneth cell-depleting hypersecretory response to obesity. However this seems improbable considering that during a chronic, steady state both enhanced mRNA and protein levels are expected. Therefore, we hypothesized the discordance between antimicrobial protein expression and mRNA expression in Paneth cells of obese subjects to be a consequence of a translational block caused by ER stress.

Paneth cells have been shown to be susceptible to ER stress due to their highly secretory nature [24]. In addition, excessive nutrient intake, increased need for protein synthesis, and excess lipid accumulation associated with obesity is known to be a chronic stimulus in causing ER stress [25-27]. The latter fact could, via its

influence on intestinal microbiota, result in a vicious circle. In this regard, the UPR has been shown to be activated in both liver and adipose tissue of obese subjects. Alternatively, it could be envisaged that the altered microbiota are causative for ER stress resulting in reduced Paneth cell antimicrobial expression.

Here, we provide the first evidence for UPR activation in the gut of obese subjects. We show that obese subjects display increased protein levels of BiP, an important initiator of the UPR pathway, and of ATF4, a player in the PERK axis of the UPR, which is required for induction of a translational block resulting in overall reduced protein synthesis [12]. It should be noted that goblet cells might contribute to these results since they are also susceptible to ER stress, although to a lesser extent than Paneth cells [24]. In addition, using EM we observed indications of ER stress in Paneth cells of obese subjects. Although we show clear activation of the UPR in the gut of obese subjects, downstream elements in the UPR pathway, including CHOP (data not shown) and GADD34, were unaltered.

In support of our findings, it has been shown that adaptation to chronic ER stress induces persistent expression of BiP and other proteins concerned with alleviating protein folding stress, whereas expression of both pro-apoptotic CHOP and GADD34 are unchanged [28]. These findings represent a fascinating aspect of the UPR; the ability to either facilitate adaptation to stress or to induce apoptosis, depending upon the nature and severity of the stressor. In obesity, a chronic condition, cells chronically exposed to ER stress must survive and adapt. Recent studies have provided insight on the mechanisms by which cells translate acute or chronic stress signals from the ER into a life-or-death response [28,29]. The involvement of such mechanisms in the gut of obese subjects is suggested by our findings that upstream players of the PERK pathway, constituting an adaptive response concerned with alleviating protein folding stress, are upregulated while pro-apoptotic CHOP and GADD34 are unchanged.

Our results, indicating a relationship between activation of the UPR in the small intestine and altered Paneth cell function, are supported by Kaser et al. who showed diminished antimicrobial protein secretion by Paneth cells in a mouse model displaying ER stress [24].

In conclusion, our findings provide important new insight into the involvement of Paneth cells in obesity. We identified these cells as a conceivable host factor responsible for the shift in microbiota composition accountable for many obesity-associated disorders. In addition, we show for the first time activation of the UPR in the intestine of obese subjects and indications of ER stress in Paneth cells which may cause the observed Paneth cell compromise. Further studies on the role of altered Paneth cell function in relation to the obesity-associated intestinal microbiota shift are warranted.

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Chapter 5

Small intestinal alterations in severely obese hyperglycemic subjects

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is associated with small intestinal hyperplasia and hypertrophy in rodents. Moreover, the small intestine is increasingly acknowledged to play a role in the pathophysiology of T2DM. The objective of this study was therefore to investigate the relation between plasma markers of small intestinal function and chronic hyperglycemia in man.

We conducted a cross-sectional observational study of 40 severely obese subjects with chronic hyperglycemia and 30 severely obese subjects without chronic hyperglycemia who were indicated for bariatric surgery. We assessed plasma levels of citrulline, representing small intestinal enterocyte mass, intestinal fatty acid binding protein (I-FABP), a marker of enterocyte loss, and glucagon-like peptide-2 (GLP-2), an intestinotrophic factor, and related them to glycated hemoglobin (HbA_{1c}) levels.

Plasma citrulline and I-FABP levels were both significantly elevated in subjects with chronic hyperglycemia (HbA_{1c}>6.0%) compared with subjects with a normal HbA_{1c} (≤6.0%) (for citrulline 35±2.1μM vs. 26±1.4μM, p=0.001; I-FABP 140±22pg/ml vs. 69±14pg/ml, p=0.001). Moreover, plasma citrulline and I-FABP levels correlated with HbA_{1c} levels (for citrulline r_s=0.30, p=0.02; I-FABP r_s=0.33, p=0.005). The I-FABP:citrulline ratio was higher in subjects with an elevated HbA_{1c} (4.0 vs. 3.1, p=0.03). Plasma GLP-2 levels were not related to citrulline or I-FABP levels (r_s=0.06, p=0.67; r_s=0.08, p=0.54, respectively).

In conclusion, chronically elevated glucose levels in obese individuals are associated with increased small intestinal enterocyte mass and increased enterocyte loss. These findings argue for further exploration of the role of the intestine in the pathophysiology of T2DM.

5.1 INTRODUCTION

Type 2 diabetes mellitus (T2DM) affects over 285 million people worldwide, a number rapidly increasing in this era of obesity [1]. Traditionally, studies on the pathogenesis of T2DM have mainly focused on insulin sensitivity of liver, muscle, and adipose tissue. However, there is increasing evidence for an important role of the small intestine in the control of glucose homeostasis and in the pathogenesis of T2DM. For example, the release of various small intestinal peptides that control glucose homeostasis, such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1, is disturbed in subjects with T2DM [2]. In addition, gluconeogenesis in the small intestine was recently shown to regulate hepatic glucose metabolism and to contribute to the rapid beneficial effects of Roux-en-Y gastric bypass surgery on T2DM in mice [3]. Importantly, bypassing the proximal intestine is associated with a rapid resolution of T2DM in man [4], supporting an important role for intestinal factors in the regulation of glucose homeostasis.

Interestingly, animals with T2DM display alterations of the small intestine such as generalized small intestinal hyperplasia and mucosal hypertrophy [5]. More specifically, elevated glycated hemoglobin (HbA_{1c}), an indicator of chronic hyperglycemia, was found to coincide with longer intestinal villi and increased intestinal proliferation in rats with T2DM. Besides morphological changes, functional alterations such as increased activity of disaccharidases and enhanced nutrient absorption were reported. Importantly, the combination of small intestinal hyperplasia and increased disaccharidase activity may contribute to the postprandial hyperglycemia that characterizes T2DM.

To investigate whether chronic hyperglycemia in man is associated with similar intestinal alterations as previously described in animal models, we determined plasma levels of citrulline, intestinal fatty acid-binding protein (I-FABP), and glucagon-like peptide-2 (GLP-2), or in other words parameters indicating intestinal mass, enterocyte loss, and enterocyte proliferation [6-8], in a population of severely obese patients with and without chronic hyperglycemia.

Our results indicate that chronic hyperglycemia is associated with a higher intestinal mass and increased enterocyte loss, supporting a role for the intestine in the pathophysiology of T2DM in man.

5.2 MATERIALS AND METHODS

SUBJECTS

From June 2006 to November 2008, 70 severely obese subjects indicated for bariatric surgery were sequentially included at the Department of General Surgery of the Maastricht University Medical Center. Table 1 summarizes the patient characteristics.

	$HBA_{1C} \leq 6\%$ (SEM)	$HBA_{1C} > 6\%$ (SEM)	P-VALUE
Number of patients	30	40	
Sex (M : F)	8 : 22	12 : 28	p=0.76
Age (years)	42 (1.4)	48 (1.3)	p<0.01
BMI (kg/m^2)	46.2 (1.7)	45.7 (1.5)	p=0.72
HbA1c (%)	5.7 (0.1)	7.3 (0.2)	p<0.01
Citrulline (µmol/L)	26 (1.4)	35 (2.1)	p<0.01
I-FABP (pg/mL)	69 (14)	140 (22)	p<0.01
I-FABP:Citrulline	3.1 (0.7)	4.0 (0.6)	p=0.03
GLP-2 (ng/mL)	14 (1.6)	18 (2.1)	p=0.29

Table 1. Patient characteristics.

Patients with type 1 diabetes, other acute or chronic inflammatory diseases (e.g. M. Crohn, colitis, hepatitis, auto-immune diseases), and patients using anti-inflammatory drugs and/or reported alcohol consumption (more than 10g per day) were excluded from the study. All severely obese subjects were screened for diabetes and used appropriate medication for their diabetic status. Eleven subjects used metformin, five used insulin, eleven patients were treated for hyperlipidemia and 31 subjects used antihypertensive medication. Subjects did not follow a specific diet and were not under dietary counseling at the time of sampling. This study was approved by the Medical Ethical Board of the Maastricht University Medical Center in line with the

ethical guidelines of the 1975 Declaration of Helsinki, and informed consent in writing was obtained from each subject.

BLOOD SAMPLING

Venous blood samples were obtained after a minimum of 8 hours fasting, prior to bariatric surgery. All samples were collected in pre-chilled EDTA tubes and centrifuged at 4°C for 10 min at 2000g. The plasma was centrifuged again for 10 min at 3500g and stored in aliquots at -80°C until analysis.

BIOPSY SAMPLING AND QUANTITATIVE PCR

Small intestinal biopsies were obtained from all patients undergoing gastric bypass surgery (n=2). Biopsies were immediately snap frozen. Total RNA was isolated from 50mg tissue by homogenization in Tri reagent (Sigma) according to the manufacturer's instructions. 750ng of RNA were converted to cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Quantitative PCR was performed with the ABsolute SYBR Green Master Mix (ABgene, Leusden, the Netherlands) and the iQ5 iCycler (BioRad) using the primers listed in Table 2.

GENE	FORWARD	REVERSE
I-FABP	5'-TAGCAGACGGAACTGAACTC-3'	5'-CATAAGTCTGGACTAGTTCATCAC-3'
β2-microglobulin	5'-TCCATCCGACATTGAAGTTG-3	5'-CGGCAGGCATACTCATCTT-3'
Relative I-FABP expression was assessed in duplicate by the dCt method after normalization for β2-microglobulin expression.		

Table 2. Primers used for qPCR.

MEASUREMENTS

HbA_{1c} levels were measured at the Department of Clinical Chemistry according to the protocol of the University Medical Center. Citrulline levels were measured as previously described [9].

Plasma I-FABP levels were determined with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Hycult Biotech, Uden, the Netherlands). Samples were analyzed in the same run and the inter-assay and intra-assay coefficient of variation were less than 10%. The detection limit was 40 pg/ml. Plasma GLP-2 levels were determined with an ELISA according to the manufacturer's instructions (Biovendor, Modrice, Czech Republic). Samples were analyzed in two runs, the intra- and inter-assay coefficient variation was less than 15%.

STATISTICAL ANALYSIS

Statistical analysis was performed using Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Data are presented as mean±standard error of the mean. Correlations were calculated using Spearman's rank correlation coefficient. Differences between groups were analyzed by the Mann-Whitney test or the Chi-square test. A p-value <0.05 was considered statistically significant.

5.3 RESULTS

INCREASED PLASMA CITRULLINE LEVELS IN SUBJECTS WITH ELEVATED HBA_{1C}

The functional small intestinal mass has previously been shown to be accurately reflected by plasma levels of citrulline, an amino acid which is not incorporated into proteins, and produced by differentiated small intestinal enterocytes from glutamine [6]. Therefore, to study the relation between small intestinal mass and hyperglycemia, we first assessed citrulline plasma levels of 30 severely obese subjects with HbA_{1c}≤6.0% (the upper limit of normal [10], and of 40 severely obese subjects with HbA_{1c}>6.0%. In the total population, plasma citrulline levels ranged from 13 to 78µM, with a mean of 31±1.4µM, and a median of 30µM. Interestingly, plasma citrulline levels were significantly higher in patients with an elevated HbA_{1c} (HbA_{1c}>6.0%: 35±2.1µM vs. HbA_{1c}≤6.0%: 26±1.4µM, p=0.001, Figure 1a). Furthermore, citrulline levels correlated with HbA_{1c} levels (r_s=0.30, p=0.02, Figure 1b). These data indicate that severely obese patients with chronic hyperglycemia have an increased functional small intestinal enterocyte mass.

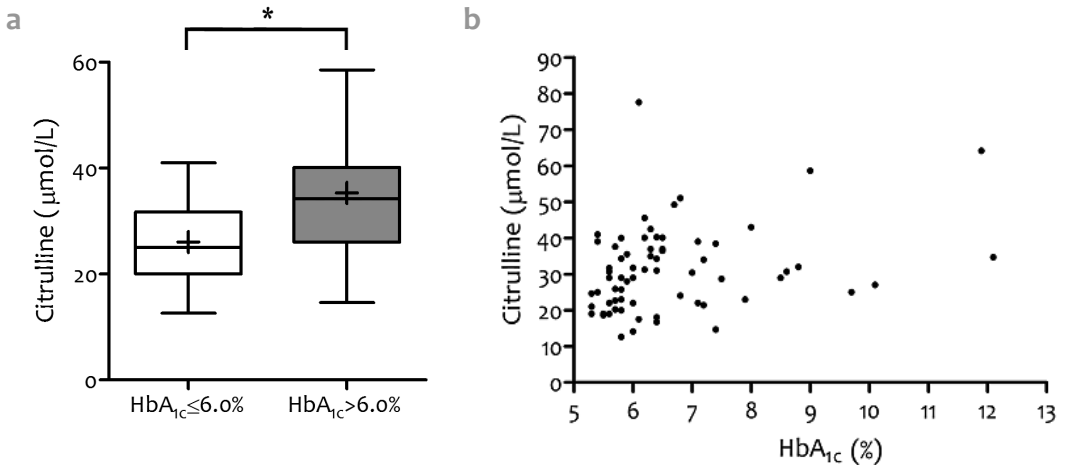

Figure 1. Enterocyte mass is increased in severely obese subjects and related to HbA_{1c}.
a) Increased levels of plasma citrulline reflecting enterocyte mass in severely obese subjects with chronic hyperglycemia. Tukey box and whiskers plot show that severely obese subjects with an elevated HbA_{1c} (>6.0%) display significantly increased plasma levels of citrulline as compared with severely obese subjects with a normal HbA_{1c} (35±2.1µM vs. 26±1.4µM, p=0.001). The horizontal line corresponds with the mean, whereas the outer boxes represent the 25th and the 75th percentiles. Whiskers show the non-outlier range. A value was defined as an outlier if it was more than 1.5 times the box height above or below the box. The + symbol indicates the mean value. **b)** The significant correlation between HbA_{1c} levels and plasma citrulline reflecting enterocyte mass in severely obese subjects (r_s=0.30, p=0.02).

HIGH HBA_{1C} IS ASSOCIATED WITH INCREASED PLASMA I-FABP IN SEVERELY OBESE SUBJECTS

To obtain additional evidence for the increased small bowel mass in subjects with chronic hyperglycemia, we next investigated small intestinal enterocyte turnover by measuring plasma I-FABP levels. I-FABP is a gut-associated cytosolic carrier protein involved in the intracellular buffering and transport of fatty acids, which plasma levels have been shown to indicate small intestinal enterocyte loss [8,11]. Assuming steady state, circulating levels of I-FABP also represent the production of enterocytes, or in other words enterocyte turnover. Like citrulline, plasma I-FABP levels were significantly higher in severely obese patients with an elevated HbA_{1c} (HbA_{1c}>6.0%: 140±22pg/mL vs. HbA_{1c}≤6.0%: 69±14pg/mL, p=0.001, Figure 2a).

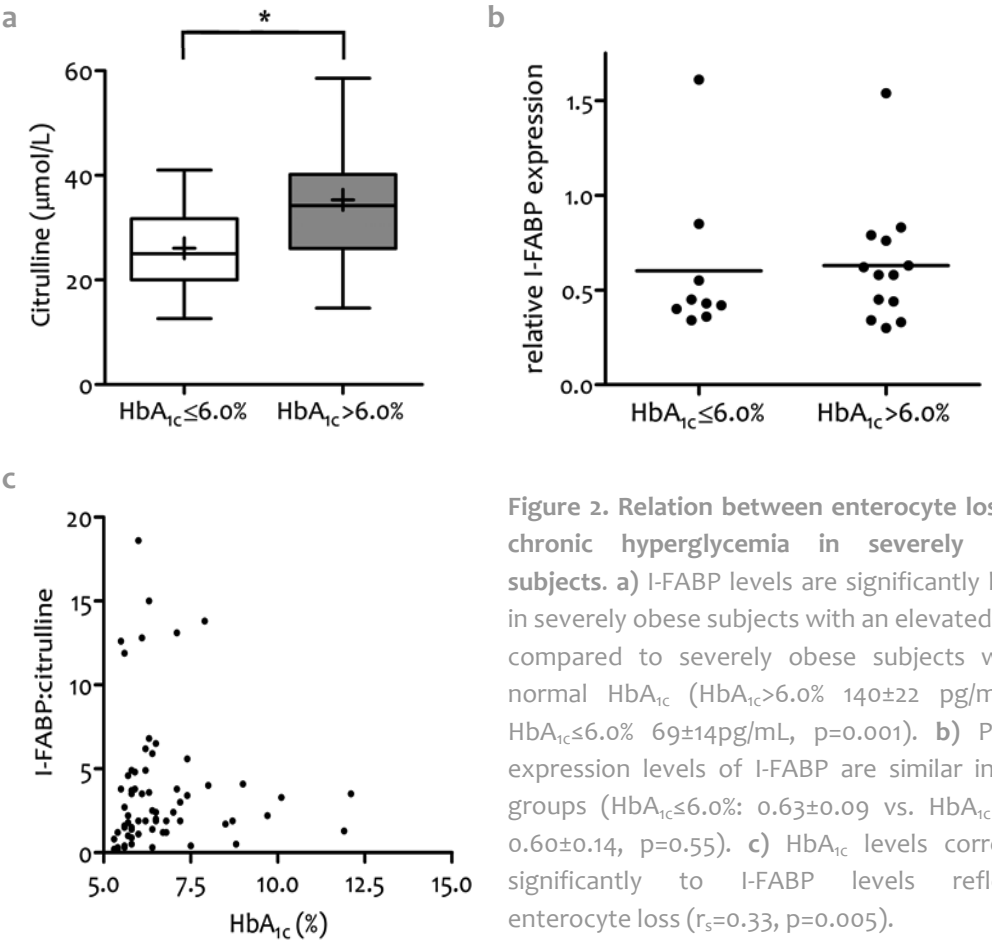

Figure 2. Relation between enterocyte loss and chronic hyperglycemia in severely obese subjects. a) I-FABP levels are significantly higher in severely obese subjects with an elevated HbA_{1c} compared to severely obese subjects with a normal HbA_{1c} (HbA_{1c}>6.0% 140±22 pg/mL vs. HbA_{1c}≤6.0% 69±14pg/mL, p=0.001). **b)** Protein expression levels of I-FABP are similar in both groups (HbA_{1c}≤6.0%: 0.63±0.09 vs. HbA_{1c}>6.0%: 0.60±0.14, p=0.55). **c)** HbA_{1c} levels correlated significantly to I-FABP levels reflecting enterocyte loss (r_s=0.33, p=0.005).

Importantly, quantitative PCR analysis showed that there was no difference in relative I-FABP expression between subjects with and without chronic hyperglycemia (0.63±0.09 vs. 0.60±0.14, p=0.55, Figure 2b). This indicates that the increased plasma I-FABP levels in subjects with elevated HbA_{1c} cannot be attributed to increased expression of I-FABP. Furthermore, I-FABP levels also significantly correlated with HbA_{1c} (r_s=0.33, p=0.005, Figure 2c). Circulating plasma I-FABP levels showed a significant positive correlation to plasma citrulline levels as well (r_s=0.26, p=0.03, data not shown). Taken together, these data suggest an increased enterocyte turnover in severely obese patients with an elevated HbA_{1c}.

PLASMA I-FABP:CITRULLINE RATIO INDICATES DISPROPORTIONALLY INCREASED ENTEROCYTE LOSS IN SUBJECTS WITH ELEVATED HBA_{1C}

To further explore the relation between enterocyte mass, enterocyte loss, and enterocyte turnover, we calculated the ratio of plasma values of I-FABP and citrulline for every subject.

The I-FABP:citrulline ratio correlated significantly with HbA_{1c} levels (r_s=0.30, p=0.04, Figure 3a). Whereas the I-FABP:citrulline ratio was 3.1 for subjects with a normal HbA_{1c}, subjects with an HbA_{1c}>6.0% displayed a significantly higher ratio of 4.0 (p=0.03, Figure 3b).

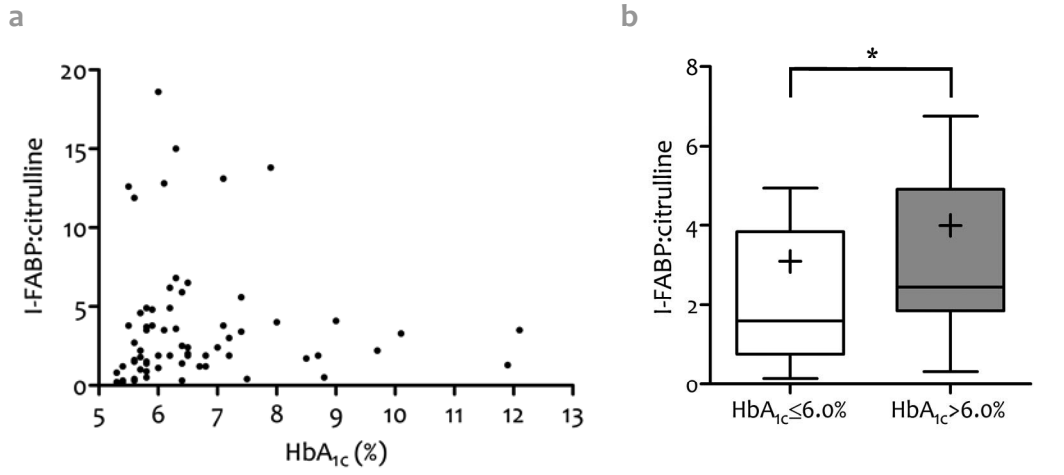

Figure 3a) I-FABP:citrulline ratio correlated significantly with HbA_{1c} levels (r_s=0.30, p=0.04), indicating that enterocyte mass is related to chronic hyperglycemia. **b)** The I-FABP:citrulline ratio is significantly higher in severely obese subjects with HbA_{1c}>6.0% as compared with severely obese subjects with a normal HbA_{1c} (p=0.03).

In short, this suggests that the increased enterocyte loss in chronically hyperglycemic subjects as indicated by I-FABP plasma levels cannot merely be explained by their relatively higher enterocyte mass. Moreover, the higher enterocyte mass despite the increased enterocyte loss suggests that severely obese subjects with an elevated HbA_{1c} have an increased enterocyte proliferation and turnover.

GLP-2 PLASMA LEVELS DO NOT CORRELATE WITH PLASMA CITRULLINE AND PLASMA I-FABP

To study a potential mechanistic factor underlying the increased enterocyte mass and turnover in subjects with T2DM, we next measured plasma levels of the incretin GLP-2. This peptide is a potent intestinal proliferative factor [7]. However, fasting GLP-2 levels did not correlate with either plasma citrulline or I-FABP levels in the severely obese population (r_s=0.06, p=0.67 and r_s=0.08, p=0.54, respectively), nor with the I-FABP/citrulline ratio (r_s=0.09, p=0.50). On the other hand, plasma GLP-2 levels did significantly correlate with HbA_{1c} levels (r_s=0.25, p<0.05), in line with the known glucose-stimulated release of GLP-2 from L cells in the small intestine [7]. These findings suggest that the increased small intestinal enterocyte mass and turnover in severely obese subjects with elevated HbA_{1c} are not mediated by GLP-2.

5.4 DISCUSSION

In the present study, we obtained the first evidence that chronic hyperglycemia in man is associated with both an increased small intestinal enterocyte mass and increased enterocyte loss. The increase in small intestinal enterocyte mass is in line with findings in rat models of T2DM which show increased intestinal proliferation and longer villi [5]. Moreover, streptozotocin-induced hyperglycemia was also found to be associated with small intestinal hyperplasia and hypertrophy in rats [12].

An increased small intestinal enterocyte mass can have important implications in the context of diabetes. First of all, it may account for the enhanced intestinal capacity to absorb glucose, as observed in T2DM [13]. This enhanced carbohydrate absorption is likely to be involved in the postprandial hyperglycemia and elevated HbA_{1c} levels that characterize T2DM. Moreover, a higher number of small intestinal enterocytes results in an increased potential for intestinal gluconeogenesis, a phenomenon that has been shown to contribute up to one-third of the total glucose production in diabetic rats [14].

The mechanisms responsible for the higher small intestinal mass in subjects with chronic hyperglycemia remain unknown, although their relatively increased I-FABP:citrulline ratio indicates that intestinal epithelial proliferation must be

augmented in these subjects. It was previously shown that hyperphagia is one of the factors promoting intestinal hyperplasia in streptozotocin-induced diabetic rats [12]. However, it seems unlikely that hyperphagia is responsible for the increased intestinal proliferation observed in this study since both study groups had a similar BMI. Nevertheless, it would be of interest to relate enterocyte mass and turnover to appetite and caloric intake in future studies.

Next to hyperphagia, enhanced secretion of the intestinotrophic peptide GLP-2 by entero-endocrine cells could be a factor driving the increased enterocyte proliferation in chronically hyperglycemic patients. However, GLP-2 levels were comparable between both study groups indicating that the observed proliferative alterations cannot be attributed to GLP-2 either. The similar GLP-2 levels may also suggest that, in contrast to the enterocytes, entero-endocrine cells are not affected in T2DM. Further studies are required to unravel potential alterations in enterocytes and entero-endocrine cells in T2DM in more detail.

Interestingly, next to the increased intestinal mass in subjects with elevated HbA_{1c} levels, both their small intestinal enterocyte loss, as reflected by plasma I-FABP levels, and their I-FABP:citrulline ratio were also higher. It was recently shown that T2DM is accompanied by significant alterations in tight junction distribution and intestinal permeability in mice, thereby promoting endotoxin-induced low grade inflammation [15,16]. Subjects with T2DM have also been shown to display elevated plasma endotoxin levels that may be related to increased intestinal permeability [17]. It is tempting to speculate that the increased enterocyte loss that we observed in subjects with chronic hyperglycemia may contribute to the impaired intestinal barrier function in T2DM. However, we cannot rule out that the elevated plasma I-FABP levels in obese subjects with T2DM reflect increased expression by enterocytes rather than increased enterocyte loss. In fact, some evidence was recently reported for an association between bodyweight and increased I-FABP expression after small bowel resection in pigs [18].

In summary, we presented evidence for both an increased functional enterocyte mass and increased enterocyte loss and turnover in severely obese subjects with elevated HbA_{1c} levels. Our findings indicate for the first time significant alterations in the pathofysiology of the intestine in human obesity-induced T2DM.

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5

Chapter 6

Novel evidence for chronic exposure to endotoxin in human non-alcoholic steatohepatitis

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ABSTRACT

Endotoxin is hypothesized to play an important role in the activation of inflammatory pathways associated with non-alcoholic steatohepatitis (NASH). However, demonstration of hepatic endotoxin exposure is challenging due to the inaccessibility of the portal circulation. Furthermore, reliable measurement of relatively low endotoxin levels in plasma of patients with liver disease and subsequent interpretation remain difficult.

In this study, we employed the EndoCab assay, which measures endogenous antibodies to the core region of endotoxin, to estimate hepatic endotoxin exposure over time.

IgG levels against endotoxin were measured in peripheral plasma obtained from 21 severely obese subjects with NASH and 9 severely obese subjects with healthy livers.

Plasma IgG levels against endotoxin were significantly elevated in subjects with NASH compared to subjects with healthy livers (48±63 vs. 10±13 GMU/ml). Moreover, these IgG levels progressively increased with NASH grade (grade 1, 29±37; grade 2, 58±51; grade 3, 84±132GMU/ml, $p<0.05$). There was no relation between plasma IgG levels and NASH stage.

Plasma IgG levels against endotoxin were found to be increased in biopsy proven human NASH, and increased with aggravated inflammation in NASH, suggesting a relationship between chronic endotoxin exposure and the severity of human NASH.

6.1 INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a liver disorder with an increasing incidence and a prevalence of 5.7% in the US [1]. Several lines of evidence support an important role for chronic exposure of the liver to bacterial endotoxin in the pathogenesis of NASH in man. First of all, small intestinal bacterial overgrowth has been shown to be associated with NASH [2-4]. In addition, patients with NASH display an impaired intestinal barrier function [2,5]. Together, this may lead to the reported increased plasma endotoxin levels in NASH [5,6].

However, there are important concerns with respect to the analysis of endotoxin in the context of NASH. First, endotoxin levels in peripheral blood may not necessarily reflect hepatic endotoxin exposure, since endotoxin is rapidly cleared from the portal circulation by the liver [7]. In line, the reported increase in endotoxin levels in peripheral plasma of patients with NASH is relatively minor [5]. Second, the commonly used for the quantification of endotoxin, the Limulus Amoebocyte Lysate [LAL] assay, suffers from several limitations. For example, it is sensitive to variations in endotoxin neutralizing factors, detergents, urea, and pH [8,9]. In addition, cell wall products of fungi and Gram-positive bacteria as well as polynucleotides can account for false positive test results [9,10]. Furthermore, due to its high sensitivity, the LAL assay is prone to exogenous endotoxin contamination [11,12]. Last but not least, interpretation of endotoxin levels in patients with liver disease is particularly challenging since they may be related to a reduced clearing efficiency of the hepatic reticuloendothelial system and/or an increased presence of liver-derived ß-1,3-D-glucans, which can account for false positive results [13,14].

In view of these concerns, there is an urgent need for an alternative method to assess endotoxin exposure of the liver in human NASH using peripheral blood. For this purpose, we here assessed the level of Immunoglobulin G (IgG) antibodies in the systemic circulation directed against the inner core of endotoxin, using the Endotoxin Core Antibodies assay (EndoCab) [15-17]. The Endocab IgG levels reflect an integrated antibody response to the endotoxin load over time. This assay has previously been used to study IgG antibodies against endotoxin in other chronic inflammatory diseases, such as inflammatory bowel disease [18]. Our data show that patients with NASH display elevated plasma EndoCab IgG levels, supporting the association between increased endotoxin plasma levels and NASH.

6.2 MATERIALS AND METHODS

SUBJECTS

Thirty severely obese subjects undergoing primary bariatric surgery were sequentially included at the department of General Surgery of the Maastricht University Medical Centre (Table 1). These subjects had no previous history of bariatric surgery or other gastrointestinal tract surgery. In case of acute, recent, or chronic inflammatory diseases (e.g. M. Crohn, Colitis), degenerative diseases, >10g alcohol consumption per day, other known liver diseases (e.g. auto-immune hepatitis or hepatitis B, C) or use of anti-inflammatory drugs, patients were excluded from the study.

	HEALTHY LIVER		NASH		P-VALUE
Number of patients	9		21*		
Age (years)	43.7 ± 2.1		47.3 ± 2.1		0.36
Sex (M : F)	3 : 6		9 : 12		0.63
BMI (kg/m^2)	47± 2.3		50.2 ± 1.9		0.33
T2DM (yes : no)	1 : 8		5 : 16		0.43
HT (yes : no)	3 : 6		12 : 9		0.47
Fasting glucose (mmol/L)	6.0 ± 0.44	(n=6)	6.7 ± 2.1	(n=17)	0.11
Total cholesterol (mmol/L)	4.6 ± 1.0	(n=5)	5.0 ± 1.3	(n=14)	1.00
LDL (mmol/L)	2.8 ± 1.3	(n=5)	3.2 ± 1.2	(n=14)	0.93
HDL (mmol/L)	0.82 ± 0.11	(n=5)	0.90 ± 0.18	(n=14)	0.96
FFA (mmol/L)	0.62 ± 0.23	(n=5)	0.78 ± 0.31	(n=14)	0.27
AST (IU)	24 ± 11	(n=8)	36 ± 17	(n=16)	0.13
ALT (IU)	29 ± 18	(n=8)	39 ± 32	(n=16)	0.60

Data are presented as mean value ± SD (n=number of patients). No significant differences with respect to age, sex distribution, BMI, T2DM, hypertension or plasma parameters were observed between the groups. * The 21 NASH patients were subdivided according to the Brunt classification in NASH grades (grade 1, n=11; grade 2, n=6; grade 3, n=4) and NASH stages (stage 0, n=3; stage 1, n=11, stage 2 and 3, n=7), respectively.

Table 1. Patient characteristics.

This study was approved by the Medical Ethical Board of the Maastricht University Medical Center in line with the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent was obtained in writing from each subject.

SAMPLES

Venous blood samples and liver biopsies of all patients were obtained and processed as previously described [19]. Plasma was stored in endotoxin-free vials (Sigma-Aldrich, St. Louis, MO). All 30 liver biopsies were at least 15mm in length, and had a sufficient number of portal tracts to allow correct evaluation of histological features. NASH was classified according to the Brunt score [20] and the NAS score according to Kleiner et al. [21], and NASH severity was assessed according to the criteria of Brunt by an experienced liver pathologist blinded to the clinical context of the biopsies and laboratory parameters. The livers of severely obese subjects without NAFLD (<5% steatosis) or with a NAS score of 0-2 were considered healthy livers. All subjects with NASH had a NAS score of ≥5.

MEASUREMENTS

Plasma IgG levels against endotoxin were measured using a commercially available sandwich ELISA for human EndoCab (Hycult Biotech, Uden, the Netherlands) according to the manufacturer's protocol. All plasma samples were analyzed in duplicate in the same run. The intra- and interassay coefficients of variance were <10%.

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation. Statistical analysis was performed using Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Differences between groups were analyzed by the Mann Whitney test or the Kruskal Wallis-test followed by Dunn's post-testing. Correlations were calculated using Spearman's correlation coefficient. A p-value <0.05 was considered statistically significant.

6.3 RESULTS

IgG antibodies directed to the core region of endotoxin were detected in all 30 subjects, with concentrations ranging up to 281 EndoCab standard median-units IgG (GMU/ml). The mean EndoCab IgG level was 37±55GMU/ml, with a median of 28GMU/ml.

Interestingly, plasma EndoCab IgG was significantly increased by almost five-fold in patients with NASH compared to subjects with healthy livers (48±63 vs. 10±13GMU/ml, $p<0.01$; Figure 1a). Moreover, plasma IgG levels against endotoxin progressively increased with NASH grade (grade 1 n=11, 29±37; grade 2 n=6, 58±51; grade 3 n=4, 84±132GMU/ml, $p<0.05$, Figure 1b), suggesting a relation between chronic endotoxin exposure and liver inflammation. NASH stage was not related to plasma EndoCab IgG levels (stage 0, n=3, 57±27; stage 1, n=11, 35±42; stage 2 and 3, n=7, 64±97GMU/ml, p=0.38, Figure 1c).

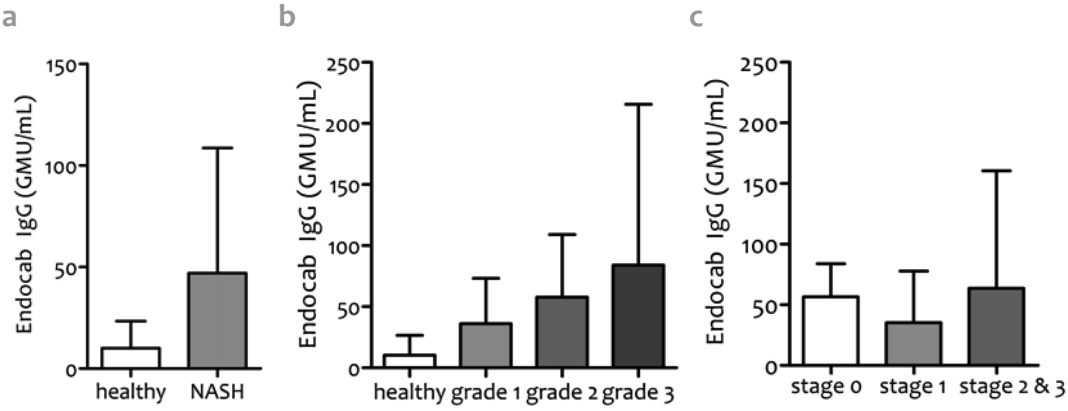

Figure 1. Elevated plasma EndoCab IgG levels are associated with NASH.
a) Plasma IgG levels against endotoxin are almost five-fold increased in severely obese patients with NASH (n=21) compared to severely obese controls with healthy livers (n=9, NASH 48±63 vs. healthy 10±13 GMU/ml; $p<0.01$). **b)** Plasma IgG levels against endotoxin increase with NASH severity as defined by the Brunt criteria (healthy n=9, 10±13.4; grade 1 n=11, 29±37; grade 2 n=6, 58±51; grade 3 n=4, 84±132 GMU/ml, $p<0.05$). **c)** Plasma IgG levels against endotoxin are unrelated to NASH stage as scored by the Brunt criteria (stage 0, n=3, 57±27; stage 1, n=11, 35±42; stage 2&3 n=7 64±97 GMU/ml, p=0.38).

Because immunoglobulin levels in general are known to increase with age, we next investigated the relation between EndoCab IgG and age. There was no correlation between EndoCab IgG levels and age (r_s=0.15, p=0.37), indicating that EndoCab IgG elevation in NASH is a specific phenomenon that cannot be attributed to increasing age. In line with this, the age distribution of the healthy liver and the NASH group was comparable (Table 1). Furthermore, since NASH is associated with type 2 diabetes mellitus (T2DM), we also investigated whether plasma Endocab IgG levels were higher in diabetic subjects. Diabetic subjects did not show significantly increased plasma Endocab IgG levels compared to subjects without diabetes (40±13 vs. 36±12GMU/ml, p=0.45).

6.4 DISCUSSION

Gut-derived endotoxin is suggested to be an important player in the pathogenesis of NASH [2,4]. Hence, there is an increasing interest in assessment of endotoxin exposure of the liver in man. However, the analysis of endotoxin is complicated due to technical and sampling issues. Here, we have studied the potential of the EndoCab assay as a indicator of chronic hepatic endotoxin exposure in human NASH. Our data reveal an association between biopsy proven human NASH and elevated plasma levels of IgG antibodies against the core region of endotoxin, supporting a possible role for chronic hepatic endotoxin exposure in the development of NASH.

The analysis of immunoglobulins against endotoxin has several advantages over the direct detection of endotoxin. First of all, it is not affected by contamination, and it detects a physically more stable molecule. Furthermore, because of the relatively long half-life (21-days) of IgG antibodies, measurement of IgG against endotoxin provides an indication of endotoxin exposure over time. Importantly, systemic plasma Endocab IgG levels were previously found to be correlated to plasma endotoxin levels [17]. In addition, plasma levels of IgG against endotoxin may also reflect the responsiveness of the immune system towards this potent immunostimulatory compound, which in the pathogenesis of NASH, could be more relevant than measuring endotoxin directly. Indeed, we found that plasma IgG levels

against endotoxin progressively increased with NASH severity, suggesting a relation between chronic endotoxin exposure and liver inflammation. However, taking into consideration that the study population size is limited, these findings will need to be validated in a larger cohort.

Anti-endotoxin IgG levels have previously been used to study the antibody response to endotoxin in other chronic inflammatory diseases associated with chronic endotoxin load, such as inflammatory bowel disease and colitis. In line with our results, these patients displayed significantly elevated plasma IgG levels [18]. To our knowledge, a potential association between plasma Endocab IgG levels and obesity-associated chronic inflammatory disorders such as diabetes or atherosclerosis has not been previously reported. However, in our study population, no relation between Endocab IgG and T2DM, hypertension, or gender was observed.

Of note, IgG antibodies against endotoxin might also have a functional role with respect to activation of innate immunity in NASH. In particular, immune complexes between IgG antibodies and endotoxins may cause deposition of complement component C1q. This C1q deposition in turn may further aggravate liver inflammation by initiating complement activation and promoting neutrophil infiltration, as we previously showed in human NASH [19]. Future studies will therefore focus on the identification of EndoCab IgG in the liver in relation to complement activation.

Taken together, our findings support the hypothesis that hepatic endotoxin exposure contributes to the development of NASH. The detection of plasma IgG levels against endotoxin may represent a novel method to assess chronic hepatic endotoxin exposure in patients with NASH.

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6

Chapter 7

Non-alcoholic steatohepatitis; a non-invasive diagnosis by analysis of exhaled breath

Submitted as: Non-alcoholic steatohepatitis; a non-invasive diagnosis by analysis of exhaled breath. Froukje Verdam, Jan Dallinga, Ann Driessen, Charlotte de Jonge, Edwin Moonen, Joep van Berkel, Jakobus Luijk, Nicole Bouvy, Wim Buurman, Sander Rensen, Jan Willem Greve, Frederik Jan van Schooten.

ABSTRACT

Histological evaluation of a liver biopsy is the current gold standard to diagnose non-alcoholic steatohepatitis (NASH), but the procedure to obtain biopsies is associated with morbidity and high costs. Hence, only subjects at high risk are biopsied, leading to underestimation of NASH prevalence and undertreatment. Since analysis of volatile organic compounds in breath accurately identifies subjects with inflammatory pulmonary diseases, we investigated its potential as a noninvasive tool to diagnose NASH.

Wedge-shaped liver biopsies from 65 subjects (BMI 24.8-64.3kg/m^2) were obtained during surgery and histologically evaluated. The profile of volatile organic compounds in pre-operative breath samples was analyzed by gas chromatography-mass spectrometry and related to liver histology scores and plasma parameters of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Three exhaled compounds were sufficient to distinguish subjects with (n=39) and without NASH (n=26), with an area under the ROC curve of 0.80. The negative and positive predictive values were 82% and 81%. In contrast, elevated ALT levels or increased AST/ALT ratios both showed negative predictive values of 43%, and positive predictive values of 88% and 70%, respectively. The breath test reduced the hypothetical percentage of undiagnosed NASH patients from 67-79% to 10%, and of misdiagnosed subjects from 49-51% to 18%.

Analysis of volatile organic compounds in exhaled air is a promising method to reflect NASH presence and absence. In comparison to plasma transaminase levels, the breath test significantly reduced the percentage of missed NASH patients as well as the number of unnecessarily biopsied subjects.

7.1 INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide [1,2], affecting one in three adults, and one in ten adolescents in the USA [1,3]. NAFLD is present in the majority of patients with metabolic risk factors such as obesity and type 2 diabetes mellitus (T2DM) [1]. Whereas steatosis, the early stage of NAFLD, is considered to be benign, progression towards more advanced stages often occurs. These advanced stages, referred to as non-alcoholic steatohepatitis (NASH), are characterized by inflammation [4-6]. Importantly, NASH is in turn associated with the development of hepatic fibrosis, cirrhosis, hepatocellular carcinoma, and an increased risk of liver failure and liver-related mortality [4,6-8]. It is therefore clinically relevant to differentiate between patients with hepatic steatosis alone as opposed to those suffering from NASH in an early stage.

Currently, a liver biopsy remains necessary to accurately diagnose NASH and to assess its severity [9-11]. However, the procedure to obtain a liver biopsy is invasive and associated with considerable discomfort, costs and morbidity; significant complications are encountered in 0.5% [12-17]. In order to optimize the risk-benefit ratio, it is advocated to obtain a per-operative biopsy from morbidly obese patients undergoing abdominal surgery, and a needle biopsy from obese patients with clinical risk factors [18]. Acknowledged risk factors for NASH are the presence of obesity, elevated plasma levels of alanine aminotransferase (ALT), an elevated ratio of aspartate aminotransferase (AST) to ALT (AST/ALT ratio), insulin resistance, hypertension, sleep apnea, and increased plasma levels of triglycerides [19-22].

In clinical practice, performing liver biopsy procedures based upon these risk factors leads to a selection bias. On one hand, plasma levels of aminotransferases often maintain within the normal range despite advanced disease [23,24], resulting in a considerable underestimation of NASH prevalence, as well as undertreatment of this disease [25,26]. On the other hand, if the indication to obtain a liver biopsy is based upon elevated aminotransferases, a large proportion of biopsies are obtained from subjects who do not suffer from NASH.

In view of 1) the clinical relevance of this liver disease, 2) the difficulties of selecting the appropriate population to biopsy, and 3) the biopsy related burden, a less invasive method to identify patients with NASH is urgently warranted. Such a method could be the analysis of volatile organic compounds (VOC) in exhaled breath.

VOC are considered as markers for oxidative stress, and can indicate the presence of reactive oxygen species that derive for example from peroxidation of polyunsaturated fatty acids [27-29]. Components in exhaled air have previously been shown to reflect the presence of inflammatory diseases affecting the airways [30-34], and the liver [35-37]. Moreover, lipid products have been detected in exhaled breath in the context of cardiac surgery [38], while lipid products are also associated with hepatic inflammation [4,5]. Hence, analysis of VOC in exhaled air may be useful for predicting the presence of NASH. We here report that subjects with NASH can be accurately distinguished from those without NASH based on analysis of VOC in exhaled breath.

7.2 PATIENTS AND METHODS

STUDY DESIGN

Overweight and obese subjects (n=65) undergoing laparoscopic cholecystectomy or primary bariatric surgery were consecutively included between October 2007 and May 2011. Surgery was performed either at the Maastricht University Medical Centre or the Atrium Medical Centre Parkstad.

Exclusion criteria were acute, recent, and chronic inflammatory diseases (e.g. M. Crohn, Colitis), other known liver diseases, consumption of >10g alcohol daily, and use of medication associated with NAFLD (e.g. steroids, amiodarone, valproate, methotrexate).

Established risk factors for NASH such as BMI, hypertension, and sleep apnea were evaluated. This study was approved by the Medical Ethical Committees of both the Maastricht University Medical Centre and the Atrium Medical Centre Parkstad, and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent was obtained from all subjects.

SAMPLE COLLECTION AND ANALYSIS

BREATH SAMPLES

Prior to the surgical intervention, breath samples from all 65 patients were obtained and analyzed as previously described [30,39]. In short, subjects exhaled into a resistance-free 5L plastic bag (Tedlar bag, SKC Ltd, Dorset, UK). Within 24 hours, the VOC were trapped by deflating the bag into a sorption tube filled with carbograph 1TD/Carbopack X (Markes International Inc, Cincinnati, OH). For analysis, the VOC were released by thermal desorption and injected into a gas chromatograph (Trace GC, Thermo Fischer Scientific, Austin, TX) connected to a time-of-flight mass spectrometry (Tempus Plus, Thermo Fischer Scientific) [39]. The complete analytical procedure and instrumental analysis has been published previously [30].

BLOOD SAMPLES

Preoperatively, from 61 subjects fasting venous blood samples were obtained and processed as previously described [40]. plasma levels including c-reactive protein (CRP), ALT, AST, glucose, insulin, HbA_{1c}, total cholesterol, HDL, LDL, triglycerides, and free fatty acids were measured according to the protocol of the department of clinical chemistry of the Maastricht University Medical Centre. the upper limit of normal alt levels was 35IU/L for women and 45IU/L for men [41], while an AST/ALT ratio >1 was considered to be elevated [42].

LIVER BIOPSIES

Wedge-shaped liver biopsies of at least 15mm in length were obtained intra-operatively from all patients by the same surgeon (JWG), and processed as previously described [43]. All biopsies contained a sufficient number of portal tracts to allow for correct evaluation of the hepatic architecture. No overt pathologic conditions other than NAFLD were observed. Steatosis, hepatocellular ballooning, lobular and portal inflammation, Mallory's hyaline, and fibrosis were scored according to both the Brunt scoring system [9] and the NAS activity score according to Kleiner et al. [10] by an experienced liver pathologist (AD) blinded to the clinical context and laboratory parameters. Liver biopsies that were evaluated as healthy or steatotic livers did not show any sign of portal or lobular inflammation, hepatocyte ballooning, or fibrosis (n=26). In contrast, those livers that showed signs of inflammation were defined as NASH (n=39, Brunt score of 2), and further evaluated according to Brunt and Kleiner.

DATA PROCESSING AND ANALYSIS

PROCESSING OF DATA

Detailed descriptions of the data handling procedures have been previously reported [39]. In short, gas chromatography and mass spectrometry (GC-MS) chromatograms of all breath samples were recorded. Retention times were normalized by calculating retention indices, relative to toluene and using easily recognizable component peaks, to correct for chromatographic drifting. the The beginning and end of each run (retention index either <0.15 or >2.8) were removed because of noisy mass spectra at the beginning of the chromatograms and column bleeding at the end of each run. The remaining data containing almost 4800 different chromatographic peaks as determined by retention time and mass spectrum combined with a relative intensity, were transformed into excel files. the The measured mass spectra were compared to one another at the same retention time. the The resemblance of the original spectra determines whether or not peaks at the same retention time represent the same component. Intensities under the detection limit were set at 0%.

STATISTICAL ANALYSIS OF THE GC-MS DATA

The data matrix was analyzed by a stepwise discriminant analysis by a leave-one-out cross-over approach, using statistical package for social sciences 19.0.0 (ibm spss software inc., chicago, il). All but one of the chromatograms were included to construct the discriminant function. the one that was left out was subsequently used to predict the group to which it belonged. This was repeated until every chromatogram had been left out once; all samples have been classified. Based upon 33 components, the discriminant functions that are optimal in terms of differentiation between both groups are not necessarily the best predictors for unknown samples, because of obvious overfitting. Therefore, the number of variables was gradually diminished until a reasonable small number of components with sufficient predictive power remained. This reduction in components was reached by repeating the analysis from the original large dataset by leaving the least informative components out, one by one.

STATISTICAL ANALYSIS OF CLINICAL DATA

Statistical analysis was performed using SPSS and Prism 5.0 (GraphPad Software Inc., San Diego, CA) for Windows. Differences between groups were analyzed by the Mann Whitney U test or the Kruskal Wallis-test followed by Dunn's post-testing. A p-value <0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean.

7.3 RESULTS

POPULATION CHARACTERISTICS

The body mass index (BMI) of the population ranged from 24.8 to 64.3kg/m^2, with a mean of 43.7kg/m^2. Population characteristics are summarized in Table 1.

	SUBJECTS WITHOUT NASH	SUBJECTS WITH NASH
No. of patients	26	39
Age (years)	45 ± 2	44 ± 2
Sex (M : F)	8 : 24	14 : 26
BMI (kg/m^2)	41.0 ± 1.3	45.2 ± 1.4
HbA1c (%)	6.4 ± 0.3	6.8 ± 0.3
HT (yes : no)	10 : 22	18 : 22
CRP (mg/dL)	6.0 ± 0.44	6.7 ± 2.1
ALT (IU/L)	22.9 ± 2.0	29.6 ± 2.5*
AST (IU/L)	18.8 ± 1.5	27.1 ± 2.2*
AST/ALT ratio	0.9 ± 0.1	1.1 ± 0.1

Data are presented as mean ± SEM.
* ALT and AST levels differed significantly between the groups (for both p<0.05), whereas no significant differences were observed for all other parameters.

Table 1. Characteristics of the study population.

Histological NASH was diagnosed in 39 subjects (60%), and the extent of NASH was further specified by means of the Brunt classification and NAS activity score according to Kleiner (Table 2). The average plasma ALT and AST levels were higher in subjects with NASH compared to subjects without NASH. Importantly, parameters such as gender, BMI, age and HbA_{1c} did not differ significantly.

BRUNT SCORE		NASH SUBJECTS (N=39)
Grade	0	12
	1	16
	2	10
	3	1
Stage	0	30
	1	5
	2	3
	3	1
KLEINER SCORE		
Steatosis	Grade 0	11
	Grade 1	14
	Grade 2	12
	Grade 3	2
Ballooning	Grade 0	23
	Grade 1	15
	Grade 2	1
Lobular inflammation	Grade 0	17
	Grade 1	14
	Grade 2	6
	Grade 3	2
Fibrosis	Stage 0	31
	Stage 1	4
	Stage 2	3
	Stage 3	1

Table 2. Activity, stages and grades of NASH.

ASSESSMENT BASED ON VOLATILE ORGANIC COMPOUNDS

Analysis of VOC showed that subjects with NASH could be distinguished from those without NASH with a sensitivity of 90% and a specificity of 69%, based upon a combination of three components. These three most discriminating compounds were 1) n-tridecane, 2) 3-methyl-butanonitrile and 3) 1-propanol as identified by means of their mass spectrum.

When less compounds were taken into account, the specificity of the breath analysis diminished accordingly (Figure 1a). The optimal relation between sensitivity and specificity was calculated at various cut-off values for the discriminant function, as visualized in the receiver operating characteristic (ROC)-curve. For the three described components, the area under the curve was 0.80 (Figure 1b).

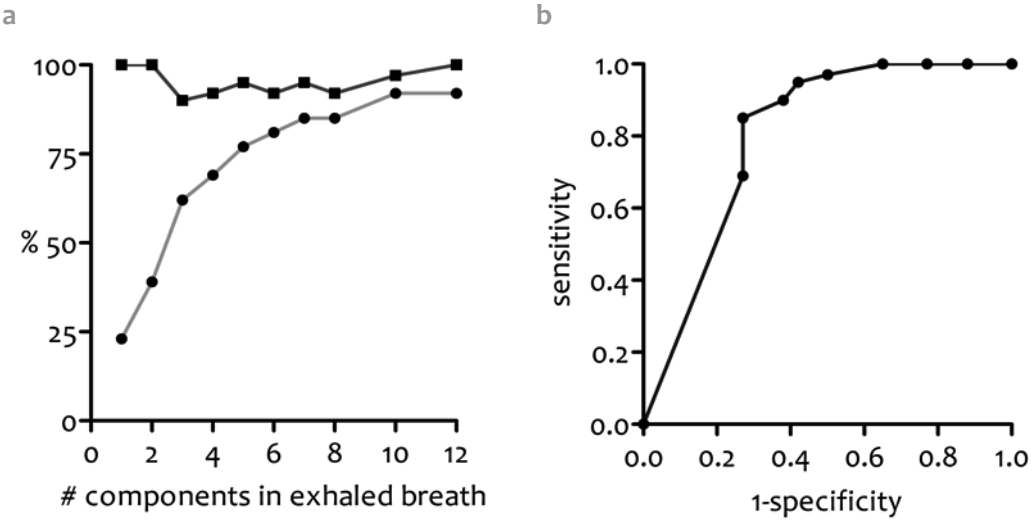

Figure 1. Analysis of the number of breath components and ROC curve.
a) The sensitivity (line with squares) and specificity (light grey line with dots) of exhaled breath related to the number of components incorporated in the discriminant function. With 1 or 2 components, the sensitivity of exhaled breath to predict NASH presence in overweight and obese subjects is 100% with a specificity of 23% and 39% respectively, whereas the optimum was with three components (sensitivity 90%, specificity 69%).
b) The ROC curve for discriminant function based on n-Tridecane, 3-methyl-butanonitrile and 1-Propanol, the area under curve is 0.80.

In order to interpret the clinical value of this test, likelihood ratios (LR) and predictive values (PV) were calculated [44-46]. The positive LR is the probability that a positive test result actually reflects the presence of disease. This was 2.90 for the three components (Table 3), whereas the negative LR, a measure for the probability that a negative test result reflects the absence of NASH, was 0.15. The actual prevalence was taken into account by calculating the positive PV, the percentage of subjects with a positive test result who actually suffer from NASH, which was 81%. The negative PV, the proportion of patients with a negative test result that do not suffer from NASH, was 82% (Table 3).

	VOC	ALT ↑	AST/ALT>1
Sensitivity	90%	19%	32%
Specificity	69%	96%	79%
Positive LR	2.90	4.54	1.52
Negative LR	0.15	0.85	0.85
Positive PV	81%	88%	70%
Negative PV	82%	43%	43%

Table 3. Test accuracy of VOC analyses and plasma parameters.

If the decision to obtain a liver biopsy in the study population would have been based upon the three mentioned components in exhaled breath, 43 patients would have been biopsied, of whom eight did not have NASH (19%), whereas four subjects with NASH (10%) would have been missed. In total, 12 out of the 65 subjects (18%) would have been misdiagnosed (Figure 2).

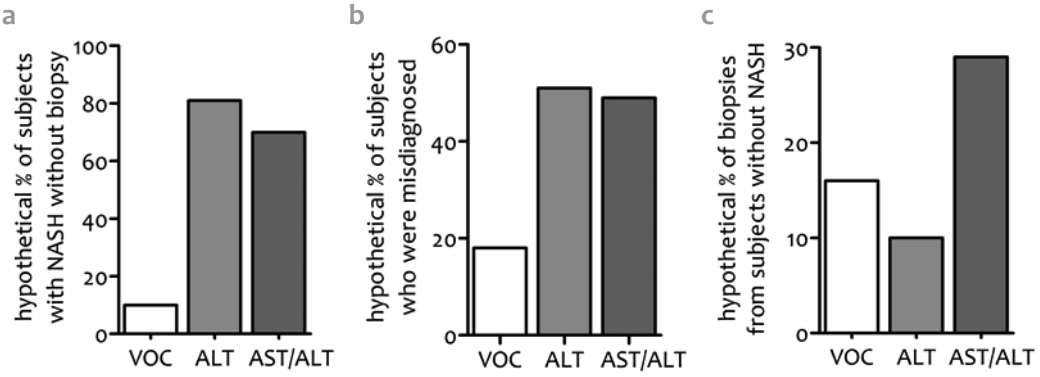

Figure 2. Number of (mis)diagnosed subjects based upon breath and plasma.
a) The percentage of patients who were falsely predicted not to have NASH by VOC (4/39 or 10%), increased ALT values (30/37 or 81%), and an increased AST/ALT ratio (25/37 or 68%).
b) The percentage of obtained biopsies from patients without NASH would be 7/29 (16%) for VOC, 1 out of 8 (13%) for ALT, and 5 out of 17 (29%) for AST/ALT if the indication to obtain a biopsy would have been based hereupon.
c) The percentage of misdiagnosed patients based upon VOC was 12/65 (18%), 31/61 (51%) based upon increased plasma ALT and 30/61 (49%) based upon AST/ALT ratio>1.

ASSESSMENT OF NASH BASED ON ELEVATED PLASMA TRANSAMINASE LEVELS

Currently, the decision to obtain a liver biopsy in a high risk population is largely based upon either elevated ALT levels or increased AST/ALT ratio. In order to assess the clinical value of the breath test, we compared the obtained data to the predictive properties of the current clinical protocol. Hence, we calculated the proportion of the population that would have undergone a liver biopsy based upon plasma transaminases levels. Plasma could not be obtained from four subjects (of whom two suffered from NASH and two did not), the 61 remaining subjects were analyzed. If the indication to obtain a biopsy would have been based upon elevated ALT levels, eight biopsies (of seven patients with and one without NASH) would have been obtained. Perhaps more importantly, 30 out of the remaining 37 subjects with NASH (81%) would have been missed (Figure 2). Increased ALT levels showed a sensitivity of 19% and a specificity of 96%. The positive PV for increased ALT was 88% while the negative PV was 43% (Table 3).

Next, AST/ALT ratios were calculated. If a liver biopsy would have been obtained from subjects with an increased AST/ALT ratio, 17 biopsies would have been performed in total; 12 of subjects with NASH and 5 of subjects without NASH. Moreover, 25 patients or 68% of all subjects with NASH would have been missed (Figure 2). The sensitivity for an increased AST/ALT ratio was 32%, the specificity 79%, the positive PV 70% and the negative PV 43%, respectively (Table 3).

The consequences of assessing NASH presence either based upon breath or plasma in the study population are provided in Figure 2. The percentage of the population that would have undergone liver biopsies based upon either VOC, increased plasma ALT, or AST/ALT ratio is illustrated. It can be concluded that the diagnostic value of VOC is much higher than that of plasma transaminases, resulting in less misdiagnosed patients. More specifically, the prediction did not match the actual histological hepatic evaluation in 18% of subjects (12 out of 65) based upon VOC, in 51% based upon ALT (31 out of 61) and in 49% (30 out of 61) based upon AST/ALT ratio. The inconsistent predictive properties of plasma parameters were further emphasized by the observation that only one patient had both elevated ALT levels and an increased AST/ALT ratio. Patients who were correctly identified with NASH by means of plasma parameters were also identified by means of exhaled breath test. In addition, the breath test identified a considerable proportion of NASH patients with normal plasma parameters. In order to assess the potential additive value of considering other known risk factors, multivariate analysis was performed. The evaluated factors included BMI, age, gender, hypertension, and plasma HbA_{1c}, ALT, AST, glucose, and insulin. Statistical significance was not reached; multivariate analysis showed no additive value to assess NASH presence (Table 4).

	B	S.E.	WALD	P-VALUE
ALT	.01	.07	.03	.86
AST	-.01	.04	.13	.72
sex (m/f)	-1.31	.89	2.1	.14
age (y)	.00	.04	.00	.99
BMI (kg/m^2)	.03	.06	.26	.61
HbA1c (%)	.45	.32	1.97	.16
glucose	.39	.30	1.68	.19
Insulin	.07	.06	1.13	.29
TG	.04	.43	.01	.93
HT (y/n)	.28	.90	.09	.76
Apnoe (y/n)	1.07	1.23	.76	.38

The β-coefficient reflects the importance of the parameter potentially predicting NASH presence, whereas the Wald value is used to test the true value of this importance; whether it is statistically significant. None of these parameters reached statistical significance; therefore none of them facilitate the diagnosis of NASH.

Table 4. Multivariate analysis.

7.4 DISCUSSION

In order to find a less invasive method to diagnose NASH, VOC in exhaled breath were examined in relation to the evaluation of wedge-shaped liver biopsies in a cohort of 65 overweight and obese subjects. The feasibility to predict the presence of NASH based upon three components in exhaled breath (n-Tridecane, 3-methyl-butanonitrile and 1-Propanol) was high compared to plasma and clinical parameters. Moreover, by means of these three exhaled breath components almost all of the subjects who would have been missed based upon plasma ALT and AST/ALT ratio, could be identified. The fact that many subjects are missed in case of risk assessment based upon ALT levels or AST/ALT ratio is supported by others. Previous studies have shown

that patients with NASH still have normal AST/ALT ratios [47,48], and that plasma aminotransferase levels do not correlate well with underlying disease activity and can remain normal despite advanced disease [49-51].

It is essential to diagnose NASH in an early phase and to prevent potential progression into fibrosis and cirrhosis. According to recent longitudinal studies, patients with NASH already have a high risk to both develop liver related complications and suffer liver-related mortality [52-54]. The Nonalcoholic Steatohepatitis Clinical Research Network recently stated that clinical and laboratory parameters are insufficient to reliably diagnose NASH [21]. However, given the associated discomfort, complications and costs, the burden of screening for this emerging liver disease by means of percutaneous liver biopsies would be disproportional to the benefit. The necessity to find minimal invasive cost-effective ways of screening will further intensify given the rising incidence of obesity and NASH. Current research therefore focuses on less invasive methods to differentiate between the various stages of NAFLD [21,55-58]. For example, plasma markers of epithelial cell damage, e.g. cytokeratin-18 and cytokeratin-18 fragments, are promising for assessing NASH presence [59-63]. When these markers are combined with acknowledged risk factors for NASH, the area under the curve to predict NASH presence can be improved [64-66], but validation in independent patient populations is still warranted to optimize their potential diagnostic value. Moreover, radiological modalities such as ultrasonography [67] and transient elastography [68,69] may assist in diagnosing NASH. Transient elastography measures tissue elasticity based on ultrasound technology, and was found to aid in predicting the presence of fibrosis [68] and hepatic inflammation prior to the development of fibrosis [69]. A recently developed clinical score combining ultrasound with the plasma parameters total cholesterol, ALT, AST/ALT ratio, and gammaglutaril-transferase (γGT) showed a positive PV of 64% in obese subjects [22]. However, differentiation between steatosis and NASH by means of ultrasound remains challenging [22], and the sensitivity of ultrasound further diminishes in case of less than 40% hepatic steatosis [70]. Furthermore, the accuracy of radiological techniques is operator-dependent and subject to an important intra- and interobserver variability [71].

The classical way to diagnose NASH by means of histopathological examination of a percutaneous needle biopsy, consisting of a tiny portion of the total liver mass (an estimated 1/50,000), is vulnerable to variability [15,72-74]. This variability could not be completely excluded in our study, but was reduced because we obtained relatively large wedge-shaped liver biopsies, allowing a more thorough histological evaluation [75]. Furthermore, whereas a small part of the liver is considered in the evaluation of biopsies, the breath test used in this study is a non-invasive method to reflect total liver function. Previously, other breath tests have been used to indicate the extent of liver disease. For example, the so-called caffeine breath test was reported to predict the presence of hepatic fibrosis in a study of 48 subjects [76]. In addition, by analysis of breath after administration of stable isotopes in 39 patients with increased ALT levels, microsomal and mitochondrial dysfunction was observed [77].

VOC analysis in exhaled breath has been used to discriminate between patients with and without inflammatory airway diseases [30-32,34], lung cancer [78], breast cancer [79], and active and passive smokers [80]. Based upon findings in these studies, we here investigated the potential of VOC analysis to diagnose NASH, and showed that subjects with and without NASH could be successfully differentiated in a high risk population. By means of three volatile breath components, the absence of NASH was more accurately predicted; less false positive and less false negative results were obtained than by use of plasma parameters. More specifically, n-Tridecane, 3-methyl-butanonitrile and 1-Propanol in exhaled breath were strongly related to the presence of NASH. Even though the exact origin of these compounds still needs to be investigated, it is tempting to speculate that they are of inflammatory origin, since NASH is characterized by inflammation with cellular infiltration and activation of neutrophils [81]. Interestingly, 1-propanol has also been associated with pulmonary cancer [82,83] and may be associated with lipid peroxidation.

After validation of our data in a larger cohort, the diagnostic value of the breath test may be even further enhanced by combining it with other plasma markers such as the previously mentioned epithelial damage markers. Analysis of non-invasive breath test could be clinically implemented as a first line screening tool for this serious and emerging liver disease. It would also be of interest to test the potential of VOC analysis to evaluate the effect of NASH treatment. The cornerstones of this

treatment are lifestyle modifications [84], and novel pharmacological therapies are under investigation [85]. Moreover, bariatric surgery has been shown to improve NAFLD in 92% and NASH in 81% of cases, and complete remission of NASH in 69% [86]. Possibly, VOC analysis can be used to prioritize patients undergoing bariatric surgery with NASH over those without NASH. In conclusion, we here report that analysis of exhaled breath seems a suitable non-invasive method to accurately predict which subjects suffer from NASH, and to diminish the number of missed diagnoses of NASH.

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Chapter 8

General discussion: the intestine is involved in obesity, type 2 diabetes mellitus and fatty liver disease in man

8.1 THE ROLE OF THE INTESTINE IN OBESITY

Obesity is recognized as the most prevalent pandemic disorder of the 21st century. Over 200 million school-age children are overweight; this is the first generation predicted to have a shorter lifespan than their parents [1,2]. In order to allow for the development of new prevention and treatment modalities for obesity, it is important to gain more insight into the underlying pathophysiology. Research has traditionally focused on adipose tissue, liver, and skeletal muscle. More recently, the role of the intestine as an important player in the development of obesity and the metabolic syndrome is progressively acknowledged [3-7].

Various aspects illustrating the involvement of the intestine in obesity and its comorbidity have been emphasized in animal studies. More specifically, an increased intestinal permeability [8,9], elevated translocation of bacterial products through the intestinal barrier [9-13], and an altered microbiota inhabiting the intestine [12,14-17] have been shown to contribute. In this thesis, thanks to the availability, willingness, and cooperation of many volunteers, we investigated whether these findings from animal studies could be extrapolated to the human situation. Intestinal microbiota were hypothesized to be altered in human obesity, and it was studied whether microbiota affect intestinal permeability and inflammation in obesity. Subsequently, Paneth cells, which are known to regulate intestinal microbiota composition, were investigated in lean versus obese subjects. Regarding obesity-associated comorbidity, two important topics were addressed: first, intestinal epithelial proliferation in the context of obesity-induced diabetes, and second the association between antibody titers to endotoxin and the degree of non-alcoholic fatty liver disease (NAFLD).

8.2 INTESTINAL MICROBIOTA IN OBESITY ARE ASSOCIATED WITH INTESTINAL AND SYSTEMIC INFLAMMATION

In **Chapter 2,** an overview of methods to assess gut wall integrity is provided. Next, a multi-sugar test was used for the assessment of gastro-duodenal, small, and large intestinal permeability in lean and obese subjects.

As described in Chapter 3, gastro-duodenal permeability was found to be increased in obesity, whereas small and large intestinal permeability were comparable. These results are supported by the study by Brignardello et al., showing similar small and large intestinal permeability in lean and obese subjects [18]. However, this is in contrast to studies in mice on a high fat diet, reporting a more permeable jejunum and colon compared to controls [19]. Other murine studies also described an augmented intestinal permeability in obesity, which was in turn related to increased bacterial translocation and systemic inflammation [9,11]. Whereas intestinal microbiota was suggested to modify intestinal permeability in mice [9], microbiota composition was not found to be related to intestinal permeability in our study with human volunteers. However, microbiota composition was related to the extent of obesity. More specifically, the ratio of Firmicutes and Bacteroidetes -the two most dominant commensal phylae inhabiting the human intestine- was strongly correlated to BMI. The microbiota composition observed in human obesity could be further specified and was characterized by a decreased amount of Bacteroidetes and an increase in Firmicutes, especially Clostridium cluster XIVa. The ratio of Firmicutes to Bacteroidetes was increased in the obese population, which is in line with recent reports in man [16,20], and with studies in both genetically and diet-induced obese animals [15,17].

Next, the microbiota composition was related to parameters of intestinal and systemic inflammation. Interestingly, intestinal inflammation as reflected by fecal calprotectin levels was observed only in subjects with an obese microbiota profile. In line, intestinal inflammation has been observed to precede the development of obesity and insulin resistance in mice on a high fat diet [21]. Our findings are also supported by a study reporting intestinal inflammation together with microbial shifts in rats prone to develop obesity [14]. Generally, commensal bacteria are considered to be critical for controlling inflammation in intestinal epithelia [22,23]. In our population, the Firmicutes/Bacteroidetes ratio was also related to systemic inflammation. This is in agreement with recent evidence showing that intestinal microbiota influence the immune response [24]. In summary, the data in Chapter 3 suggest that the observed obesity-specific microbiota profile underlies intestinal and systemic inflammation in obese subjects.

8.3 PANETH CELL FUNCTION IS DETERIORATED IN OBESITY

The role of Paneth cells in controlling intestinal microbiota composition and limiting bacterial translocation has become increasingly clear in the past decade [25-29]. Paneth cell-derived bactericidal α-defensins are secreted into the small intestinal lumen, and remain functional throughout the intestinal tract [30]. Interestingly, mice incapable of producing functional forms of these bactericidal peptides are more susceptible to intestinal inflammation [31]. Intestinal inflammation has in turn been shown to coincide with diminished Paneth cell derived protein levels [32] and impaired Paneth cell differentiation [33]. Moreover, mice with diminished α-defensin protein levels showed a shift in microbiota composition at the expense of Bacteroidetes and in favor of Firmicutes. A similar microbiota shift is observed in human obesity in both in Chapter 2 and in other studies [16,20]. We therefore investigated Paneth cells in small intestinal biopsies of severely obese and lean subjects (Chapter 4).

Obese subjects showed reduced levels of two different Paneth cell derived antimicrobials, α-defensin 5 and lysozyme, whereas the number of Paneth cells was similar in lean and obese subjects. Semi-quantitative analysis confirmed an inverse relation between Paneth-cell-derived antimicrobial proteins and BMI. Interestingly, in spite of the lowered protein levels in obesity, gene expression levels were upregulated. The phenomenon of upregulated gene expression levels contrasted by lower protein production is typically present in case of Endoplasmic Reticulum (ER) stress. ER stress has previously been reported in obesity and in relation to obesity-related factors such as excessive nutrient intake, increased need for protein synthesis, and accumulation of lipids [34-37]. In addition, Paneth cells are acknowledged to be susceptible to ER stress since they are highly secretory cells [38]. Indeed, the presence of ER stress in the small intestine of obese subjects was confirmed at the protein level. Moreover, hallmarks of ER stress, such as the presence of vacuoles and enlargement of the ER, were visualized in Paneth cells of obese subjects by means of electron microscopy. In short, our results indicate a relation between ER stress and Paneth cell malfunction in obesity, which is supported by a recent study showing diminished antimicrobial protein secretion in a mouse model displaying ER stress [38].

8.4 INCREASED ENTEROCYTE MASS AND TURNOVER IN OBESE SUBJECTS WITH TYPE 2 DIABETES MELLITUS

Intestinal homeostasis, epithelial proliferation, and epithelial turnover are known to be influenced by intestinal microbiota composition [24,39-43]. Germ-free animals exhibit a slower intestinal turnover and altered morphologic properties, such as atrophic crypts, longer villi [44], and defective angiogenesis [45]. Interestingly, the small intestine of rats with T2DM is characterized by longer villi, mucosal hypertrophy, and hyperplasia [46]. This led us to investigate intestinal enterocyte mass and turnover in obese subjects with and without T2DM in Chapter 5.

Enterocyte mass was indicated by assessing plasma citrulline levels as previously described [47]. Citrulline levels were found to be significantly higher in severely obese subjects with T2DM compared to severely obese subjects without T2DM. Enterocyte loss was investigated by measuring plasma levels of Intestinal Fatty Acid Binding Protein (I-FABP), a protein previously shown to reflect enterocyte loss and intestinal barrier damage in acute situations [48,49]. Enterocyte loss was also increased in subjects with T2DM. In addition, the ratio of enterocyte loss and enterocyte mass was higher in obese subjects with T2DM, suggesting increased proliferation. Both enterocyte mass and turnover were also positively related to chronic hyperglycemia as indicated by glycated hemoglobin levels.

Overall, these findings strongly suggest that the small intestinal aberrations observed in animals with T2DM [46] are also present in obese subjects with T2DM. In animals, this increased intestinal proliferation was shown to coincide with increased food absorption [50] and enhanced disaccharidase activity [46]. In line, the observed higher enterocyte mass in our population may account for the high postprandial glucose levels characteristic of T2DM [46,51].

8.5 ELUCIDATING THE PATHOGENESIS AND FACILITATING THE DIAGNOSIS OF NASH

Intestinal permeability and gut-derived endotoxin have been shown to be involved both in the development of T2DM [9-11] and in the pathogenesis of NASH in animal models [8,52]. A positive relation was observed between hepatic inflammation and enhancedintestinal permeability, modified tight junction distribution, and the presence of LPS in portal blood of obese mice [8]. In Chapter 6, the putative involvement of LPS in human NASH was investigated by histologic evaluation of liver biopsies in relation to systemic antibody titers to endotoxins. This approach was chosen because the commonly used assay to measure LPS levels in biological fluids, the limulus amoebocyte lysate assay, is notorious, easily contaminated, and affected by the presence of for example β-D-glucans of hepatic origin [53-57].

The data indeed revealed an association between NASH severity and the levels of antibodies to LPS, supporting the hypothesis that hepatic endotoxin exposure plays a role in the progression of human NAFLD. This is further substantiated by other human studies reporting respectively increased LPS levels [58,59], enhanced levels of LPS-binding protein [60], increased intestinal permeability [59], and diminished expression levels of duodenal tight junction proteins [61] in human NASH.

Histological scoring of a liver biopsy is the current gold standard for the diagnosis of NASH [62,63]. A liver biopsy is particularly essential to differentiate between simple steatosis and NASH [64] and to indicate the severity of NASH [65]. Due to the morbidity and costs, not all patients at risk undergo this invasive procedure. It has been advocated to perform a liver biopsy in all morbidly obese patients undergoing bariatric surgery, and in all obese patients with elevated plasma levels of aminotransferases, γGT, and fasting glucose [66]. Since NASH is usually asymptomatic, risk assessment is based on these plasma levels. However, these plasma aminotransferase levels often remain within the normal range despite advanced disease [67,68]. This leads to an underestimation of the prevalence of both steatosis and NASH, even when combined with ultrasound [64,69]. Therefore, current research focuses on investigating the potential of less invasive methods to discriminate between simple steatosis, NASH, fibrosis, and cirrhosis [65,70-73].

Human NASH is not only related to antibody levels to endotoxin as described in Chapter 6, but also characterized by an accumulation of oxidative products, hepatic production of chemokines, and infiltration and activation of neutrophils [74]. Interestingly, specific compounds in exhaled breath have been shown to differ between subjects with and without inflammatory pulmonary [75-78] and inflammatory liver diseases [79-83]. It was therefore investigated whether exhaled breath could predict the presence or absence of NASH as reported in Chapter 7. In comparison to the current risk assessment based upon plasma transaminases, analysis of volatile organic compounds in exhaled breath reduced the percentage of undiagnosed patients with NASH from 67-79% to 10%, and diminished the number of misdiagnosed patients from 47-50% to 18%. The negative and positive predictive values of this breath test were 82% and 81%, whereas plasma levels showed a negative predictive values of 43%, and positive predictive values of 88% and 70%, respectively. By means of this breath test, the presence of NASH may be detected at an earlier stage, prior to the development of fibrosis or cirrhosis, and regardless of normal plasma transaminase levels. Our data need validation in a larger cohort before clinical implementation. Thereafter, this breath test could be used to screen for NASH, and to monitor the effect of treatment. This is of particular interest patients who are currently excluded from liver biopsy procedures, such as children and adolescents, or for candidates who refuse to undergo this procedure. In addition, since NASH can improve and even go into remission by means of bariatric surgery [84], this test may be applied to indicate the urgency of surgical treatment for morbidly obese patients.

8.6 SUMMARY, POTENTIAL IMPLEMENTATIONS, AND FUTURE RESEARCH

The findings described in this thesis contribute to the accumulating scientific evidence underlining the role of the intestine in the pathophysiology of human obesity and its comorbidity. First, we observed the intestinal microbiota composition to be related to BMI, and to intestinal and systemic inflammation. In obese animals, it has been shown that the obesity-associated shift in microbiota can be reversed by supplementation of probiotics [85]. In animal studies, pro- and/or pre-biotic supplements in turn are reported to have beneficial effects on body weight [86-88], systemic [9,13,87], hepatic, and adipose tissue inflammation [89], insulin resistance

[13,86-88], and lipid profile [86]. In man, positive effects of pre- and probiotics on satiety [90], weight loss [91,92], and glucose metabolism [91] have been reported. It would be relevant to study whether intestinal and systemic inflammation in the context of human obesity can also be reduced by modifying microbiota composition with pre- or probiotics.

In animal models of obesity-induced T2DM, increased intestinal proliferation has been described to coincide with enhanced food- and glucose absorption [46,50]. An increased absorptive capacity can be induced by microbiota [45]. Notably, microbiota alterations in mice are related to changes in plasma glucose levels [93]. In addition, an altered intestinal microbiota composition has been reported in obese mice [94] and subjects with T2DM [93]. Moreover, transplantation of microbiota can induce increased appetite and insulin resistance [5]. It would be interesting to investigate whether our observations of an increased intestinal cell mass and turnover in human T2DM also coincide with increased absorption, and whether this can be influenced by modulating intestinal microbiota.

Furthermore, it is tempting to assume that the disturbed Paneth cell function observed in obesity is related to the observed obesity-specific microbiota profile. Paneth cells prevent luminal microbes from invading the mucosa and triggering inflammation [32,95], and to limit bacterial translocation [26,96]. Increased bacterial translocation has been reported in mice on a high fat diet, in combination with mucosal adherence of intestinal microbiota [97], and in human T2DM [98] and NASH [58,59]. This increased bacterial translocation may be related to Paneth cell function, since Paneth cells have been shown to prevent translocation of bacterial products following intestinal ischemia and reperfusion [96]. Future studies will have to elucidate the exact impact of Paneth cell malfunction on bacterial translocation in human obesity and its comorbidity.

The final part of this thesis contributes to the reported evidence that hepatic endotoxin exposure is involved in of human NASH. It is important to further elucidate the pathogenesis of this increasingly prevalent liver disease, in order to develop suitable treatment options. However, due to a current lack of accurate non-invasive diagnostic tools, it is difficult to identify obese subjects with NASH. We found that a

breath test can be of additive value over the currently used plasma parameters to assess NASH presence, and showed that analysis of exhaled breath to be a promising aid in screening for NASH.

Overall, this thesis reports factors related to the intestine, such as intestinal inflammation, intestinal permeability, Paneth cell function, and microbiota composition to be involved in human obesity. Moreover, intestinal epithelial proliferation and antibodies to LPS were observed to be related to obesity-associated comorbidity. It would be relevant to investigate whether the discovered obesity-associated alterations in the intestine can be restored, for example by influencing the microbiota composition, or by bariatric surgery.

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Chapter 9

Summary

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9.1 SUMMARY

This thesis focuses on the role of factors related to the intestine in human obesity and its comorbidity. The introduction emphasizes the extent of the epidemic of obesity, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD). Chapter 1 also provides an overview on current treatment options for severe obesity and an update of the literature on the putative role of the intestine in obesity. Various methods to assess intestinal permeability are reviewed in Chapter 2. Intestinal permeability can be either assessed directly, for example by means of protein or sugar-based tests, or indirectly, for example by measuring the extent of bacterial translocation, or antibodies against bacterial components.

Intestinal permeability of various parts of the gastrointestinal tract was then assessed by means of a multi-sugar mixture in obese and lean individuals, as described in Chapter 3. Gastroduodenal permeability was found to be increased in obesity. In line with previous reports, the microbiota composition in obesity was characterized by an increased Firmicutes/Bacteroidetes ratio. Interestingly, a positive relation between microbiota composition and markers for intestinal and systemic inflammation was observed, implying that the microbiota inhabiting the intestine in obesity might have a pro-inflammatory effect. This is supported by animal studies showing that a shift towards a 'lean microbiota profile' by means of pro- or prebiotic administration induces diminished bacterial translocation and subsequent inflammatory tone.

Microbiota composition is known to be regulated by Paneth cells by secretion of antimicrobial peptides. Interestingly, it was previously reported that mice deficient for one of these peptides have a microbiota composition similar to that observed in obesity (in Chapter 3). Therefore, Paneth cell characteristics were investigated in Chapter 4. A reduced number of bactericidal peptides was observed in Paneth cells of obese subjects. In spite of these lowered protein levels, gene expression levels were upregulated. This phenomenon has been associated with a cellular stress response related to the endoplasmic reticulum (ER). The presence of this stress response in the small intestine of obese subjects could indeed be confirmed both at the protein level and by means of electron microscopy.

The second part of this thesis describes the potential association between the comorbidity of obesity and factors related to the intestine. Literature data show that rats with T2DM have an increased intestinal cell mass, longer villi, and mucosal hypertrophy. Therefore in Chapter 5, plasma markers for intestinal cell mass and turnover were investigated in the context of human T2DM. These markers showed an increased enterocyte mass, enterocyte loss and turnover in subjects with obesity-induced diabetes. Moreover, these parameters were positively related to the extent of chronic hyperglycemia.

In Chapter 6 NAFLD, another comorbidity of obesity, is investigated. NAFLD ranges from from hepatic steatosis, which is considered to be benign, to more severe stages such as non-alcoholic steatohepatitis (NASH). Based upon the fact that hepatic inflammation in obese mice is related to enhanced intestinal permeability and increased levels of microbial compounds (lipopolysaccharide, LPS) in the portal vein, we investigated the potential involvement of LPS in human NASH in Chapter 6. Since measuring relatively low plasma levels of LPS is notoriously challenging and easily confounded by ß-glucans derived from the liver, we measured systemic antibodies to LPS. These antibody titers were observed to be higher in obese subjects with NASH compared to obese subjects with healthy livers. This strongly suggests that hepatic endotoxin exposure, as reflected by increased systemic antibody levels to LPS, plays a role in the development of human NASH.

Histological scoring of a liver biopsy is currently the gold standard to diagnose NAFLD and NASH; there is an evident need for less invasive diagnostic methods. Previous studies illustrated that NASH is characterized by accumulation of oxidative products, production of chemokines, and hepatic infiltration and activation of neutrophils. Since inflammatory compounds have also been shown to be detectable in exhaled air, we investigated the feasibility of analyzing exhaled air as a diagnostic instrument to predict the absence or presence of NASH (Chapter 7). A panel of three volatile organic compounds was shown to accurately reflect the presence of hepatic inflammation. These data first need to be confirmed in a larger cohort, but could lead to less invasive screening in high risk populations for NASH, and contribute to an earlier identification of NASH.

In Chapter 8, all findings are placed in the context of the current literature and future research lines are provided. Overall, this thesis emphasizes two important aspects: microbiota composition, Paneth cells, and intestinal and systemic inflammation are involved in obesity. Secondly, intestinal epithelial proliferation is likely to play a role in the postprandial increased glucose levels in obesity-induced diabetes, and bacterial compounds may well play a role in the pathogenesis of human NASH.

9.2 SOMMAIRE

Cette thèse s'est concentrée sur le rôle des facteurs intestinaux dans la genèse de l'obésité et des troubles associés tels le diabète de type 2 (T2DM) et la stéatose hépatique non alcoolique, dont l'ampleur épidémique est soulignée dans le Chapitre 1. Il décrit aussi les traitements possibles de l'obésité sévère puis présente des données récentes chez l'animal comme chez l'homme montrant l'implication de l'intestin dans le développement de l'obésité.

La genèse de cette pathologie semble être ainsi associée à une augmentation de la perméabilité intestinale, dont plusieurs méthodes d'évaluation sont décrites dans le Chapitre 2. En effet, la perméabilité intestinale peut être évaluée soit directement au moyen de sucres ou de protéines, soit indirectement en mesurant par exemple l'étendue de la translocation bactérienne ou des anticorps contre des composés d'origine bactérienne.

Au cours de nos expériences décrites au Chapitre 3, la perméabilité intestinale du tractus gastro-intestinal fut évaluée au moyen d'un mélange de plusieurs sucres dans une population de patients minces et obèses. Nous avons pu observer que les patients obèses montraient une perméabilité gastroduodénale accrue et une modification de la flore intestinale caractérisée par une augmentation du ratio Firmicutes / Bactéroidetes, en accord avec des études précédemment publiées. Nos expériences ont également pu mettre en évidence qu'une relation existait entre la composition de la flore intestinale et des marqueurs de l'inflammation locale mais aussi systémique. Ces résultats suggèrent que des changements de composition de la flore peuvent induire une réponse inflammatoire intestinale au cours de l'obésité. Il a été montré chez des animaux obèses qu'une normalisation de la flore bactérienne altérée réduisait la translocation bactérienne mais aussi le développement d'une réponse inflammatoire.

Le Chapitre 4 présente la fonction des cellules de Paneth chez l'homme. Ces cellules régulent la composition de la flore en sécrétant des peptides antimicrobiens. L'importance de leurs rôles dans le maintien de la flore intestinale a été confirmée par des études récentes montrant que des souris déficientes en au moins un de ces

peptides présentaient une modification de la flore intestinale similaire à celle observée au cours de l'obésité au Chapitre 3. Nous avons pu observer que ces cellules chez les patients obèses contenaient moins de peptides bactéricides malgré des niveaux augmentés de leurs expressions géniques. Ce phénomène était associé à un stress du réticulum endoplasmique, une structure cellulaire constituée de tubules, de vésicules et de citernes, responsable de la synthèse des protéines. Cette modification cellulaire au niveau de l'intestin grêle des patients obèses fut confirmée par des observations en microscopie électronique mais aussi par des mesures d'expression protéique.

La deuxième partie de cette thèse décrit la relation entre les troubles associés à l'obésité et des facteurs de risques intestinaux. Des études précédentes ont montré que des rats diabétiques de type 2 présentaient une augmentation de la masse du tissu intestinal, caractérisée par une augmentation du nombre des villosités et une hypertrophie de la muqueuse. Le Chapitre 5 détaille les résultats des mesures de concentrations de marqueurs plasmatiques associés à la perte ainsi qu'au renouvellement des cellules absorbantes intestinales dans le contexte du diabète de type 2 humain. Les patients obèses et diabétiques présentaient une muqueuse plus abondante caractérisée par une augmentation simultanée de la perte et de renouvellement des entérocytes, phénomène non observé chez les gens obèses mais non diabétiques. Ces marqueurs intestinaux étaient de plus positivement corrélés à une hyperglycémie chronique.

Ensuite, nous nous sommes intéressés à un autre trouble associé à l'obésité décrit au cours du Chapitre 6, la stéatose hépatique non alcoolique (NAFLD). Les premiers stades de cette pathologie du foie sont caractérisés par une stéatose et considérés comme bénins, mais peuvent progresser jusqu'a des stades plus sévères tels que la stéato-hépatite non alcoolique (NASH). Observée chez des souris obèses, l'inflammation hépatique semble être liée à l'augmentation de la perméabilité intestinale résultante probablement d'une modification des jonctions serrées, et des niveaux accrus d'une endotoxine d'origine bactérienne, le lipopolysaccharide (LPS), dans la veine porte. Chapitre 6 résume donc notre volonté de comprendre l'implication potentielle du LPS dans la NASH humaine. Nous avons choisi de mesurer

les anticorps systémiques dirigés contre le LPS car sa mesure directe très sensible est perturbée par la présence de bêta-glucanes dérivés du foie. Les anticorps dirigés contre le LPS étaient plus élevés chez les patients obèses présentant une stéato-hépatite non alcoolique en comparaison avec les niveaux mesurés chez les patients obèses avec des foies sains. Ces résultats suggèrent que l'exposition hépatique aux endotoxines joue un rôle dans le développement des stéato-hépatites non alcooliques chez l'homme.

Les stéatoses hépatiques non alcooliques et stéato-hépatites non alcooliques sont actuellement diagnostiquées par des scores histologiques de biopsies du foie, et il semblait nécessaire de mettre au point une méthode moins invasive. De précédentes études ont montré que la stéato-hépatite non alcoolique est associée à une inflammation hépatique caractérisée par l'accumulation de produits d'oxydation, la production de chimiokines et l'activation des neutrophiles. Comme des composés inflammatoires sont également détectables dans l'air expiré, nous avons étudié la faisabilité de l'analyse de l'air expiré comme un outil de diagnostic pour prédire la présence d'une stéato-hépatite non alcoolique (Chapitre 7). Les composés organiques volatils pourraient ainsi refléter la présence d'inflammation hépatique et permettre une détection précoce de la maladie. Ces données doivent néanmoins être confirmées sur une plus grande population.

Dans le Chapitre 8, l'ensemble des résultats fut replacé dans le contexte de la littérature actuelle, dans le but de discuter l'orientation de futures recherches. Premièrement, cette thèse met l'accent sur l'importance de la composition de la flore intestinale, la fonction des cellules de Paneth, et l'inflammation systémique au cours du développement de l'obésité. Dans un second temps, il a pu être mis en évidence que le renouvellement de l'épithélium intestinal est important au cours de l'obésité associée au diabète, pouvant jouer un rôle notamment dans l'élévation des niveaux de glucose postprandiale. Finalement, le rôle prépondérant des endotoxines bactériennes fut démontré dans la genèse des stéato-hépatites non alcooliques chez l'homme.

9.3 NEDERLANDSE SAMENVATTING

Overgewicht en obesitas worden geclassificeerd middels de internationaal erkende standaard 'Body Mass Index' (BMI, het aantal kilogram gedeeld door lengte in meter in het kwadraat), een begrip dat in de 19e eeuw geïntroduceerd werd door de Belgische statisticus Adolphe Quételet. Bij het Kaukasische ras spreken we van overgewicht vanaf een BMI van 25kg/m^2 of meer en van ernstig overgewicht of obesitas vanaf een BMI van 30kg/m^2.

Volgens de Wereldgezondheidsorganisatie zijn er naar schatting in 2015 ruim twee miljard volwassenen met overgewicht en ruim 700 miljoen met obesitas, men spreekt van een zogenaamde pandemie (wereldwijde epidemie). In Nederland heeft ruim de helft van de mensen overgewicht en het aantal mensen met obesitas is de afgelopen drie decennia verdubbeld. De provincie Limburg kent de hoogste incidentie van obesitas (ruim 13% in 2008-2009 volgens het Centraal Bureau van de Statistiek).

In de inleiding van dit proefschrift (Hoofdstuk 1) wordt de omvang en de ernst beschreven van de overgewicht-epidemie en van de aandoeningen die gepaard gaan met overgewicht. Deze aandoeningen, bijvoorbeeld diabetes type 2 (T2DM) en niet-alcoholische vette leverziekte (NAFLD), worden ook wel co-morbiditeiten genoemd. T2DM wordt gekenmerkt door verhoogde glucosewaarden in het bloed ten gevolge van een toegenomen glucoseproductie door de lever en verminderde opname van glucose in de weefsels. Een chronisch verhoogde bloedglucosewaarde kan onder meer leiden tot bloedvatafwijkingen, blindheid en voetproblemen zoals infecties en misvormingen. Bovendien gaat dit vaak samen met een verstoring van het vetmetabolisme en met de ontwikkeling van de leverziekte NAFLD. Deze aandoening kan variëren van relatief onschuldige vervetting tot ernstige ontsteking van de lever, die op haar beurt weer kan resulteren in het ontwikkelen van leverfalen en leverkanker.

In Hoofdstuk 1 wordt ook ingegaan op de behandelingsmogelijkheden voor obesitas en op de huidige literatuur over de vermeende rol van de darm. Belangrijke functies van de darm zijn enerzijds de absorptie van vocht en de opname en vertering van

voeding. Anderzijds vormt de darmwand de barrière tussen het lumen van de darm (het externe milieu) en de bloedbaan (het interne milieu). Deze barrière is essentieel om de gastheer te beschermen tegen de invasie van potentieel schadelijke stoffen, zoals toxinen, bacteriën en virussen. In proefdieren is aangetoond dat een verhoogde darmwand-doorgankelijkheid, een verhoogde passage van bacteriële producten door de darmwand en een veranderde darmflora een rol spelen bij de ontwikkeling van overgewicht.

In **Hoofdstuk 2** worden verschillende testen beschreven die een indicatie geven van de doorgankelijkheid van de darmwand. Deze doorgankelijkheid kan gekwantificeerd worden met behulp van directe en indirecte methoden. Een voorbeeld van een directe methode is het bepalen van de doorlaatbaarheid van de darmwand voor specifieke suikers of eiwitten. Een indirecte methode is bijvoorbeeld het meten van de hoeveelheid antilichamen gericht tegen bacteriële producten in het bloed. Aansluitend wordt in **Hoofdstuk 3** bij proefpersonen met en zonder overgewicht de doorgankelijkheid van verschillende delen van het maagdarmstelsel gerapporteerd. Deze doorgankelijkheid, gemeten met een suikertest, bleek in vergelijking met slanke proefpersonen bij mensen met ernstig overgewicht verhoogd voor wat betreft de maag en de twaalfvingerige darm.

In dierstudies zijn aanwijzingen gevonden dat de doorgankelijkheid van de darmwand beïnvloed wordt door de darmflora. Met de term darmflora worden de micro-organismen (voornamelijk bacteriën) bedoeld die zich in het maag-darmstelsel bevinden. De humane darmflora bestaat met name uit twee groepen bacteriën; de Firmicutes en de Bacteroidetes. Gebaseerd op bevindingen van een verhoogde darmwand-doorgankelijkheid en een veranderde darmflora bij dieren met overgewicht, onderzochten we bij proefpersonen de bacteriële samenstelling in de ontlasting. Mensen met ernstig overgewicht bleken een karakteristieke darmflora te hebben, die gekenmerkt wordt door een veranderde verhouding tussen Firmicutes en Bacteroidetes. In de ontlasting van deze mensen werden meer Firmicutes ten opzichte van Bacteroidetes aangetroffen. Dit komt overeen met resultaten uit andere humane studies en werd bovendien gesterkt doordat we ook een verband vonden tussen de verhouding van deze bacteriën en de BMI.

Aansluitend bepaalden we de hoeveelheid calprotectine, een eiwit dat geproduceerd wordt door witte bloedcellen, in de ontlasting. In het geval van ontsteking reageren witte bloedcellen door calprotectine uit te scheiden. Het gehalte calprotectine in de ontlasting wordt daarom beschouwd als een maat voor de ernst van ontsteking in de darm. In Hoofdstuk 3 werden met name bij die mensen aanwijzingen voor ontsteking gevonden, die gekarakteriseerd werden door een verhoogde verhouding van Firmicutes ten opzichte van Bacteroidetes in de flora. Dit suggereert dat het hebben van obesitas en van een darmflora zoals die in obese mensen wordt gevonden, samengaat met de aanwezigheid van ontsteking in de darm.

Tenslotte werd ook in het bloed het gehalte van een eiwit gemeten dat de mate van ontsteking weergeeft (C-reactief proteïne ofwel CRP). Er bleek ook een verband te bestaan tussen enerzijds de hoeveelheid CRP in het bloed en anderzijds de verhouding van Firmicutes ten opzichte van Bacteroidetes. De darmflora van mensen met obesitas lijkt zowel samen te gaan met de aanwezigheid van locale ontsteking (in de darm) als met algehele ontsteking (in het bloed). Dit is van belang omdat in muizen is aangetoond dat de co-morbiditeiten van obesitas zich ontwikkelen in aanwezigheid van systemische ontsteking. Bovendien bleek recent dat de ernst van deze ontsteking teruggedrongen kan worden door beïnvloeding van de darmflora met zogenaamde pre- of probiotica in muizen. Toekomstig onderzoek moet uitwijzen of dit bij mensen ook mogelijk is.

Zoals hierboven beschreven is de darmflora veranderd in dieren en mensen met obesitas. Omdat de zogenaamde Panethcellen in de darm een grote invloed hebben op de samenstelling van de darmflora, was het onderzoeken van deze cellen een logische volgende stap. Panethcellen zijn gelokaliseerd in de crypten van de dunne darm en produceren bacterie-dodende ofwel antimicrobiële eiwitten. Deze eiwitten spelen een rol in de bescherming van de gastheer tegen de bacteriën die zich in het lumen van de darm bevinden. De Panethcellen van obese patiënten bleken minder antimicrobiële eiwitten te bevatten, zoals beschreven in Hoofdstuk 4. De oorzaak hiervan zou gelegen kunnen zijn in een verstoring van de functie van het Endoplasmatisch Reticulum (ER). Het ER waarborgt de correcte aanmaak van eiwitten in een cel. Onder meer door middel van electronenmicroscopie zagen we dat

de functie van het ER in Panethcellen van obese patiënten verstoord was, dit wordt ook wel ER stress genoemd. Omdat er een verband werd gevonden werd tussen enerzijds deze verstoorde functie en anderzijds de hoeveelheid antimicrobiële eiwitten, is het aannemelijk dat een oorzaak van het lagere eiwitgehalte in obese proefpersonen gelegen is in de verminderde functie van het ER van Panethcellen.

In proefdieren met overgewicht en in proefdieren met T2DM zijn kwantitatieve en kwalitatieve veranderingen in de darmflora beschreven. Bovendien bevat het bloed van muizen die T2DM ontwikkelen naar aanleiding van het consumeren van een vetrijk dieet, een hogere concentratie bacteriële producten. Deze producten zijn oorspronkelijk afkomstig uit het darmlumen. Daarnaast hebben ratten met T2DM langere darmvlokken, meer en grotere dunne darmcellen, en grotere cellen in de crypten van de dunne darm. Op basis van deze resultaten uit dierstudies werd de darmmassa en het verlies van darmcellen in obese mensen met en zonder T2DM onderzocht (Hoofdstuk 5). Obese personen met T2DM bleken -in vergelijking met obese personen zonder suikerziekte- een verhoogde massa en een verhoogd verlies van dunne darmcellen te hebben. De verhouding tussen de massa en het verlies van dunne darmcellen was ook groter bij obese mensen met T2DM in vergelijking met obese mensen zonder T2DM. Dit wijst erop dat er sprake is van een snellere vervanging van afgestorven darmcellen (turnover) bij obese mensen met T2DM. Juist deze dunne darmcellen zijn verantwoordelijk voor de absorptie van voedingsstoffen. Dieren met een verhoogde celmassa hebben inderdaad ook een verhoogde absorptiecapaciteit. Op basis van de resultaten beschreven in Hoofdstuk 5 is het plausibel dat de dunne darm van obese mensen met T2DM ook een verhoogde absorptiecapaciteit heeft.

In de laatste hoofdstukken van dit proefschrift wordt dieper ingegaan op de meest voorkomende leverziekte NAFLD. Leververvetting, het vroege stadium van NAFLD, wordt beschouwd als goedaardig. Een aanzienlijk deel van de patiënten ontwikkelt echter een gevorderd stadium dat wordt gekenmerkt door leverontsteking, ook wel niet-alcoholische steatohepatitis (NASH) genoemd. Deze leverontsteking kan leiden tot levercirrose, leverfibrose en leverfalen. Levercirrose treedt op als leverweefsel dusdanig ernstig beschadigd is, dat herstel niet meer mogelijk is. Naar aanleiding van deze beschadiging sterven levercellen af en ontstaat fibrose oftewel littekenweefsel.

Zowel de oorzaken van NAFLD als de factoren die ten grondslag liggen aan de ontwikkeling van NASH zijn nog niet volledig opgehelderd. Het is wel aangetoond dat dieren met NASH een verhoogde doorgankelijkheid van de darmwand hebben. Hierdoor kunnen bacteriële producten vanuit het lumen van de darm (het externe milieu) door de darmwand heen de gastheer binnendringen (het interne milieu). Normaal gesproken worden deze bacteriële producten via de bloedvaten naar de lever getransporteerd en aldaar uit het bloed gefilterd. Dieren met NASH hebben inderdaad een hogere concentratie van bepaalde bestanddelen van bacteriën, ook wel lipopolysaccharide (LPS) of endotoxine genoemd, in het bloed.

In **Hoofdstuk 6** is onderzocht of LPS ook betrokken is bij de progressie van de vroege stadia van NAFLD naar NASH in mensen. Het is echter gecompliceerd om LPS in het bloed betrouwbaar te meten. Bovendien zijn de bloedvaten die van de darm naar de lever leiden zijn slecht toegankelijk. Om op een zo min mogelijk belastende manier een indruk te krijgen van de concentratie LPS in het bloed tussen de darm en de lever, hebben we gebruik gemaakt van het feit dat het lichaam op de aanwezigheid van LPS reageert met het aanmaken van antilichamen. De concentratie van deze antilichamen tegen LPS in het bloed weerspiegelt de activiteit van het immuunsysteem tegen LPS. Bovendien is voor het meten van de concentratie antilichamen tegen LPS niet perse bloed nodig uit de bloedvaten tussen de darm en lever. Bij patiënten die middels een operatie werden behandeld voor hun overgewicht is toestemming verkregen om bloed en een leverbiopt af te nemen om te kunnen onderzoeken of er een relatie is tussen de ernst van de leverziekte en de hoogte van de concentratie antilichamen tegen LPS. De ernst van NAFLD is door middel van microscopische beoordeling van het leverbiopt geclassificeerd, en vervolgens afgezet tegen de concentraties van antilichamen tegen LPS. Die concentraties in het bloed bleken inderdaad hoger te zijn bij mensen met NASH vergeleken met mensen zonder NASH. Dit suggereert dat LPS een rol speelt bij de ontwikkeling van NASH.

Het beoordelen van een leverbiopt onder de microscoop is op dit moment de gouden standaard om NAFLD en de ernst ervan te diagnosticeren. Echter, de afname van een leverbiopt gaat gepaard met ongemak, een risico op complicaties en hoge kosten. Daarnaast is mede gezien het vele voorkomen van deze ziekte het nemen van een

leverbiopt niet geschikt om te screenen op NAFLD. De recente literatuur adviseert om een leverbiopt af te nemen bij elke obese patiënt die geopereerd wordt en bij alle obese patiënten met risicofactoren. Erkende risicofactoren zijn bijvoorbeeld een hoge bloeddruk en bepaalde verhoogde bloedwaarden, zoals van glucose, insuline, en bepaalde enzymen die de leverfunctie weerspiegelen. Echter, indien er alleen biopten afgenomen worden bij patiënten met deze risicofactoren, blijkt een groot aantal patiënten met NASH te worden gemist. Bovendien wordt er dan ook soms een leverbiopt afgenomen bij mensen die wel risicofactoren hebben, maar geen NASH. Het is dus van belang om een nauwkeuriger, minder invasieve methode te vinden om zowel de aanwezigheid als de afwezigheid van NASH beter te kunnen voorspellen.

Eerder bleek dat de aanwezigheid van ontstekingsprocessen in de longen kan worden aangetoond door het meten van organische stoffen in uitgeademde lucht. Omdat bij NASH ontstekingscomponenten ook een prominente rol spelen, richtten we ons in **Hoofdstuk 7** op de mogelijkheid om de aanwezigheid van NASH te voorspellen op basis van ademanalyse. De ernst van deze leverziekte in obese patiënten werd gerelateerd aan meetbare componenten in de uitademingslucht, zogenaamde Volatile Organic Compounds. De aanwezigheid van NASH bleek op deze manier met een nauwkeurigheid van 90% te voorspellen en de afwezigheid met een nauwkeurigheid van 69%. Deze getallen zijn hoog in vergelijking met de eerder beschreven bloedwaarden, hetgeen betekent dat op basis van deze ademhalingstest veel nauwkeuriger kan worden voorspeld of iemand wel of geen NASH heeft. Deze bevindingen zullen nog uitvoerig moeten worden getoetst voordat van klinische toepassing sprake kan zijn, maar deze test zou in de toekomst als hulpmiddel ingezet kunnen worden om te screenen op NASH en om te beoordelen of een behandeling van een patiënt met NASH aanslaat.

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9.5 SCIENTIFIC OUTPUT

- An update on less-invasive and endoscopic techniques mimicking the effect of bariatric surgery. **FJ Verdam,** R Schouten, JW Greve, GH Koek, ND Bouvy *(accepted in Journal of obesity)*.

- Endoscopic duodenal-jejunal bypass liner rapidly improves type 2 diabetes. C de Jonge, SS Rensen, **FJ Verdam,** RP Vincent, SR Bloom, WA Buurman, CW le Roux, N Schaper, ND Bouvy, JW Greve *(submitted)*.

- Richtlijn voor de behandeling van morbide obesitas. **FJ Verdam,** C de Jonge, JW Greve (*accepted in Nederlands Tijdschrift voor Geneeskunde*).

- Expression of the adipokines LEP, RARRES2, and ANGPT2 in human adipose tissue is associated with non-alcoholic steatohepatitis. MGM Wolfs, SS Rensen, **FJ Verdam,** JW Greve, C Wijmenga, WA Buurman, L Franke, L Scheja, TW van Haeften, MH Hofker, J Fu *(submitted)*.

- Endobarrier tegen obesitas en het metabool syndroom. **FJ Verdam,** PR Liedorp, N Geubbels, R Schouten, IM Janssen, GH Koek, JW Greve. Nederlands Tijdschrift voor Geneeskunde 2012;156(13):A3844.

- Non-alcoholic steatohepatitis; a non-invasive diagnosis by analysis of exhaled breath. **FJ Verdam,** JW Dallinga, A Driessen, C de Jonge, EJC Moonen, JBN van Berkel, J Luijk, ND Bouvy, WA Buurman, SSM Rensen, JWM Greve, FJ van Schooten *(submitted)*.

- Intestinal microbiota composition is related to local and systemic inflammation in human obesity. **FJ Verdam,** S Fuentes, E Zoetendal, H van Eijk, N Bouvy, JW Greve, SS Rensen, W de Vos, WA Buurman (*submitted)*.

- Decreased nucleotide excision repair in steatotic livers associates with myeloperoxidase-immunoreactivity. MA Schults, PW Nagle, SS Rensen, RW Godschalk, A Munnia, M Peluso, SM Claessen, JW Greve, A Driessen, **FJ Verdam,** WA Buurman, FJ van Schooten, RK Chiu. Mutation Research (*2011 Nov 7 Epub ahead of print)*.

- Delayed primary closure of the septic open abdomen with a dynamic closure system. **FJ Verdam,** DE Dolmans, MJ Loos, MH Raber, RJ de Wit, JA Charbon, JP Vroemen. World Journal of Surgery 2011;35(10):2348-55.

- Reduced Paneth cell antimicrobial protein levels correlate with activation of the unfolded protein response in the gut of obese individuals. CM Hodin, **FJ Verdam,** J Grootjans, SS Rensen, FK Verheyen, CHC Dejong, WA Buurman, JW Greve, K Lenaerts. Journal of Pathology 2011;225(2):276-84.

- Small intestinal alterations in severely obese hyperglycemic subjects. **FJ Verdam,** JW Greve, S Roosta, H van Eijk, N Bouvy, WA Buurman, SS Rensen. Journal of Clinical Endocrinology and Metabolism 2011;96(2):E379-83.

- Novel Evidence for Chronic Exposure to Endotoxin in Human Nonalcoholic Steatohepatitis. **FJ Verdam,** SS Rensen, A Driessen, JW Greve, WA Buurman. Journal of Clinical Gastroenterology 2011;45(2):149-52.

- Non-invasive assessment of barrier integrity and function of the human gut. J Grootjans, G Thuijls, **F Verdam,** JP Derikx, K Lenaerts, WA Buurman. World Journal of Gastrointestinal Surgery 2010;2(3):61-9.

- Co-expressed immune and metabolic genes in visceral and subcutaneous adipose tissue from severely obese individuals are associated with plasma HDL and glucose levels: a microarray study. MG Wolfs, SS Rensen, EJ Bruin-Van Dijk, **FJ Verdam,** JW Greve, B Sanjabi, M Bruinenberg, C Wijmenga, TW van Haeften, WA Buurman, L Franke, MH Hofker. BMC Medical Genomics 2010;5(3);34.

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- Free cartilage grafts and healing by secondary intention; a viable reconstructive combination following the excision of non melanoma skin cancer in the nasal alar region. PA van der Eerden, **FJ Verdam,** SCR Dennis, H Vuyk. Archives of Facial Plastic Surgery 2009;11(1):18-23.

- Spread of excitation measurements for the detection of electrode array foldovers: a prospective study comparing 3-dimensional rotational x-ray and intraoperative spread of excitation measurements. W Grolman, A Maat, **FJ Verdam,** Y Simis, B Carelsen, N Freling, RA Tange. Journal of Otology & Neurootology 2009;30(1):27-33.

- Osteomyelitis van de fibula - een bijzondere oorzaak van een compartiment-syndroom bij kinderen. **FJ Verdam,** AJP Joosten. Nederlands tijdschrift voor Orthopedie, maart 2008.

- Een vrouw struikelt met haar handtas en wordt binnengebracht met een haemorragische shock. **FJ Verdam,** DI Vos. Nederlands tijdschrift voor Heelkunde, 3^{e} editie 2008.

- How we do it: Reinsertion of the stylet into the nucleus contour cochlear implant to facilitate second insertion. W Grolman, **FJ Verdam,** RA Tange. Clinical Otolaryngology 2006;31(3); 230–232.

9.6 ABOUT THE AUTHOR

The author of this thesis was born the eldest of three daughters of Jan Verdam and Anneke Verdam-de Witte on October 12th, 1979 in Amsterdam. After graduating from high school (Gemeentelijk Gymnasium, Hilversum), she studied French in Amboise in 1997 and started Pre-medical Studies at the University of Massachusetts in Dartmouth in 1998.

She returned to the Netherlands to obtain her medical degree in Groningen, where she became president of the student council. She finished her general internships at the Martini Hospital in Groningen. She volunteered in hospitals in Kenya and participated in post-graduate courses at the Departments of Reconstructive Surgery and Gastrointestinal Surgery at the Westmead Hospital in Sydney. Thereafter, she returned for her final internship at the Department of Otorhinolaryngology / Head and Neck Surgery at both the University Hospital Groningen and the Academic Medical Centre in Amsterdam, and obtained her medical degree in the summer of 2005.

She started working for the Royal Dutch Football Association (KNVB) and assisted the Netherlands women's national football team under-18. Thereafter, she worked as a resident in the Department of General Surgery of the Amphia Hospital in Breda and Oosterhout. In December 2007, she started this PhD research at the Department of General Surgery at the Maastricht University Medical Centre, led by dr SS Rensen, professor WA Buurman and professor JW Greve. Professor Greve pursued his further career at the Atrium Medical Centre. Hence, these studies were carried out in Maastricht, Heerlen and Brunssum. Financial support for this research was granted by the Transnational University Limburg; a cooperation between the Maastricht University and the Hasselt University.

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