An obese brain and an inflamed body: Central and peripheral consequences of obesity

de Weijer, B.A.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
AN OBESE BRAIN AND AN INFLAMED BODY
Central and peripheral consequences of obesity

AN OBESE BRAIN AND AN INFLAMED BODY
Central and peripheral consequences of obesity

AN OBESE BRAIN
AND AN INFLAMED BODY

Barbara Anna Maria de Weijer

Uitnodiging
Voor het bijwonen van de openbare verdediging van het proefschrift

AN OBESE BRAIN AND AN INFLAMED BODY
Central and peripheral consequences of obesity

Door
Barbara de Weijer

Donderdag 18 februari 2016
om 14.00 uur
In de Agnietenkapel
Oudezijds Voorburgwal 231
Amsterdam

Paranimfen
Nicolette Lammers
Nicolettelammers@hotmail.com
Karin Koopman
Kem.koopman@gmail.com
Barbara de Weijer
Tweede Jacob van Campenstraat 129-3a
1073XR Amsterdam

http://www.proefschriftmaken.nl/ebooks/barbara_de_weijer/
AN OBESE BRAIN AND AN INFLAMED BODY

Central and peripheral consequences of obesity

Barbara Anna Maria de Weijer
COLOFON
AN OBESE BRAIN AND AN INFLAMED BODY
Central and peripheral consequences of obesity
Academic Thesis, Universiteit of Amsterdam, The Netherlands


Copyright © 2015 by B.A.M. de Weijer, Amsterdam, The Netherlands
All rights reserved. No part of this these may be reproduced, stored, or transmitted in any
form or by any means, without prior permission of the author.

Author: Barbara A. de Weijer
Cover design: Proefschriftmaken.nl || Uitgeverij BOXPress
Printed & Lay Out by: Proefschriftmaken.nl || Uitgeverij BOXPress
Published by: Uitgeverij BOXPress, ’s-Hertogenbosch

Printing of this thesis was financially kindly supported by: Universiteit van Amsterdam,
Rijnstate vriendenfonds, Nederlandse obesitas kliniek, Goodlife Healthcare,
ChipSoft, Novo Nordisk and Fresenius-Gabi.
AN OBESE BRAIN AND AN INFLAMED BODY

Central and peripheral consequences of obesity

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 18 februari 2016, te 14.00 uur

door

Barbara Anna Maria de Weijer
geboren te Hilversum
Promotiecommissie

Promotores  Prof. dr. E. Fliers  Universiteit van Amsterdam
Co-promotores  Dr. M.J.M. Serlie  Universiteit van Amsterdam
               Dr. M.C. van Eijk  Universiteit van Amsterdam
Overige leden  Prof. dr. E. Lutgens  Universiteit van Amsterdam
               Prof. dr. T. van der Poll  Universiteit van Amsterdam
               Prof. dr. H. Pijl  Universiteit Leiden
               Dr. L.M. de Brauw  Slotervaart Ziekenhuis
               Prof. dr. C.J.J. Tack  Radboud Universiteit Nijmegen
               Prof. dr. U.H.W. Beuers  Universiteit van Amsterdam

Faculteit der Geneeskunde
Experience is the hardest kind of teacher, it gives you the test first and the lessons afterward.

Oscar Wilde
# TABLE OF CONTENTS

Chapter 1  General introduction 9

**PART I: Obesity and the brain**

Chapter 2  Lower striatal dopamine D$_{2/3}$ receptor availability in obese compared to non-obese subjects. 33

Chapter 3  Striatal dopamine receptor binding in obese women before and after gastric bypass surgery. 41

Chapter 4  Striatal dopamine D$_{2/3}$ receptor availability increases after long-term bariatric surgery-induced weight loss 53

**PART II: Obesity, metabolism and inflammation**

Chapter 5  Hepatic and peripheral insulin sensitivity do not improve 2 weeks after bariatric surgery. 71

Chapter 6  Influx of macrophages and T cells in visceral and subcutaneous adipose tissue of morbidly obese women is not associated with insulin sensitivity. 83

Chapter 7  Serum Retinol Binding Protein-4 Is Inversely Associated with Insulin Action in Adipose Tissue, Skeletal Muscle and Liver in Obese Women. 101

Chapter 8  Morbid obesity is associated with hepatic inflammation independent of liver fat content and insulin sensitivity. 115

Chapter 9  Summary and General discussion 147

Chapter 10  Appendix  
Nederlandse Samenvatting 148  
Author Affiliations 151  
Dankwoord 154  
PhD portfolio 157  
About the author 161
1

General Introduction
Introduction

According to the World Health Organization, the prevalence of obesity has nearly doubled since 1980. Approximately 1.9 billion adults were overweight (BMI ≥ 25 kg/m² and < 30 kg/m²) in 2014 while at least 600 million were obese (1). In the Netherlands, 31.5% of the population is overweight and 10.1% is severely obese (statline.cbs.nl). The potential medical consequences of obesity include development of the metabolic syndrome (2) with complications such as cardiovascular disease, diabetes mellitus type 2 (T2DM), non-alcoholic fatty liver disease (NAFLD), respiratory complications (obstructive sleep apnoea), osteoarthritis of large and small joints and some types of cancer (3; 4; 5). Obesity itself has recently been recognized as a disease by the Obesity Society Council (6).

Not all obese people develop disease since about 20-30% of the obese population remains relatively healthy, and is characterized by preserved insulin sensitivity, absence of hypertension and a favourable lipid, inflammation, hormonal, liver enzyme and immune profile (7; 8). This group is often referred to as metabolically healthy obese (MHO), in contrast with obese people that develop complications referred to as the metabolically abnormal obese (MAO). The MHO group shows a lower lean body mass (LBM), lower visceral adipose tissue content, less fat accumulation in the liver and lower muscle fat infiltration compared to the MAO group, whereas no difference in amount of subcutaneous adipose tissue has been reported (7; 9). Moreover, high birth weight and childhood/adolescence-onset obesity are associated with a MHO profile (10; 11). Mechanisms explaining the differences between the two metabolic phenotypes are largely unknown. Dynamic aspects of adipose tissue (AT) may explain part of the differences because many metabolic abnormalities in obesity-induced insulin resistance correlate with adipocyte characteristics, including adipocyte-size, degree of inflammation within adipose tissue and the secretion of adipokines involved in insulin sensitivity, energy expenditure and fuel handling (12). A causal relationship between dysfunctional AT in the setting of obesity and the metabolic phenotype therefore seems plausible.

To study whether metabolic function and phenotype of adipose tissue contributes to the metabolic derangements in obesity, we performed several studies in morbidly obese subjects undergoing bariatric surgery. We performed in depth metabolic phenotyping combined with tissue analyses and studied independent variables associated with metabolic health.

Obesity has a multifactorial pathogenesis and studies in twins, analyses of familial aggregation, adoption studies and animal models of obesity all indicate that both genetic and environmental factors are determinants of obesity (13; 14; 15; 16). Obesity results from an energy imbalance between calories consumed, i.e. food intake and calories burned, i.e. energy expenditure. The two main reasons why numbers of obesity grow globally are the increased availability of foods high in sugars and fat, and a decrease in physical activity due to the ever more sedentary nature of many jobs, changing modes of transportation, and upcoming urbanization. It has become clear that the decrease in energy expenditure is not
accounted for in energy intake in large percentages of the population leading to increasing body weight. Why food intake is not adjusted in accordance with caloric need is subject of extended basic and clinical research. Studying the underlying mechanisms obviously involves the brain because the brain is the master regulator of energy intake and metabolism. Extended brain areas and circuits control food intake by integrating metabolic and hormonal signals of hunger and satiety and dysregulation of this feedback system contributes to obesity (17).

To study whether there are differences in brain regions involved in food intake between lean and obese subjects and whether the assumed differences are reversible after weight loss, we performed brain imaging and analysed feeding behaviour questionnaires in lean and obese subjects after weight loss.

**PART I Obesity and the brain**

Food intake is essential for survival and it is not surprising that there are multiple pathways, which are coordinated by the brain, that regulate energy intake (figure 1). Food intake is initiated by both external (smell and sight of food) and internal (metabolic and hormonal signals) factors. The homeostatic control of food intake is driven by hunger and satiety signals which inform the brain on energy status resulting in an appropriate feeding response and adjustment in energy expenditure (17; 18). The central homeostatic control of energy metabolism is orchestrated by the hypothalamus. The major neurons involved in homeostatic control are the orexigenic neuropeptide Y (NPY) and agouti related protein (Agrp) neurons and the anorexigenic proopiomelanocortin (POMC) and cocaine amphetamine related transcript (CART) neurons in the arcuate nucleus (ARC). Additionally, input from the nucleus of the solitary tract (NTS) in the brainstem and the afferent vagal nerves from the gut provide information on energy status (19). The control of food intake through homeostatic mechanisms is disturbed in obesity. For example, in rodent models of obesity the anorexigenic effects of leptin are reduced showing a state of leptin resistance. Indeed, despite the fact that most adults are aware of the consequences of eating too much food, it has proven to be difficult to maintain a healthy energy balance illustrated by the obesity epidemic. This might be due to the fact that food is a powerful positive reinforcer caused by the rewarding or hedonic properties of (palatable) food. Individual differences in the reinforcing value of food may provide a mechanism to explain the excess intake and positive energy balance in some, but not all persons (20, 21). Using functional magnetic resonance imaging (fMRI) it has been shown in obese individuals that images of high caloric food trigger an enhanced response in brain areas involved in reward (22) independent of hunger and satiation compared to lean controls (23).

The augmenting value of food is related to activity of the dopaminergic system and it has been shown that dopamine (DA) is the primary neurotransmitter involved in food reinforcement (24; 25). DA is synthesized from phenylalanine in the midbrain ventral tegmental area (VTA) and the substantia nigra. There are 5 dopamine receptor subtypes (D1R-
D5R) besides dopamine transporters responsible for DA re-uptake. The main DA projections run from the VTA towards the nucleus accumbens (NAc) and other regions including dorsal striatum (caudate and putamen), cortical (orbitofrontal cortex (OFC), the cingulate gyrus (ACC), the limbic regions (hippocampus and amygdala) and the lateral hypothalamus (for review 26). Drugs that block dopamine D2 receptors (D2R) increase appetite and result in significant weight gain in rats and humans (27; 28), on the other hand, drugs that increase brain dopamine concentration act anorexigenic (29). Also in humans, using single photon emission and positron emission computed tomography (SPECT/PET) imaging, a decrease in striatal D2/3R was observed in obese individuals compared to lean controls (30) and lower striatal D2/3R availability has been linked to decreased activity in the OFC and ACC in obese humans (31). Moreover, in obese individuals D2/3 receptors negatively correlate with BMI and some but not all human brain imaging studies showed an increase in D2/3R availability after weight loss but the results are inconsistent and the sample sizes low (30; 32; 33). The relationship between feeding behaviour and reward has been studied extensively in rodents and to a smaller extent in humans (for review 34) and those studies show that macronutrient composition, eating pattern and exposure to high palatable food all can modulate the brain circuitry involved in food related reward resulting in overeating. A second neurotransmitter involved in the regulation of food intake is serotonin (5-hydroxytryptamine, 5-HT) (35). An inverse relationship between brain serotonin and food intake has been described (36). We earlier showed that serotonin transporter (SERT) binding within the diencephalon in lean men decreases after a short term hypercaloric high fat high sugar snacking diet while an increase in meal size did not (37; 38). The recently approved 5-HT2c receptor agonists further show the involvement of brain serotonin signalling in regulation of food intake and body weight. Discussion of how serotonin modulates food intake, body weight and energy metabolism is outside the focus of this theses (34).

In summary, obesity is the result of a disbalance between energy intake and expenditure. Overeating results from disrupted hedonic and homeostatic brain circuits and although many underlying mechanisms have been studies in rodent models, translational studies are relatively scarce due to the relative inaccessibility of the human brain. Imaging studies showed that dopaminergic and serotonergic systems might be altered in human obesity but the pathogenesis of these observations remains unclear. Moreover whether the differences between lean and obese subjects are caused by obesity or induce obesity remains matter of debate.

In this thesis, we describe studies on striatal dopamine receptor availability (D2/3R) in lean and obese subjects before and after weight loss to investigate whether lower receptor availability is reversible and whether that relates to healthier eating behaviour (assessed with questionnaires).
PART II Obesity, metabolism and inflammation

Glucose metabolism

Glucose is the major fuel for many organs including the brain and therefore blood glucose concentrations are tightly regulated through peripheral hormones and the autonomic nervous system. After blood glucose levels rise in response to exogenous nutrient supply from the gastrointestinal tract, insulin is secreted from the pancreatic islets and flows via the pancreatic vein through the portal vein exposing the liver to a high concentration of insulin, while a lower concentration of insulin is presented to peripheral tissues. In insulin sensitive tissues like adipose tissue and skeletal muscle insulin binds to its receptor and activates an insulin signalling cascade resulting in recruitment of the glucose transporter 4 (GLUT
4) on the cell membrane resulting in cellular glucose uptake. Glucose is then either stored as glycogen or oxidized. In the liver, insulin suppresses glucose production and activates glycogen synthesis. Lipolysis, the process of degradation of triglycerides within adipocytes resulting in FFA and glycerol efflux is also regulated by insulin. Insulin suppresses lipolysis through inhibition of hormone sensitive lipase. Insulin release is augmented by incretins, gut hormones released after food ingestion. About 50% of the total amount of insulin released in response to ingestion of glucose is attributed to mainly glucagon-like peptide (GLP-1) (39). During fasting, hypoglycemia is prevented by an increase in glucose production, a reduction in peripheral glucose uptake and an increase in lipolysis induced by increased glucagon, cortisol and growth hormone levels as well as activation of the autonomic nervous system. Obesity increases the risk of insulin resistance (IR), which is defined as an impairment of insulin’s capacity to increase peripheral glucose uptake into insulin responsive tissues such as adipose tissue, and muscle, a reduction in suppression of liver endogenous glucose production (EGP) and a reduction in insulin-mediated suppression of lipolysis. IR might also be present in the brain although conflicting data exist (40; 41). IR increases the risk for impaired fasting glucose, glucose intolerance and finally diabetes mellitus type 2. Insulin resistance in obesity is caused by several factors, including increased release of FFA and pro-inflammatory cytokines from adipose tissue, genetic factors (42), lipotoxicity, ectopic lipid accumulation, mitochondrial dysfunction, ER stress (43) as well as eating pattern and macronutrient composition (38; 44). Although many of these pathways have been shown in rodent models of obesity, in humans it is less clear whether and to what extent these pathways contribute to whole body insulin resistance. Finally, as described above brain circuitries involved in glucose metabolism might also be affected by obesity and hyperphagia. In conclusion, obesity-induced insulin resistance has a multifactorial etiology and the exact contribution of each component still needs to be elucidated.

Bariatric surgery

Caloric restriction and weight loss are well known to improve insulin sensitivity (45). Treatment of obesity by a calorie restricted diet, physical exercise, a combination of both, or medication results in a partial reversal of the obesity-induced metabolic alterations when subjects adhere to the intervention. The sequential order of restoration of healthy metabolism is unknown since most studies are performed in a cross-sectional way. Longitudinal studies would be of help, but are very difficult to perform because repeated measurements within a limited time frame are time-consuming, expensive and might overburden the study subjects. Moreover, weight reduction with above mentioned interventions is often modest and difficult to maintain. A more effective method to rapidly lose weight and maintain significant weight loss is bariatric surgery. At present, bariatric surgery is the most effective treatment modality to induce sustained weight loss and reversal of the obesity-induced changes in lipid and glucose metabolism. It reduces cardiovascular risk factors and decreases mortality rates (46; 47; 48). Bariatric procedures result in either reduced food intake (restrictive surgery) and/
or reduced food uptake (malabsorptive surgery). Roux-en-Y gastric bypass surgery (RYGB) and biliopancreatic diversion are the most effective methods in terms of sustained control of weight loss and glucose homeostasis (46). RYGB is the most commonly performed bariatric procedure, and is considered the ‘gold standard’ treatment for morbid obesity (BMI > 40 kg/m² or > 35 kg/m² with obesity-related complications) (49; 50). Current techniques involve the use of a surgical stapler to create a small and vertically oriented gastric pouch, the volume of which is usually less than 30 cm³. The pouch is completely divided by the gastric remnant and is anastomosed to the jejunum (between 30 and 75 cm from the ligament of Treitz), through a narrow gastrojejunal anastomosis in a Roux en-Y fashion (Fig. 2). Bowel continuity is restored by an entero-entero anastomosis between the excluded biliary limb and the alimentary limb. This anastomosis is usually created at 75–100 cm distal to the gastrojejunostomy, although it has also been performed at 100–250 cm in patients with BMI above 50 kg/m². RYGB usually results in 60–70% excess weight loss and most of this effect is maintained (51; 52; 53; 54). Therefore, it can be concluded that bariatric surgery for morbid obesity is associated with long-term weight loss and decreased all-cause mortality and a reduction in morbidity such as cardiovascular disease, hypertension and diabetes.

Figure 2. Schematic representation of RYGB.
The included figure is the property of Johnson and Johnson and Ethicon Endo-Surgery (Europe). Reprint with permission from: KJ Neff, T Olbers and CW le Roux. Bariatric surgery: the challenges with candidate selection, individualizing treatment and clinical outcomes. Copyright © 2013 Neff et al; licensee BioMed
Because of its major effect on weight loss and metabolism, bariatric surgery can be used as a model to study alterations that contribute to improvement in insulin sensitivity. Recently, reports have been published on amelioration of insulin sensitivity within 10 days after malabsorptive bariatric surgery. This phenomena occurred in the absence of significant weight loss (55; 56). Alterations in more traditional glucoregulatory factors, like free fatty acids (FFA) and adiponectin could not explain this early improvement. Compared to similar weight loss during a very low caloric diet (VLCD), RYGB has a greater effect on glucose metabolism which indicates that other mechanisms besides weight loss must be involved (57). One concept is that by bypassing the proximal gastrointestinal tract from the nutrient flow an increase in release of gut hormones such as GLP-1 and peptide YY (PYY) is induced (58; 59). GLP-1 has insulinotropic effects by stimulating proliferation of β-cells of the pancreas and additionally GLP-1 inhibits glucagon secretion, suppresses endogenous glucose production (EGP), slows gastric emptying and promotes satiety (60; 61). Although the effect on weight loss is not dependent on GLP-1 since GLP-1 knock out animals still show weight loss after bariatric surgery (59; 62). PYY has an anorectic action on the central nervous system (CNS) via the vagal nerve and reduces gastric emptying and inhibits pancreatic exocrine function (63). Another result of bypassing a part of the gastrointestinal (GI) tract is adaptation and restructuring of cells of the intestine (for review 64). Duodenum-jejunal bypass, leaving the stomach intact and bypassing the upper gut, in Zucker rats leads to atrophy of the bypassed gut and hyperplasia in the jejunum (65). In normal physiology the duodenum and jejunum resorb most macronutrients, whereas the ileum absorbs micronutrients. As such the proximal intestine takes up most of the dietary glucose by the sodium-D-glucose co-transporter 1 (SGLT1). SGLT1 overexpression is associated with obesity in murine models (66), leading to increased glucose transport. Furthermore, SGLT1 is also overexpressed three- to four fold in human and animal models of DM2 (67; 68). In a rodent RYGB model it was shown that intestinal glucose uptake is reduced (69), providing a possible mechanism for some of the antidiabetic effects of RYGB.

Another mechanism of bariatric surgery-induced amelioration of glucose metabolism is through changes in bile acids (BA). Recently bile acids (BA) have been recognized as hormones affecting whole body metabolism (70). Bile acids are required for the uptake of lipids from the intestine, they are produced in the liver and released in the duodenum in response to fat ingestion. Most bile acids are reabsorbed in the ileum by apical sodium-dependent bile acid transporters (ASBT). Serum BA levels are increased after RYGB in human and animal models (71; 72; 73). Two BA receptors have been identified, i.e. the farnesoid X receptor (FXR) (74) and the Takede G-protein-coupled receptor-5 (TGR5) (75). TGR5 is considered to increase GLP-1 release thereby improving insulin secretion and insulin sensitivity (76). FXR is the main regulator of BA metabolism and is involved in lipid metabolism, energy homeostasis and insulin sensitivity (for review 77). FXR exerts its effects on BA secretion through fibroblast growth factor 19 (FGF19 in humans and FGF15 in mice) and FGF21 (78; 79). In mice FGF19 increases metabolic rate and energy expenditure in response to a high fat diet (80). Whether
BA and FGF 19 or FGF21 mediate the quick improvements in energy metabolism observed after bariatric surgery is unclear since in humans no changes in FGF19 or BA were observed in the first week after RYGB (81). Finally, malabsorptive bariatric surgery inevitable leads to an alteration in communication between the brain and the gut, thereby possibly affecting regulation of body weight and aspects of glucose metabolism (82; 83).

In short, bariatric surgery has a sustained effect on body weight loss, obesity-related morbidity and insulin sensitivity. The short-term and long-term beneficial effects of RYGB on body weight and glucose metabolism are partly elucidated and include malabsorption, reduced food intake, gastrointestinal enteroplasticity, change in gut hormone secretion, bile acids and altered communication between the gut and the brain. Moreover it is not clear whether the improvement in glucose metabolism is due to an increase in β-cell function, an increase in hepatic or peripheral insulin sensitivity, or a combination of these factors. In the present thesis, we aimed to investigate the short-term effect of bariatric surgery on basal glucose metabolism, insulin sensitivity, lipolysis and striatal D2/3R availability.

**Adipose tissue as an endocrine organ**

Adipose tissue is composed of pre-adipocytes, mature lipid filled adipocytes and the stromal vascular fraction, containing adipose tissue macrophages (ATM), lymphocytes and vascular endothelial cells (84; 85). The primary function of adipose tissue is to store excessive calories as triglycerides (TG) and to release free fatty acids during fasting. In the 1990’s adipose tissue was also recognized as an endocrine organ (86) because of its ability to secrete molecules acting in distant tissues. The cytokines and hormones secreted by adipose tissue are referred to as adipokines. They exert their biological functions both in a local and systemic manner, influencing many biological processes including glucose metabolism (87). The first adipokine hormone to be discovered was leptin (86). Leptin is mainly produced in adipocytes, and correlates with body fat content (88). It plays an important role in regulating and controlling food intake and it is referred to as ‘the satiety hormone’. Since then, many new adipokines have been identified (89) among which the adipokine adiponectin, which is solely produced by adipose tissue, has a role in improving insulin sensitivity. In the contrary to leptin, adiponectin levels are inversely correlated with adipose tissue mass (90).

More recently retinol-binding protein 4 (RBP₄) has been added to the list of adipokines. RBP₄ is a transport protein for vitamin A and its main function is delivering retinol to tissues. It is synthesized mainly by hepatocytes and adipose tissue (91). Recent studies revealed that RBP₄ is increased in obesity and that overexpression of RBP₄ induces insulin resistance (graham 2006 NEJM). The precise role of RBP₄ in regulating peripheral and hepatic insulin sensitivity in obese human subjects remains to be elucidated (92).

Besides adipokines, cytokines originating from adipose tissue have been shown to be associated with insulin sensitivity. A link between adipose tissue inflammation, insulin resistance and obesity was made a decade ago (84; 93) while tumor necrosis factor alpha (TNF-α) was recognized as one of the first pro inflammatory cytokines secreted by...
adipose tissue (94). Hereafter other cytokines were identified including interleukin-6 (IL-6), monochemoattractant protein-1 (MCP-1), II10, serum amyloid A (SAA), interleukin-1β (IL-1β) and others (86; 95; 96; 97; 98; 99).

Pro-inflammatory profiles of adipose tissue, high serum leptin and RBP4 and lower adiponectin all have been linked to insulin resistance and most studies show that adipose tissue function is important in maintaining glucose homeostasis. Disrupted secretion or function of adipocytes hampers whole body insulin sensitivity. Whether tissue specific inflammatory phenotypes independently contribute to insulin resistance in obesity in humans is still not known.

This thesis describes studies on mRNA expression levels of inflammatory proteins and RBP4 in insulin sensitive tissue in relation to basal glucose metabolism, insulin sensitivity and lipolysis in morbidly obese women undergoing bariatric surgery.

**Inflammation in obesity**

Obesity is associated with a state of chronic low grade inflammation with higher circulating C-reactive protein (CRP) (100). In the early nineties Hotamisligil et al reported TNF-α to be locally produced in adipose tissue in states of obesity and interfere with insulin sensitivity (94). Since then it has been shown in numerous studies in animals and humans that adipose tissue in obesity is characterized by an influx of bone marrow-derived immune cells including macrophages resulting in a shift in balance between anti-inflammatory and pro-inflammatory cytokines favoring the pro-inflammatory state (84; 101; 102). In the lean condition, adipose tissue resident macrophages (ATM) are alternatively activated, referred to as an M2 state, with predominant production of anti-inflammatory proteins aimed for tissue repair, extracellular matrix modeling and adipogenesis (103).

When obesity develops, adipose tissue expands due to hypertrophy of adipocytes. When the adipocyte reaches a critical cell size it starts to secrete growth factors to induce pre-adipocyte proliferation (104), leading to hyperplasia. The efficiency and capacity of adipocytes to store lipids within the lipid droplets are controls by lipid droplet proteins like CIDEA (105) perilipin and adipophilin (also known as adipocyte differentiation relate protein, ADRP) (106). It has been shown that macrophage content of adipose tissue is positively related to adipocyte size with macrophages organized in crown like structures surrounding adipocytes (84). Whether cell size is the triggering factor in the induction of inflammation is still matter of debate but blocking hormone sensitive lipase in adipose tissue resulting in large lipid-loaded adipocytes also triggers an inflammatory state (107). The overall hypothesis is that when adipose tissue becomes dysfunctional in terms of balancing lipid storage and lipid breakdown due to the inflammatory state, fatty acids are released from adipose tissue and stored in non-adipose tissue like muscle and liver. This so called ectopic lipid accumulation contributes to whole body insulin resistance.

There are several pathways that contribute to the inflammatory state of adipose tissue in obesity. One pathway involves tissue hypoxia because of inadequate absence of increased
vascularization and vascular endothelial growth factor (VEGF) (108) despite an increase in adipose mass. This leads to a cascade of events including induction of hypoxia inducible factor-1 (HIF-1) (109) recruitment of numerous immune cells and production of pro-inflammatory proteins (110). The pro-inflammatory cytokines negatively impact the insulin signaling pathway either directly or indirectly by stimulating inflammatory pathways (111) and reduce insulin-mediated suppression of lipolysis (112) (figure 3). However, despite tissue hypoxia, adipocyte cell death is increased some (107) but not all studies in obese humans (113). Besides, ATM can cause tissue damage and inhibit cell proliferation (114). Other pathways involved in triggering an inflammatory state in adipose tissue in obesity include among others ER-stress, adipocyte necrosis, altered adipokine secretion, upregulation of MCP-1 and abnormal extracellular matrix remodeling leading to fibrosis of adipose tissue (for review: 115).

Besides recruited and resident macrophages, other immune cells are invading adipose tissue in obesity. Mast cells and natural killer T cells (NKT) within adipose tissue contribute to IR in diet-induced obesity (116; 117) and recent data showed an increase in recruited B and T lymphocytes into adipose tissue in obesity (figure 3) (118). Regulatory T cells (Tregs), especially the CD4+ population, serve to suppress the immune response of other inflammatory cells in order to protect the human body from excessive innate immune reactions. Therefore it is not surprising that Tregs are increased in adipose tissue in obese conditions (118). Tregs positive for CD4, FOXP3 and CD25 cells represent activated T cells and secrete the anti-inflammatory cytokine IL10. IL10 inhibits TNF-α production by macrophages and surprisingly it has been shown that in obese visceral adipose tissue the amount of Tregs is diminished (118; 119; 120) therefore contributing to an ongoing pro-inflammatory state. Additionally, high levels of insulin inhibit IL10 production by Tregs (121), suggesting that the hyperinsulinemic state occurring in obesity might further contribute to the development of the state of low-grade inflammation.

Finally it has been shown that modulation of inflammation in rodents and weight loss in humans reduces adipose tissue inflammation and increases insulin sensitivity (122). Inflammatory changes within adipose tissue occur both in visceral and subcutaneous compartments but most mechanistic studies in rodents focus on visceral fat. It has been shown that increased visceral fat increases the risk for insulin resistance and comparing subjects matched for BMI, those with higher percentage of visceral adipose tissue (VAT) are more insulin resistant compared to subjects with a higher percentage of subcutaneous adipose tissue (SAT) (123). VAT shows a higher expression of MCP-1, which is produced by macrophages and endothelial cells, and recruits monocytes, leukocytes and other inflammatory cells (for review 124). In addition, the macrophage markers CD68 and CD14 show greater expression in VAT compared to SAT (125). It is of interest studying whether inflammatory changes within VAT are more pronounced compared to subcutaneous adipose tissue and whether that predicts IR in humans. On the other hand one could hypothesize that as long as subcutaneous adipose tissue is able to sequester the caloric surplus adequately,
insulin sensitivity is preserved and the occurrence of inflammation in adipose tissue offsets IR. In this thesis we therefore study inflammatory changes within both adipose tissue compartments and relate these to insulin sensitive metabolic fluxes.

In summary, caloric excess is stored as triglycerides within adipocytes that become enlarged. Long term obesity is associated with influx of immune cells into adipose tissue, creating a pro-inflammatory environment and a low grad inflammatory state. Inflamed adipose tissue is associated with adipose tissue dysfunction and insulin resistance. Ongoing lipolysis leads to ectopic lipid accumulation resulting in further reduction of insulin sensitivity. Most mechanistic studies on this topic are performed in rodent models of obesity and it remains to be clarified if and to what extent inflammation in different adipose tissue compartments contribute to insulin sensitivity in obese humans. Therefore in this thesis we aimed to study inflammatory expression profiles in omental and subcutaneous adipose tissue compartments and to correlate these findings with basal glucose metabolism as well as whole body, liver and adipose tissue insulin sensitivity.

**Figure 3.** Inflammatory changes in expanding adipose tissue leading to a reduction in metabolic control. Reprinted with permission from: Adipokines in inflammation and metabolic disease. Noriyuki Ouchi, Jennifer L. Parker, Jesse J. Lugus & Kenneth Walsh Nature Reviews Immunology 11, 85-97 (February 2011). Permission number: 3567900536371.

**Liver steatosis, hepatic insulin sensitivity and inflammation in obesity**

The liver plays an essential role in glucose and lipid metabolism and disrupted liver function in the setting of obesity leads to hyperglycemia and dyslipidemia. Obesity is associated with increased storage of liver fat, i.e. liver steatosis defined as a triglyceride storage of > 5.7% (126). Liver steatosis increases the risk for nonalcoholic fatty liver disease (NAFLD),
nonalcoholic steatohepatitis (NASH) and cirrhosis, which can lead to liver failure and hepatocellular carcinoma (127). An non-invasive method to quantify the lipid content of the liver is magnetic is resonance spectroscopy (1H-MRS) (126). Lipid accumulation in the liver can be higher due to an increased uptake of FA from dietary fat, increased FA released from adipose tissue and from de novo lipogenesis or reduced FA oxidation (128; 129). In addition, adiponectin, an insulin sensitizing adipokine, is lower in subjects with NAFLD compared to BMI matched controls (130; 131) and replenishment of adiponectin in obese mice reverses insulin resistance and alleviates NAFLD (132). In animal models short term high fat feeding leads to NAFLD and hepatic insulin resistance without peripheral insulin resistance (133). Excessive TG storage in the liver is associated with insulin resistance in some but not all studies (134). A major role for diacylglycerol with subsequent activation of protein kinase C (PKC) has been described underlying the association between liver steatosis and insulin resistance. (135; 136). And recently heme oxygenase-1 has been proposed as a pro-inflammatory signal linking inflammation to insulin resistance in mice on a high fat diet (137). Moreover lipid-induced endoplasmic reticulum (ER) stress has been associated with insulin resistance in mice (138). In parallel with inflammatory changes occurring in adipose tissue, obesity is associated with inflammation in liver in rodents. In vivo models in mice showed that chronic systemic inflammation leads to increased lipid accumulation in liver (139; 140). Whether liver steatosis per se contributes to liver inflammation or vice versa remains a matter of debate. So far it has been shown that inflammatory changes in NAFLD have been attributed to a reduction in peroxisome proliferator-activated receptor α (PPARα) expression, lower circulating adiponectin, exposure to cytokines derived from adipose tissue as well as lipotoxicity induced by fatty acid overload (141; 142). Finally, Kupffer cells (the resident macrophages of the liver) decrease in rodents on a high fat diet while recruited myeloid cells invade the liver through a mechanism involving the C-C chemokine receptor type 2 (CCR2). Subsequently the invaded immune cells trigger hepatic fat accumulation which is dependent on chemokine (C-C motif) ligand 2 (CCL2)/CCR2 (15). In support, short term infusion of oleate increases hepatic recruitment of myeloid cells, suggesting that increased portal delivery of FFA both induces inflammation and triglyceride (TG) accumulation through induction of chemotaxis (143). Finally, increased levels of TNFα induce upregulation of the nuclear transcription factor SREBP-1 and the enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) and increase de novo lipogenesis (139; 141).

To study the relation between liver inflammation, hepatic fat content and hepatic insulin sensitivity in humans, we assessed glucose metabolism, liver fat and expression profiles of inflammatory markers in liver biopsies from morbidly obese women undergoing bariatric surgery and describe the outcome of these studies in this thesis.
Aims and outline of this thesis

The main aims of this thesis were to study:

1. Striatal dopamine receptor availability in obese women and controls as well as the reversibility of lower striatal dopamine receptor availability after short and long term weight loss.

2. The role of inflammation in insulin resistance.

Part 1 of the thesis describes three studies on striatal dopamine D_{2/3} receptor availability. In chapter two we compared striatal dopamine D_{2/3} receptor availability between obese and lean women. In chapter three we studied changes in striatal dopamine D_{2/3} receptor availability in obese women before and shortly after Roux-en-Y gastric bypass surgery, and in chapter four we studied long-term changes in striatal dopamine D_{2/3} receptor availability, i.e. more than three years after bariatric surgery in the same cohort.

Part 2 of this thesis describes four studies on inflammation in adipose tissue and liver of obese women and its relation to insulin sensitivity. In chapter five we focussed on short-term effects of RYGB surgery on hepatic, peripheral and adipose tissue insulin sensitivity. In chapter six we studied the expression of pro- and anti-inflammatory markers in adipose tissue in relation to metabolic fluxes. In chapter seven we describe the results of the relation
between the adipokine retinol binding protein 4 (RBP4) in adipose tissue and liver and insulin sensitivity in obese women. Finally, in chapter eight we studied the relationship between inflammatory changes in liver and hepatic insulin sensitivity as well as liver fat content.

References


Chapter 1

GENERAL INTRODUCTION


PART I

OBESITY AND THE BRAIN
Lower striatal dopamine $D_{2/3}$ receptor availability in obese compared to non-obese subjects

Barbara A. de Weijer*, Elsmarieke van de Giessen*, Thérèse A. van Amelsvoort, Erik Boot, Breg Braak, Ignace M. Janssen, Arnold van de Laar, Eric Fliers, Mireille J. Serlie and Jan Booij

* These authors contributed equally to this work

Abstract

**Background:** Obesity results from a relative excess in energy intake over energy expenditure. These processes are controlled by genetic, environmental, psychological and biological factors. One of the factors involved in the regulation of food intake and satiety is dopaminergic signalling. A small number of studies have reported that striatal dopamine D2/D3 receptor (D2/3R) availability is lower in morbidly obese subjects.

**Methods:** To confirm the role of D2/3R in obesity, we measured striatal D2/3R availability, using [123I]IBZM SPECT, in 15 obese women and 15 non-obese controls.

**Results:** Striatal D2/3R availability was 23% (p = 0.028) lower in obese compared to non-obese women.

**Conclusions:** This study is an independent replication of the finding that severely obese subjects have lower striatal D2/3R availability. Our findings invigorate the evidence for lower striatal D2/3R availability in obesity and confirm the role of the striatal dopaminergic reward system in obesity.
Introduction

Over the last decades, the average body mass index (BMI) has increased world-wide. The prevalence of obesity (BMI ≥ 30 kg/m²) in the US is now over 30% among adults [1]. This leads to a substantial increase in obesity-related diseases and costs. Obesity is the result of an imbalance between energy intake and energy expenditure, and these processes are normally controlled by genetic, environmental, psychological and biological factors. Excessive caloric intake of highly palatable food can be regarded as compulsive-like feeding behaviour [2]. The mechanisms underlying disturbed appetite regulation and overeating, are poorly understood. However, a role for several neurotransmitters and hormones has been proposed (for a review see [3]). There is a large body of evidence that suggests that overeating in obesity involves the neurotransmitter dopamine. Dopaminergic agonists induce anorexigenic effects, while treatment with dopamine D₂ receptor (D2R) antagonists (neuroleptics) induces obesity [4]. Moreover, a high prevalence of the TaqIA A1 allele for the D2R, an allele known to moderate food reward, has been found in obesity [5; 6]. Finally, a role for dopamine and the D2R has been established in animal models of obesity [2]. Interestingly, two imaging studies by the same group showed lower striatal D2/3R availability in obese versus non-obese subjects [7; 8], although in another study statistically significant lower availability in obese subjects was only found by a voxel-based and not by region-of-interest (ROI) analysis [9]. D2/3R imaging studies in obese humans are scarce and inconclusive. Therefore we evaluated whether earlier findings of lower striatal D2/3R availability in obesity can be replicated, in order to increase our understanding on the potential role of dopamine in obesity.

Material and methods

Subjects

We included 15 obese (BMI ≥ 35 kg/m²) women who were matched with 15 non-obese historical female controls who participated in previous studies [10, 11]. Exclusion criteria for all subjects were: (1) age below 18 years, (2) current or past psychiatric disease, (3) current or past exposure to dopaminergic medication, (4) lifetime history of alcohol/drug abuse, (5) concomitant or past severe medical conditions, including diabetes mellitus, (6) pregnancy. The 15 obese subjects participate in an on-going study on the early metabolic effects of Roux en Y gastric bypass surgery. Here we report on the assessment of striatal D2/3R availability before surgery. Each participant gave written informed consent. The protocol was approved by the ethics committee of the Academic Medical Center of Amsterdam.

Neuropsychological assessment

The obese subjects underwent neuropsychological assessment by the team involved in the pre-assessment for surgery and filled out the Beck Depression Inventory version II (BDI-II) for assessment of depressive symptoms.
**SPECT protocol**

The subjects underwent a measurement of D2/3R binding potential (BP_{ND}) with SPECT and the selective radiolabeled D2/3R antagonist \([^{123}I]IBZM\), using the sustained equilibrium/constant infusion technique [12]. The applied protocol has been described in detail previously [11]. SPECT data were acquired for approximately 60 minutes, starting from 120 minutes after the initiation of \([^{123}I]IBZM\) administration. SPECT studies were performed using a 12-detector single slice brain-dedicated scanner (Neurofocus, Inc., Medfield, Massachusetts, USA). The obese subjects were scanned in the morning after an overnight fast, the lean subjects were scanned at various moments of the day and they were not fasting.

**Image reconstruction and analysis**

Attenuation correction of all images was performed as earlier described [13]. SPECT data were reconstructed in 3-D mode and analysed by the same investigator (BdeW). For quantification, a ROI analysis was performed, with fixed ROIs for the striatum and occipital cortex, as described earlier [11]. Mean striatal and mean occipital binding were averaged from right and left ROIs. BP_{ND} was calculated as the ratio of specific to non-specific binding ((total activity in striatum - activity in occipital cortex) / activity occipital cortex).

**Statistical analysis**

BMI and age differences between groups were evaluated with a non-paired t-test. Between-group comparisons in striatal D2/3R BP_{ND} were performed by ANCOVA. Since in-vivo D2/3R availability is influenced by natural ageing [14], age was introduced as a co-variate. Pearson correlation coefficients were calculated with two-tailed tests of significance to investigate the relationship between striatal D2/3R BP_{ND} and BMI. A probability value of 0.05 two-tailed was selected as significance level.

**Results**

Mean BMI of the obese women was 46.8 ± 6.5 kg/m² versus 21.7 ± 2.1 kg/m² of the controls (Table 1; p < 0.0001). The obese women were older (37.8 ± 7.0 years) than the controls (28.0 ± 10.4 years; p = 0.0057). The BDI-II results showed that none of the obese women had severe depressive symptoms, only one felt in the category of mild depression (score of 14) and the others had even lower scores (scores 0-13).

Mean BP_{ND} as a measure of striatal D2/3R availability was 23% lower in the obese group: 0.86 ± 0.22 for the obese subjects and 1.12 ± 0.24 for the controls (Table 1). The ANCOVA revealed a significant main effect of group on D2/3R availability in the striatum (F(1,29) = 5.39, p = 0.028). There was no significant effect of age on BP_{ND} (F(1,29) = 0.69, p = 0.412). BMI did not correlate significantly with BP_{ND} within the obese (r = -0.392, p = 0.149) or control group (r = -0.141, p = 0.617).
Table 1. Descriptive characteristics for obese and non-obese control subjects.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>OBESE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>BMI (KG/M²)</td>
<td>21.7 ± 2.1 (19.5 – 27.6)</td>
<td>46.8 ± 6.5 (38.7 – 61.3)</td>
</tr>
<tr>
<td>AGE (YEARS)</td>
<td>28.0 ± 10.4 (20 – 60)</td>
<td>37.8 ± 7.0 (26 – 49)</td>
</tr>
<tr>
<td>BDI-II SCORE</td>
<td>n.a.</td>
<td>5.6 ± 4.2 (0 – 14)</td>
</tr>
<tr>
<td>STRIATAL D2/3R AVAILABILITY (BP_ND)</td>
<td>1.12 ± 0.24 (0.75 – 1.78)</td>
<td>0.86 ± 0.22 (0.5 – 1.28)</td>
</tr>
</tbody>
</table>

Data are shown as: mean ± standard deviation (range). BDI-II = Beck Depression Inventory version II.

![striatal D2/3R availability](image)

**Figure 1.** Striatal D2/3R availability for obese and non-obese control subjects. Horizontal line indicates mean BP_ND.

### Discussion

This study replicates earlier findings that obese subjects have lower striatal D2/3R availability than non-obese subjects. The first two studies to demonstrate this difference [7; 8] were in a largely overlapping sample of obese subjects with a mean BMI of 51 kg/m² Haltia et al. [9] replicated this finding only with a voxel-based analysis, reporting a lower D2/3R availability in obese subjects in a cluster partly covering the striatum. The major difference with the first study was that the average BMI of the obese group was lower (33 kg/m²). In the present study, we included obese women with a mean BMI of 47 kg/m² and we were able to replicate the finding with a ROI analysis. Thus, this suggests a decrease in striatal D2/3R availability with increasing BMI. This is strengthened by the finding of a negative correlation between BMI and striatal D2/3R availability in the obese groups in the previous studies [7; 9].

It should be mentioned that one study, performed in patients undergoing bariatric surgery, found no significant difference in striatal D2/3R availability between obese subjects and historical controls [15]. However, this study included only five women per group. Although no statistical test was described, absolute D2/3R availability shown in a graph was lower in the
obese than control subjects. Thus, this study may not have been able to detect a difference in D2/3R availability between obese and controls due to insufficient sample size.

The present study nor the previous ones can solve the question whether lower striatal D2/3R availability in obesity is a causal factor in obesity or rather the result of the obese condition. Carriers of the Taq1A allele in the gene encoding for the D2/3R show decreased D2/3R expression [16] and have a higher susceptibility for obesity [5]. This would suggest that lower D2/3R expression levels are a pre-existing condition that plays a role in the susceptibility. However, in rats it has been shown that downregulation of striatal D2/3R can be induced by a cafeteria diet and that this is associated with an increase in the susceptibility for reward deficits and compulsive eating behavior [2]. The available studies on effects of weight loss after bariatric surgery on D2/3R availability are scarce and show conflicting results [15; 17].

The involvement of dopamine signalling in regulation of food intake has been clearly established [3]. Its major functions are related to motivation and reward and involvement in salience attribution to food. Food intake induces a dopamine release in the striatum thereby exerting its rewarding effect [18]. This is similar to the effects of drugs of abuse [19], suggesting parallels between obesity and drug addiction [3]. Part of the etiology of both conditions could be explained by a hypodopaminergic mesolimbic system that leads to increased motivation for food and drugs, respectively [3]. In this context, it is of interest that the extent of lower striatal D2/3R availability in obese subjects is comparable to cocaine and alcohol abusers [19]. Nevertheless, lower striatal D2/3R availability is probably only one underlying mechanism in the disturbed balance between energy intake and energy expenditure present in obese subjects. Peripheral metabolic signals, e.g. leptin, ghrelin, insulin and hypothalamic neuropeptides are able to interact with the striatal dopaminergic system as well [3]. This complexity may explain the considerable overlap in striatal D2/3R availability between obese and non-obese women in the present study.

A limitation of this study is the difference in age between the obese and control subjects. To correct for this, age was added as a covariate to the statistical model. Besides, it has previously been shown that age leads to a decrease of 4.6 % to 8.2 % D2/3R availability per decade [14; 20]. As we found a 23% lower D2/3R availability in our obese subjects, this difference is too large to be explained by age per se. Therefore, we believe that the age difference does not significantly affect our results and conclusions.

The two groups were not scanned under the same conditions regarding fasting state. While the obesity patients were scanned after an overnight fast, the healthy controls were not scanned in the fasted state. As previously mentioned, food intake induces a striatal dopamine release [18], so this can transiently lead to increased dopamine levels. However, even if the fed state in the lean group would have led to increased dopamine levels, this would have resulted in a decrease of D2/3R availability, and subsequently in an underestimation of the presently observed difference between the obese and lean group.

Unlike previous studies on D2/3R availability in obesity with mixed gender samples, this study only included women. Although this may affect the extrapolation of the results to
men, it increased the homogeneity of the subjects and demonstrates that the lower D2/3R availability is also detectable in females only.

In conclusion, this study is an independent replication of the earlier finding that morbidly obese subjects have lower striatal D2/3R availability detected by ROI analysis [7]. In combination with the other available studies on this subject so far, this study invigorates the evidence for lower striatal D2/3R availability in obesity and confirms the role of the striatal dopaminergic reward system in obesity.

Reference List


Striatal dopamine receptor binding and insulin sensitivity in obese women before and after gastric bypass surgery

Barbara A. de Weijer, Elsmarieke van de Giessen, Ignace Janssen, Frits J. Berends, Arnold van de Laar, Mariette T. Ackermans, Eric Fliers, Susanne E. la Fleur, Jan Booij and Mireille J. Serlie

Diabetologia. 2014 May;57(5):1078-80.
Abstract

**Background:** In cross-sectional studies, a reduction in striatal D$_{2/3}$ receptor (D2/3R) binding has been reported in obese humans. It is unknown whether this reflects a cause or consequence of the obese state. In addition, the relation between metabolic health and striatal D2/3R is unclear at present. We therefore studied striatal D2/3R availability two weeks before and six weeks after a hypocaloric metabolic state accompanied by weight loss in morbidly obese women undergoing Roux-en-Y gastric bypass surgery (RYGB).

**Methods:** In morbidly obese women, striatal D2/3R availability was assessed using a brain-dedicated SPECT scanner and $[^{123}]$IBZM. Insulin sensitivity was determined at baseline during a two-step hyperinsulinemic euglycemic clamp using a stable glucose isotope tracer.

**Results:** We included 19 women. After RYGB, BMI was reduced, but D2/3R availability did not change significantly. Glucose production and hepatic insulin sensitivity did not correlate significantly with D2/3R availability while peripheral insulin sensitivity tended to correlate positively with D2/3R availability.

**Conclusions:** A hypocaloric state and significant weight loss following RYGB in morbidly obese women did not increase D2/3R availability. Moreover, the D2/3R did not correlate to measures of insulin sensitivity. This finding does not support an important role for striatal D2/3R in short-term changes in energy balance or weight loss in obesity.
Introduction

Unravelling the association between obesity and disturbances in lipid and glucose metabolism is necessary to improve future treatment modalities. The relationship between the increase in fat mass and the metabolic perturbations is complex and probably depends on the nature of the adaptive response to the hypercaloric milieu. Some of the metabolic adaptations are orchestrated by the brain. Areas that are involved include the hypothalamus, the prefrontal cortex and striatum (1,2). There is indeed evidence that these brain areas are functionally altered in obesity. In obese humans, we and others reported a reduction in dopamine D<sub>2/3</sub> receptor (D2/3R) binding in the striatum, an important component of the brain reward system (3,4). In addition, a hypercaloric diet for two days in lean healthy subjects resulted in a different activity pattern to visual food cues in the prefrontal cortex and hypothalamus, measured with functional MRI (fMRI), implying a central adaptation to the hypercaloric milieu (5).

Food is a powerful primary reinforcer, and individual differences in the reinforcing efficacy of food may provide a mechanism to explain the excess food intake and positive energy balance responsible for obesity (3,6). The neurotransmitter dopamine is important for the reinforcing value of food and it has been shown that food can induce a release of endogenous dopamine in the striatum (7). Obese subjects are thought to be more sensitive to food reinforcement than those who are non-obese. Using fMRI, it was shown that obese subjects show a greater hemodynamic response to visual food stimuli in dopamine-rich regions such as the nucleus accumbens/ventral striatum, caudate nucleus and putamen (8,9). This may underlie the notion that obese humans experience increased craving for food (10). In addiction, striatal D2/3R availability has been linked to craving and diet induced obesity (11,12). Similarly, the trait impulsiveness has been linked to striatal D2/3R availability (13-15). Therefore, it is plausible that the dopamine related mechanisms underlying craving and impulsiveness play a role in the development and pathophysiology of obesity. Dopamine deficiency in obese subjects may constitute a compensatory eating pattern to make up for decreased activation in the reward circuitry (3). It remains difficult, however, to dissect what components contribute to lower striatal D2/3R availability during the transition of the lean towards the obese state. Does lower D2/3R availability predispose to obesity, or is D2/3R availability reduced through a positive energy balance or an increase in fat mass? In addition, it is unknown at present whether reduced D2/3R availability is reversible after losing clinically significant fat mass or during a hypocaloric state. Therefore we studied D2/3R availability before and 6 weeks after Roux-en-Y gastric bypass (RYGB) surgery in morbidly obese women. We hypothesized that the reduction in striatal D2/3R availability observed in a hypercaloric condition, i.e. the obese state, would be restored by a negative energy balance and weight loss after bariatric surgery. Insulin receptors are widely expressed in the human brain (16) and a relationship between insulin sensitivity and central dopamine signaling has been suggested (17,18). Whether lower striatal D2/3R availability contributes to this observation is currently unknown. To study the possible coherence between striatal D2/3R and glucose metabolism, we correlated the
striatal D2/3R binding potential to hepatic and peripheral insulin sensitivity in these morbidly obese women. We expected striatal D2/3R availability to be positively correlated to measures of insulin sensitivity.

Material and methods

Subjects

Twenty obese women, who were scheduled for bariatric surgery, were studied two weeks before and six weeks after RYGB surgery. They participated in a study on the short term metabolic effects of RYGB surgery (NTR1548) (19). We reported earlier on the imaging findings of the preoperative data of 15 patients of this study population (4). None of these subjects had a history of neuroleptic or other dopaminergic treatment, childhood onset obesity, current or past psychiatric disease, lifetime history of alcohol/drug abuse, concomitant or past severe medical conditions, including diabetes mellitus. Informed consent was obtained in all subjects and the study was approved by the local medical ethics committee of the Academic Medical Center in Amsterdam.

SPECT acquisition

All subjects were studied after a 12-h fast from 22:00 PM the day before and all scans were scheduled at the same time in the morning. SPECT studies were performed using a 12-detector brain-dedicated scanner (Neurofocus 810, Inc., Medfield, Massachusetts, USA) with a full-width at half-maximum (FWHM) resolution of 6.5 mm, throughout the 20 cm field-of-view. After positioning of the subjects with the head parallel to the orbitomeatal line, axial slices parallel and upward from the orbitomeatal line to the vertex were acquired in 5 mm steps (300 sec scanning time per slice). The energy window was set at 135–190 keV. In all participants, approximately 80 MBq [123I]IBZM was given as an intravenous bolus, followed by continuous infusion of 20 MBq/h to achieve unchanging regional brain activity levels. Acquisition of the images was started 2 h after the bolus injection, the infusion continued until the scan was finished (after approximately 60 minutes). The SPECT scan was repeated under the same conditions and following the same protocol at 6 weeks after surgery. This timeframe was chosen based on the estimated turnover time of striatal D2/3 receptors, and this timeframe was used in previous preliminary studies (20,21).

SPECT processing

Attenuation correction of all images was performed as described earlier (22). Images were reconstructed in 3-D mode. For quantification, two techniques were used. In the first analysis, a classic region-of-interest (ROI) analysis was performed with fixed ROIs for the striatum and occipital cortex, as described earlier (4). Briefly, on four consecutive transverse slices representing the most intense striatal binding, the average striatal and occipital binding (representing nonspecific binding) was measured by positioning ROIs manually. Then the non-displaceable binding potential (BP_{ND}) was calculated as follows: (total striatal binding
occipital binding)/occipital binding. In the second analysis, the individual SPECT images were registered with the individual MRI images as described earlier (23). Then ROIs were manually drawn on the MR images for the whole striatum, caudate nucleus and putamen and occipital cortex. Just like the first analysis, we then calculated the BPND.

**MRI acquisition**

Prior to the SPECT scan, a MRI scan of the brain was completed to exclude anatomic abnormalities and to enable anatomic mapping. Brain scans were performed on an open 1.0 Tesla MR scanner with a 160 cm-wide patient aperture and a height of 45 cm (Panorama HFO, Philips Healthcare, Best, The Netherlands) using the sense head coil (scan time 6 minutes). Images were acquired using a T1 weighted 3D gradient echo sequence with full brain coverage and high spatial resolution (voxelsize: 0.88 x 0.88 x 0.80 mm³, 256 x 256 x 160 matrix) with TE/TR=25/6.9 ms.

**Surgical procedure**

The surgical procedures were carried out in two medical centers (Rijnstate Hospital, Arnhem and Slotervaart Hospital, Amsterdam, the Netherlands) and performed by experienced bariatric surgeons. During surgery, the gastric volume was reduced by stapling off a 30-mL proximal gastric pouch and connecting the antecolic alimentary limb in a gastroenterostomy. The biliopancreatic limb with a length of 45–50 cm from the ligament of Treitz was connected to this alimentary limb at a distance of 100–150 cm as an enterointerostomy. This procedure resulted in a bypass of the distal stomach, duodenum, and proximal part of the jejunum.

**Hyperinsulinemic euglycemic clamp**

The subjects were studied two weeks before and two weeks after RYGB. The differences in glucose metabolism before versus 2 weeks after surgery are reported separately (19). They were admitted to the Metabolic Clinical Research Unit of the AMC after an overnight fast and were studied in the supine position. After a 10-h fast from 22:00 PM the day before, a catheter was inserted into the dorsal vein of each hand or distal vein of each arm. One catheter was used for sampling of arterialized blood using a heated hand box (60°C). The other catheter was used for infusion of [6,6-²H₂]glucose, glucose 20%, and insulin. At T=09:00 h AM (t= -2), after drawing a blood sample for background enrichment of plasma glucose, a continuous infusion of [6,6-²H₂]glucose (99% enrichment; Cambridge Isotopes, Andover, MA), Andover, MA) was started at a rate of 0.11 µmol/kg*min after a priming dose equivalent to 2 hours of infusion. After 110, 115 and 120 min, blood samples were drawn for determination of glucose enrichment, and insulin. Subsequently, at T=11:05 h AM (t=0), a continuous infusion of insulin (Actrapid 100U/ml; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2 h at a rate of 20 mU/m² body surface area. At T=2 h PM, the infusion rate of insulin was increased to 60 mU/m² body surface area min⁻¹. Plasma glucose was measured every 10 min and glucose 20% was infused at a variable rate to maintain plasma glucose at 5.0 mmol/L.
[6,6-2H2]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to minimize changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. At t = 2 h and t = 4 h, blood samples with a 5 minutes interval were drawn to measure glucose enrichment and 2 samples were drawn to measure insulin. During the study the participants were only allowed to drink water.

**Glucose and insulin measurements**

Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barbleben/Magdeburg, Germany). [6,6-2H2]glucose enrichment (tracer-to-tracee ratio) was measured as described earlier (24). The [6,6-2H2]glucose intra-assay variation was 0.5 – 1% with an inter-assay variation of 1% and a detection limit of 0.04%. Insulin was determined on an Immulite 2000 system (Diagnostic Products, Los Angeles, CA, USA). Insulin was measured with a chemiluminescent immunometric assay with intra-assay variation of 4–5%, inter-assay variation of 5% and detection limit of 15 pmol/l.

**Resting energy expenditure (REE)**

REE was measured during the final 10 min of the basal state of the hyperinsulinemic euglycemic clamp by indirect calorimetry using a ventilated hood system (Sensormedics model 2900; Sensormedics, Anaheim, USA).

**Statistical analysis**

Data were analyzed using parametric tests. Comparison of the data before surgery compared to the data obtained six weeks after surgery were analyzed using the paired student’s t-test. SPSS version 16.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Data are presented as mean ± sd (minimum - maximum). Comparisons were considered statistically significant when \( P < 0.05 \) and as a trend with \( P < 0.1 \). Correlations between striatal D2/3R availability and insulin sensitivity were determined with Pearson’s correlation. Resting energy expenditure (REE), was calculated from VO2 and VCO2 as reported previously (25).

**Results**

In one subject, acquisition of the SPECT failed due to claustrophobia during the scanning procedure and she was excluded from the analyses. The descriptive characteristics for the 19 remaining obese women are shown in table 1. Weight loss 6 weeks after RYGB was 13.8 ± 4.5 kg [range 8-24 kg] which resulted in a significant reduction in BMI after surgery (Table 1). No correlation could be found between BMI and D2/3R availability before surgery (\( p = 0.59; r^2 0.017 \)).
**SPECT analysis**

The region-of-interest analysis showed no significant change in D2/3R availability before versus 6 weeks after RYGB (0.81 ± 0.23 [0.39 – 1.28] vs. 0.79 ± 0.16 [0.54-1.07]) respectively; \( p = 0.666; \) Fig. 1a). Also, in the MRI-driven analysis, the D2/3R availability in the whole striatum as well as in subregions of the striatum (caudate nucleus and putamen) did not significantly change after surgery (Fig. 1b).

**Glucose metabolism**

Since one subject was already excluded from the entire analyses due to failure of the SPECT system, scan 19 subjects remained. The entire hyperinsulinemic euglycemic clamp was unsuccessful in two subjects and the second step was unsuccessful in one subject due to technical failures with the iv-lines. As a consequence, the correlation between hepatic insulin sensitivity and D2/3R availability was performed in 17 subjects and between peripheral insulin sensitivity and D2/3R availability was performed in 16 subjects. Weight loss 2 weeks after RYGB was 7.8 ±3.2 kg [range 6.2-9.4kg].

As reported earlier (19) basal endogenous glucose production (EGP) was 13.6±1.8 (10.3-18.1) \( \mu \text{mol/kg FFM*min} \). Hepatic insulin sensitivity expressed as percentage suppression of EGP by insulin was assessed during the first step of the hyperinsulinemic clamp and was 79±14 (55-99)\%. Insulin-mediated peripheral glucose uptake (Rd) was 25.4±9.5 (11.6-42.5) \( \mu \text{mol/kg*min} \). The REE measurement failed in a total of five subjects. The results of the 15 remaining subjects show that the mean REE significantly decreased from 1889±247 (1530-2413) kcal/day to 1718±230 (1339-2050) kcal/day in the basal state (\( p =0.008 \)). The metabolic parameters (EGP, Rd, basal glucose, basal insulin) were correlated to the striatal D2/3R availability and revealed a clear trend between D2/3R availability and peripheral insulin sensitivity (\( p=0.06; r^2=0.022; \) fig 2.).

### Table 1. Descriptive characteristics of the morbidly obese women before and 6 weeks after bariatric surgery.

<table>
<thead>
<tr>
<th></th>
<th>BEFORE SURGERY</th>
<th>6 WEEKS AFTER SURGERY</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>19</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td><strong>AGE (YEARS)</strong></td>
<td>40.5 ± 8 (26 - 50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI (KG/M(^2))</strong></td>
<td>45.5 ± 6.3 (38.7 – 61.3)</td>
<td>38.9 ± 6.3 (34.1 – 57.6)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data are shown as: mean ± standard deviation (range).
Discussion

We studied the short-term effects of weight loss on striatal D2/3R availability in morbidly obese women after RYGB and found that despite being in a negative energy balance and after clinically significant weight loss, D2/3R availability did not change significantly. We analysed the data using two different techniques namely classic fixed ROI analysis and MRI-based analysis and the outcome of both analyses were consistent. This suggests that striatal D2/3R is not regulated by acute changes in energy balance nor influenced by fat mass per se. Glucose production rate and hepatic insulin sensitivity did not correlate with the D2/3R binding potential while there was a clear trend for a positive correlation with peripheral insulin sensitivity.
Earlier studies on D2/3R binding after bariatric surgery are contradictory and report either an increase (21) or a decrease in striatal D2/3R availability (20). It should be noted however that these two studies included less than 10 patients, whereas in this study 19 patients were included. Besides the difference in number of included patients, we used a different radioligand ([123I]IBZM SPECT versus [11C]raclopride and [18F]fallypride PET). However, this is unlikely to have affected the results because all three tracers are well-validated to measure D2/3R in-vivo in humans (26-28).

Whether an increase in D2/3R occurs after long-term weight loss is unknown. The low D2/3R availability in obesity has previously been compared to findings in drug abuse. It has been shown that drug abuse results in reductions in striatal D2/3R binding up to 4 months after the last drug abuse (29). In addition, self-administration of cocaine in monkeys depressed D2/3R binding up to one year in some but not all monkeys (30). Also, we cannot rule out that reduced D2/3R availability in our subjects was already present before the occurrence of obesity, although we excluded subjects with childhood onset obesity. Finally, striatal dopaminergic neurotransmission might be resistant to the metabolic signals involved in weight loss in chronically obese subjects as has been shown for leptin (31) and insulin in rodents. This might predispose these individuals to relapse of obesity after weight loss.

Insulin, leptin, and ghrelin are important neuroendocrine hormones which drive the homeostatic control of feeding behaviour by the hypothalamus (32; 33). In addition to this homeostatic control, these hormones also regulate non homeostatic control via receptors located on dopaminergic neurons in the striatum. Despite the fact that the dopamine system is insulin responsive (34), in this present study the striatal dopaminergic neurotransmission as assessed by D2/3R availability did not correlate convincingly with measures of insulin sensitivity besides a positive trend for peripheral insulin sensitivity. The latter is in line with the insulin sensitizing effects of dopamine agonists in obese diabetic subjects (35). In addition, dopamine antagonists are known for their diabetic side effects (36) and drug-naïve schizophrenic patients, known for their disturbed central dopamine metabolism (37), are characterized by hepatic insulin resistance (38). In a previous study (17) a correlation between peripheral insulin sensitivity, using the insulin sensitivity index (SI) and D2/3R availability in the ventral striatum was found. We measured hepatic and peripheral insulin sensitivity separately using the gold standard technique and found no correlation with hepatic insulin sensitivity but a trend for a positive correlation between D2/3R availability and peripheral insulin sensitivity. This suggests that peripheral glucose uptake, which occurs under hyperinsulinemic conditions predominantly in skeletal muscle, might be in part regulated by cerebral dopamine metabolism. These observations suggest a biological relationship between cerebral dopamine and glucose metabolism. On the other hand, systemic treatment with modulators of whole body dopamine metabolism might exert pharmacological effects on glucose metabolism without altering striatal D2/3R availability (39). Basal EGP and hepatic insulin sensitivity were not correlated to D2/3R availability making a functional connection between the striatal dopaminergic transmission and hepatic glucose metabolism unlikely, at
least in chronically obese female subjects. Although a clear difference in D2/3R availability was found between lean and obese subjects (3; 4), within our obese group no clear correlation between BMI and D2/3R availability was found. This suggests that fat mass per se is not the main determinant of D2/3R availability in obesity. This is in line with the unchanged D2/3R availability despite clinically significant weight loss.

In conclusion, surgery-induced weight loss does not significantly increase striatal D2/3R availability in morbidly obese women. This suggests that short-term changes in energy balance in morbidly obese humans do not induce profound alterations in striatal dopaminergic neurotransmission and might predispose obese individuals to weight gain after a hypocaloric diet. Moreover, the striatal dopamine receptor binding potential is not significantly correlated to hepatic insulin sensitivity but showed a trend for a positive correlation with peripheral insulin sensitivity. This confirms earlier findings on a potential role of cerebral dopamine in glucose metabolism.

Acknowledgements

We thank Dr. Aart Nederveen for his consultation on the MRI studies.

References


Striatal dopamine D$_{2/3}$ receptor availability increases after long–term bariatric surgery–induced weight loss

Barbara A. de Weijer*, Esther M. van der Zwaal*, Elsmarieke M. van de Giessen, Ignace Janssen, Frits J. Berends, Arnold van de Laar, Mariëtte T. Ackermans, Eric Fliers, Susanne E. la Fleur, Jan Booij* and Mireille J. Serlie*

*These authors contributed equally to this work.

Submitted
Abstract

**Background:** Reduced striatal dopamine D\(_{2/3}\) receptor (D\(_{2/3}\)R) availability was reported in obese subjects compared to lean controls. Recently we determined the effect of short-term bariatric surgery-induced weight loss on striatal D\(_{2/3}\)R availability in 20 morbidly obese women and found reduced striatal D\(_{2/3}\)R availability at baseline, which remained unaltered 6 weeks after surgery, despite significant weight loss. To determine whether long-term bariatric surgery-induced weight loss normalizes striatal D\(_{2/3}\)R availability.

**Methods:** In 14 morbidly obese women who participated in our previous short-term study and age-matched lean controls. Changes in striatal D\(_{2/3}\)R binding was measured using \([^{123}\text{I}]\text{IBZM SPECT}\) and were correlations with changes in body weight/composition, eating behaviour and fasting plasma levels of leptin, ghrelin, insulin and glucose.

**Results:** Mean body mass index declined from 46 ± 7 kg/m\(^2\) to 32 ± 6 kg/m\(^2\) and this was accompanied by a significant increase in striatal D\(_{2/3}\)R availability (p=0.031). D2/3R remained significantly reduced compared to the age-matched controls (BMI 22 ± 2 kg/m\(^2\); p = 0.01). Changes in striatal D\(_{2/3}\)R availability did not correlate with changes in body weight/fat, insulin sensitivity, ghrelin or leptin levels. Although food craving measures improved, they were not related to the observed changes in striatal D2/3R availability.

**Conclusions:** Striatal D\(_{2/3}\)R availability increases after long-term weight loss independent of changes in body weight, metabolic hormones or food craving measures. Our data show that reduced D\(_{2/3}\)R availability in obesity is a reversible phenomenon.
Introduction

The prevalence of obesity and its health consequences is rising, necessitating fundamental insight into the regulation of energy balance with the aim to improve future treatment modalities (1). Previous studies have implicated the brain dopamine system in the hedonic and motivational aspects of food intake and, similar to findings in addiction, obese subjects exhibited reduced striatal dopamine D_{2/3} receptor (D_{2/3}R) availability compared to lean controls in some (2, 3, 4), but not all studies (5, 6, 7, 8, 9). It remains unknown whether lower D_{2/3}R availability reflects a cause or a consequence of obesity (or both). In support of a causal role, it has been hypothesized that overeating in subjects susceptible to obesity constitutes a compensatory response to make up for decreased dopaminergic signalling in the reward circuitry caused by reduced expression of dopamine receptors due to genetic factors (10, 11). In contrast, downregulation of striatal D_{2/3} R occurring after the onset of obesity in animal studies suggests changes in the striatal dopaminergic system to be a consequence of a persistent increase in palatable food consumption, positive energy balance and/or fat mass (12, 13).

To study the reversibility of reduced striatal D_{2/3} R binding in obesity, we previously determined D_{2/3}R availability in 20 morbidly obese women 2 weeks before and 6 weeks after Roux-en-Y gastric bypass surgery (RYGB) using [123I]IBZM single photon emission computed tomography (SPECT). In that study, striatal D_{2/3} R availability was reduced by ~20% compared to lean controls and did not significantly change 6 weeks after surgery, despite significant weight loss (14). However, reductions in striatal D_{2/3} R availability caused by addiction to drugs of abuse may persist for several months after cessation of drug use, and in one study, self-administration of cocaine caused reductions in striatal D_{2/3} R availability that persisted up to 1 year in some, but not all monkeys (15). Furthermore, body weight following bariatric surgery only stabilizes after approximately 1 year (16). Therefore, we re-invited the subjects that were included in the study on the short-term effects of RYGB to repeat the striatal D_{2/3} R measurements at least 2 years after RYGB.

The dopamine system also appears to play a role in glucose control, as e.g. dopamine agonists previously showed insulin-sensitizing effects in obese diabetic subjects (17). Moreover, dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN) pars compacta express receptors for insulin, leptin and ghrelin (18, 19) and insulin sensitivity and fasting plasma levels of ghrelin and leptin were previously associated with striatal D_{2/3} R availability (20). Therefore, we additionally studied whether long-term changes in plasma levels of leptin, insulin, glucose and ghrelin and the quantitative insulin sensitivity check index (QUICKI) correlated with changes in striatal D_{2/3} R availability.

Finally, healthier eating behavior was previously reported after bariatric surgery, with reductions in hunger, disinhibition and food craving (21, 22). As dopamine signaling is known to be involved in drug craving (23) and changes in striatal D_{2/3} R are linked to the emergence of compulsive feeding behavior in obese rats (12), we hypothesized that changes in food craving after RYGB would correlate with changes in D_{2/3} R availability. To investigate this,
we compared the results of eating behavior questionnaires before and after RYGB in these subjects.

**Material and methods**

**Subjects**

Twenty women previously participated in the study on the short-term effects of RYGB on striatal D<sub>2/3</sub>R availability and insulin sensitivity (14) (NTR1548) and were therefore eligible for this follow-up study (NTR3684). One subject was excluded due to claustrophobia, one due to pregnancy, one because she had started using anti-dopaminergic drugs (after completion of the short-term study), one did not wish to participate, and two were lost to follow up. The average age of the 14 remaining subjects was 40.5 ± 8 years (range 26 - 50). They were age-matched to non-obese historical controls that participated in a previous study and similarly examined after an overnight fast (3).

RYGB surgery had been carried out between December 2009 and December 2011 in two hospitals (Rijnstate Hospital, Arnhem and Slotervaart Hospital, Amsterdam, the Netherlands) as described previously (14). Informed consent was obtained in all subjects and the study was approved by the local medical ethics committee of the Academic Medical Center in Amsterdam.

**Study protocol**

After an overnight fast from 22:00 PM the day before, all subjects were admitted for one day to the Metabolic Clinical Research Unit of the AMC. Subjects were weighed and body composition determined using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK). Blood samples were drawn after insertion of a catheter into a distal arm vein.

Striatal D<sub>2/3</sub>R availability was assessed with [123I]IBZM SPECT, using the same protocol and brain-dedicated SPECT system (Neurofocus, Inc., Medfield, Massachusetts, USA) as described for the short-term study (14). A classic region-of-interest (ROI) analysis was performed by manually positioning fixed ROIs for the striatum and occipital cortex on four consecutive transverse slices representing the most intense striatal binding. Non-displaceable binding potential (BPND<sub>ND</sub>) was calculated as follows: (striatal binding−occipital binding)/occipital binding. Additional exploratory analyses of striatal subregions were performed in a similar manner using separate standardized ROIs for the putamen and caudate nucleus.

During the 2 hours waiting time between start of the administration of [123I]IBZM and the acquisition of the SPECT data, subjects filled out the questionnaires described below (these were similarly administered at baseline in the short-term study). Until completion of the scan, participants were only allowed to drink water.

**Plasma measurements**

Leptin was measured with <sup>125</sup>I radioimmunoassay (Millipore; intra-assay variation 3.4-8.3%; total assay variation 3.6-6.2%; detection limit 0.5 ng/ml). Ghrelin was determined with <sup>125</sup>I
radioimmunoassay (Millipore; intra-assay variation 6.5-9.5%; total assay variation 9.6-16.2%; detection limit 10 pg/ml). Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barbleben/Magdeburg, Germany). Insulin was measured with a chemiluminescent immunometric assay (intra-assay variation of 3–6%; inter-assay variation of 4%; detection limit of 15 pmol/l). The quantitative insulin sensitivity check index (QUICKI) was calculated using the formula: \( I / (\log(\text{fasting insulin } \mu U/mL) + \log(\text{fasting glucose } mg/dL)) \).

**Questionnaires**

To compare different aspects of eating behavior with measures obtained at baseline, subjects were asked to repeat the following questionnaires:

1. **Dutch eating behavior questionnaire (DEBQ):** 33 items, divided into three subscores: eating behavior patterns (24) - (1) restrained eating (i.e. the degree of conscious food restriction); (2) external eating (i.e. eating in response to food-related stimuli); (3) emotional eating (i.e. eating in response to negative emotions in order to relieve stress while disregarding internal physiological signals of satiety).

2. **Eating disorder examination questionnaire (EDEQ):** 30 items with four subscores (25) - (1) Restraint; (2) Eating Concern; (3) Shape Concern; (4) Weight Concern.

3. **General food craving questionnaire - trait (GFCQ-T):** 21 items to measure food craving occurring in general, containing four factors (26) - (1) preoccupation with food (i.e., obsessively thinking about food and eating); (2) loss of control (i.e., experiencing difficulties in regulating eating behavior when exposed to food cues); (3) positive outcome expectancy (i.e., believing eating to be positively reinforcing); and (4) emotional craving (i.e., the tendency to crave food when negative emotions are present).

**Statistical analysis**

SPSS version 20.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Outliers were defined as values more than 1.5 times the interquartile range beyond the quartiles. Changes in all measured parameters were analyzed using parametric tests (paired student’s t-test, two-sided), except for leptin and the GFCQ-T, which were tested using the Wilcoxon signed rank test (two-sided), as they failed to pass the Kolmogorov-Smirnov test for normality. Comparisons were considered statistically significant when \( p < 0.05 \) for the primary outcome measures (changes in \( D_{2/3} \)R availability, plasma measurements and total scores on eating behavior questionnaires). Correlations between striatal \( D_{2/3} \)R availability and changes in body mass index (BMI), %body fat, plasma measurements, QUICKI and scores on the GFCQ-T were determined with Spearman’s rank order. These secondary outcome measures were considered statistically significant at a p-value of \( < 0.01 \) (to correct for multiple testing). Other Spearman’s rank order correlations are presented as exploratory analyses, as are the changes in the subscores of the questionnaires. Unless otherwise specified, data are presented as mean ± standard deviation (minimum - maximum).
Results

**Body weight, body composition, plasma measurements and QUICKI**

Table 1 summarizes the changes in measures of body weight/composition and metabolic parameters of the 14 subjects included. Time since RYGB ranged from 2.1 to 3.6 years (average 3.1 years). All subjects reported that body weight had stabilized by the second year after RYGB. One outlier was excluded from the analysis for ghrelin levels (baseline ghrelin of 2860 pg/ml). Changes in plasma leptin levels correlated with percentage weight loss ($\rho = -0.70, p=0.005$) and change in BMI ($\rho =0.58, p=0.03$), but not with absolute weight loss ($\rho = 0.45, p=0.1$). There was a trend for correlation of changes in insulin with changes in %body fat ($\rho=0.48, p=0.08$) and of changes in QUICKI with changes in BMI ($\rho = -0.54, p=0.07$). Plasma level changes in ghrelin and glucose were not correlated with any measure of body weight loss ($p> 0.3$).

### Table 1. Descriptive characteristics of the 14 participants at baseline and long-term follow-up after RYGB (2.1-3.6 years). Data are shown as mean ± SD (range). ** p < 0.01.

<table>
<thead>
<tr>
<th></th>
<th>BASELINE</th>
<th>LONG-TERM FOLLOW-UP</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI (KG/M²)</strong></td>
<td>45.2 ± 6.7 (38.7 – 61.3)</td>
<td>31.2 ± 5.7 (24.1 – 43.7)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td><strong>% BODY FAT</strong></td>
<td>54.0 ±3.4 (48.7 - 60)</td>
<td>40.3 ±6.6 (26.9-52.3)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td><strong>% LEAN BODY MASS</strong></td>
<td>46.1 ± 3.4 (40 - 51.3)</td>
<td>59.7 ± 6.6 (47.7 - 73.1)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td><strong>LEPTIN (NG/ML)</strong></td>
<td>69.3 ± 22.7 (37.3 - 107.8)</td>
<td>43.1 ± 23.3 (12.2 - 76.8)</td>
<td>0.001**</td>
</tr>
<tr>
<td><strong>GHRELIN (PG/ML)</strong></td>
<td>894 ± 330 (499-1456)</td>
<td>1069 ± 384 (572-1779)</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>GLUCOSE (MG/DL)</strong></td>
<td>101.2 ± 15.2 (81.1 - 127.9)</td>
<td>83.5 ± 4.0 (77.5 - 91.9)</td>
<td>0.001**</td>
</tr>
<tr>
<td><strong>INSULIN (MU/L)</strong></td>
<td>11.7 ± 5.1 (2.8-20.4)</td>
<td>7.2 ± 2.8 (2.2 - 11.1)</td>
<td>0.001**</td>
</tr>
<tr>
<td><strong>QUICKI</strong></td>
<td>0.33 ± 0.03 (0.30 - 0.40)</td>
<td>0.37 ± 0.03 (0.33 -0.44)</td>
<td>0.002**</td>
</tr>
</tbody>
</table>

**SPECT analysis**

Unfortunately, acquisition of SPECT images failed in two subjects due to technical difficulties. These were excluded from the analyses, in addition to an outlier with a baseline BPND of 1.15 (showing an unexplainable decrease to 0.69). Figure 1 depicts the changes in BPND compared to baseline (delta BPND). A significant increase in striatal D2/3R availability was observed for the entire striatum (0.76 ± 0.11 to 0.88 ± 0.13, $p=0.031$). Additional exploratory analyses of striatal subregions revealed that the increase in BPND was slightly more pronounced within the caudate nucleus (0.79 ± 0.12 to 0.90 ±0.13; $p=0.027$) than the putamen (0.76 ± 0.11 to 0.85 ± 0.18; $p=0.052$). Striatal D2/3R availability remained significantly lower than age-matched lean controls (1.05 ± 0.12; $p = 0.01$; Figure 2), however BMI of the RYGB subjects also remained significantly higher than that of controls (31.8 ± 6.1 vs 21.9 ± 2.0 kg/m² respectively, $p < 0.001$).
There were no significant correlations between striatal BP$_{ND}$ and BMI at baseline ($p=0.85$), at long-term follow-up ($p=0.59$) or in the control group ($p=0.42$; Summarized in figure 3). In addition, there were no significant correlations between changes in D$_{2/3}$R availability and changes in BMI ($p=0.54$), % body fat ($p=0.67$), plasma levels of leptin ($p=0.42$), ghrelin ($p=0.20$), glucose ($p=0.15$), insulin ($p=0.54$) and QUICKI ($p=0.47$; Figure 4).

**Figure 1.** Change in D$_{2/3}$R availability measured with [123I]IBZM SPECT, represented as change in BP$_{ND}$ compared to baseline (Delta BP$_{ND}$) for (A) the total striatum; (B) the caudate nucleus (squares) and putamen (triangles). Line and whiskers represent median and interquartile range.

**Figure 2.** Striatal D$_{2/3}$R availability measured with [123I]IBZM SPECT, represented as BP$_{ND}$ for: obese subjects before RYGB (filled circles), at long term follow-up after RYGB (open circles), and age-matched lean controls (triangles). Line and whiskers represent median and interquartile range. *$p=0.03$ (paired t-test); **$p<0.01$ (t-test).
Figure 3. Correlation between BMI and striatal D$_{2/3}$R availability measured with [123I]IBZM SPECT (BPND) for obese subjects before RYGB (filled circles), at long term follow-up after RYGB (open circles), and age-matched lean controls (triangles).

Figure 4. Correlation between changes in (A) BMI, (B) leptin, (C) ghrelin, (D) QUICKI, (E) glucose, and (F) insulin with changes in total striatal D$_{2/3}$R availability, represented as change in BPND compared to baseline. O= two overlapping data points.

**Questionnaires**

Total scores and subscores of the questionnaires are summarized in Table 2. (Sub)scores for the GFCQ-T could not be calculated for 2 subjects, due to missing values.

The lower score on the DEBQ was due to lower values on all subscores (external eating, emotional eating and restrained eating). The trend for a decrease in score on the EDEQ
was accompanied by decreases in all subscores, especially ‘weight concern’. The reduction in total score for the GFCQ-T was mainly due to reductions in factor 2 (loss of control) and factor 4 (emotional craving), and to a lesser degree reductions in factor 1 (preoccupation with food) and factor 3 (positive outcome expectancy). There were no significant correlations between changes in striatal D2/3 R availability and the total GFCQ-T score (p=0.35, p=0.29) or any subscore (p>0.2).

Table 2 Total scores and subscores for the Dutch Eating Behavior Questionnaire (DEBQ), Eating Disorder Examination Questionnaire (EDEQ) and General Food Craving Questionnaire-Trait (GFCQ-T) at baseline and long-term follow-up. Data are shown as mean ± SD. ** p < 0.01; *p < 0.05;  #p < 0.1.

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Baseline</th>
<th>Follow-Up</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DEBQ-TOTAL</strong></td>
<td>2.87 ± 0.42</td>
<td>2.36 ± 0.43</td>
<td>0.004**</td>
</tr>
<tr>
<td><strong>DEBQ-EMOTIONAL EATING</strong></td>
<td>2.53 ± 0.86</td>
<td>2.10 ± 0.72</td>
<td>0.034*</td>
</tr>
<tr>
<td><strong>DEBQ-EXTERNAL EATING</strong></td>
<td>2.98 ± 0.76</td>
<td>2.39 ± 0.56</td>
<td>0.001**</td>
</tr>
<tr>
<td><strong>DEBQ-RESTRAINED EATING</strong></td>
<td>3.10 ± 0.72</td>
<td>2.59 ± 0.52</td>
<td>0.06a</td>
</tr>
<tr>
<td><strong>EDEQ-TOTAL</strong></td>
<td>2.97 ± 0.65</td>
<td>2.24 ± 0.84</td>
<td>0.07a</td>
</tr>
<tr>
<td><strong>EDEQ-RESTRAINT</strong></td>
<td>2.54 ± 0.99</td>
<td>1.79 ± 0.85</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>EDEQ-EATING CONCERN</strong></td>
<td>1.87 ± 0.82</td>
<td>1.49 ± 0.58</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>EDEQ-SHAPE CONCERN</strong></td>
<td>3.86 ± 1.24</td>
<td>3.11 ± 1.13</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>EDEQ-WEIGHT CONCERN</strong></td>
<td>3.61 ± 0.79</td>
<td>2.59 ± 1.21</td>
<td>0.05a</td>
</tr>
<tr>
<td><strong>GFCQ-T TOTAL</strong></td>
<td>2.84 ± 1.02</td>
<td>2.13 ± 0.62</td>
<td>0.024*</td>
</tr>
<tr>
<td><strong>GFCQ-T PREOCCUPATION WITH FOOD</strong></td>
<td>2.70 ± 0.83</td>
<td>2.10 ± 0.78</td>
<td>0.076#</td>
</tr>
<tr>
<td><strong>GFCQ-T LOSS OF CONTROL</strong></td>
<td>2.88 ± 1.18</td>
<td>1.86 ± 0.63</td>
<td>0.006**</td>
</tr>
<tr>
<td><strong>GFCQ-T POSITIVE OUTCOME EXPECTANCY</strong></td>
<td>2.85 ± 1.04</td>
<td>2.38 ± 0.70</td>
<td>0.099#</td>
</tr>
<tr>
<td><strong>GFCQ-T EMOTIONAL CRAVING</strong></td>
<td>2.97 ± 1.37</td>
<td>2.14 ± 0.80</td>
<td>0.035*</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. ** p < 0.01; *p < 0.05;  #p < 0.1.

**Discussion**

To our knowledge, this is the first study to determine changes in striatal D2/3 R availability after long-term weight loss in obese subjects. Our data show that, more than 2 years after RYGB, striatal D2/3 R availability increased compared to pre-operative measures. Weight loss after RYGB was also accompanied by improvements in insulin sensitivity and eating behaviour, although these did not correlate with changes in D2/3 R availability.
Previous imaging studies assessing short-term changes in striatal D_{2/3}R binding after RYGB surgery have produced conflicting results: one preliminary positron emission tomography (PET) study in 5 women reported a decrease ± 7 weeks after bariatric surgery (27), another preliminary PET study in 5 women reported an increase 6 weeks after RYGB (9), whereas our previous SPECT study reported no significant changes 6 weeks after RYGB (14). Similar to previous reports, body weight loss in the present study was initially rapid, but stabilized in the second year after surgery (16). Therefore, the increase in striatal D_{2/3}R availability observed in this study provides a reliable estimate of the effect of long-term weight loss following RYGB. Although striatal D_{2/3}R availability increased after long-term weight loss in this study, it remained significantly lower than age-matched controls. However, there was considerable variability in the degree of long-term weight loss after RYGB, and average BMI remained in the overweight/obese range (31.2 ± 5.7 kg/m^2). Therefore, D_{2/3}R availability may have normalized completely if the all subjects had reached a BMI within the normal range.

At present, it remains uncertain to what extent reduced striatal D_{2/3}R availability precedes or follows development of obesity in humans, as there is data supporting both hypotheses (10; 12; 11; 13). In addition, it remains a matter of debate whether lower striatal D_{2/3}R availability in obese humans reflects a hyperdopaminergic or hypodopaminergic state. This is partly due to the fact that striatal D_{2/3}R availability is not only influenced by D_{2/3}R expression but also by synaptic dopamine levels, which in turn are influenced by both tonic and phasic dopamine release. Importantly, increased basal dopaminergic tone is accompanied by reduced phasic dopamine release (e.g. triggered by food-stimuli), because it is partly influenced by activation of pre-synaptically located autoreceptors (28). Thus, interpretation of the mechanisms underlying the reduced striatal D_{2/3}R availability observed in obesity is not straightforward.

As reduced expression of striatal D_{1} receptors was reported following diet-induced obesity in rodents (12), the increase in BP_{ND} observed in the present study could reflect an upregulation of striatal D_{1} receptors (i.e. reversal of downregulation). Alternatively, the increase in BP_{ND} could also be due to a reduction in basal dopaminergic tone (as subjects in this study were scanned in a fasted state). It was recently hypothesized that subjects in the morbidly obese range exhibit a higher basal dopaminergic tone with a subsequent decrease in phasic dopamine release (28). Reversal of this phenomenon after weight loss would cause a reduction in basal dopaminergic tone, and thus explain the increase in BP_{ND} observed in this study. Interestingly, we recently showed blunted phasic dopamine release in severely obese women (3). However, due to the present study design we were unable to quantify any changes in dopamine release after RYGB-induced weight loss in this cohort.

The lack of correlation between delta BP_{ND} and body weight loss after RYGB suggests the change in striatal D_{2/3}R availability might not be determined solely by (changes in) body weight. Interestingly, reduced striatal D_{2/3}R mRNA expression was observed following long-term exposure of rats to ‘junk food’ even in the absence of body weight gain (12). Furthermore, studies in which rats are exposed to different obesogenic diets suggest that the reduction in striatal D_{2/3}R availability depends on dietary composition, especially a high fat/carbohydrate
ratio (13; 29). Although we did not assess dietary caloric content or diet composition before versus after RYGB, previous studies reported lower caloric intake after bariatric surgery with reduced preference for high fat and high sucrose food (30; 31). Therefore the increase in striatal D$_{2/3}$R availability observed in this study might be (partially) explained by changes in diet composition and caloric intake.

Our additional exploratory analysis of striatal subregions revealed a slightly more pronounced increase in D$_{2/3}$R availability in the caudate nucleus compared to the putamen. Interestingly, obese subjects showed increased hemodynamic responses and glucose uptake in the caudate nucleus while viewing appetizing versus bland foods (32). Moreover, in a recent fMRI study, RYGB patients showed lower activation of brain reward systems (including the caudate nucleus) compared to gastric banding patients, with lower palatability scoring and appeal of high-calorie foods (33). Although changes in blood flow and glucose consumption do not necessarily reflect changes in dopamine signalling, taken together with our findings, this suggests that the caudate nucleus may play an important role in the hedonic aspects of food intake in obese subjects and that this may be altered by RYGB-induced weight loss. This might result from decreased phasic dopamine release in response to food-related stimuli, however, as mentioned above, it was not possible to determine changes in phasic dopamine release in this cohort.

The unchanged ghrelin levels after RYGB in this study are in line with previous findings (34) but in contrast to the study by Dunn et al., we observed no correlations between changes in BP$_{ND}$ and changes in plasma levels of ghrelin (20). This may be because we measured total ghrelin and not acylated ghrelin. In addition, no correlation was present between plasma leptin, glucose, insulin or the insulin sensitivity index (QUICKI) and changes in D$_{2/3}$R availability. However, given that circadian rhythms both affect these metabolic parameters as well as dopaminergic signalling, it is also possible that methodological differences played a role (35, 36). Subjects in the present study were scanned just before noon, after an overnight fast, whereas subjects in the study of Dunn et al. were scanned in the evening after a 6-hour fast (20). Furthermore, although in lean subjects acyl-ghrelin levels correlated with D$_{2/3}$R availability in the SN, in obese subjects it did not (37). Thus, the relationship between D$_{2/3}$R availability and levels of acyl-ghrelin and BMI does not appear straightforward and dependent on experimental circumstances. Finally, the lack of correlation between changes in BP$_{ND}$ with changes in QUICKI, plasma glucose and insulin suggest that these changes are independent effects of RYGB and that lower basal insulin and glucose levels do not affect striatal D$_{2/3}$R availability or vice versa. Thus, although dopamine agonists may improve insulin sensitivity in obese diabetics, the beneficial effects of RYGB on insulin sensitivity do not appear to depend on changes in striatal D$_{2/3}$R availability (17).

The changes we observed in eating behavior questionnaire scores are in line with previous studies reporting reductions in hunger, restraint, food craving and disinhibition after RYGB (21, 22). The reductions we observed in the G-FCQ-T and DEBQ indicate a lower tendency
to experience food cravings and to eat in response to negative emotions, stress or external food-cues. Although the GFCQ-T score decreased after RYGB, no correlation was found between the observed changes in BPND and GFCQ-T scores, suggesting that increases in basal D_{2/3} R availability did not play a role in the reductions in food craving and disinhibition. Interestingly, this is in line with a recent animal study that showed that low dopamine D_{2} receptor expression increased vulnerability to diet-induced obesity through changes in physical activity, and not through increased food motivation (38). Nevertheless, it remains possible that changes in feeding/food cue-induced striatal dopamine release underlie the reduction in food craving observed in this study.

To our knowledge, this is the first study to determine the effect of long-term weight loss on striatal D_{2/3} R availability in a human model. However, it has several limitations. Due to the study design and the relative inaccessibility of the human brain, we can only speculate on the mechanisms underlying the observed effect on striatal D_{2/3} R availability, as both D_{2/3} R expression and synaptic dopamine levels can influence this. Other limitations include the limited number of subjects and the fact that repeat scans were only performed on the obese subjects. Ideally repeat scans would also have been performed in the healthy controls or a group of obese subjects that did not undergo RYGB. However, striatal D_{2/3} R availability decreases with age (39), thus any age-related effect would only have reduced the probability to detect an increase in BPND after RYGB. Furthermore, good reproducibility was previously reported for striatal D_{2/3} R measurements using a similar IBZM-protocol (intraclass correlation coefficient of 0.74 (40) and the SPECT scans of this follow-up study were performed using the same protocol and scanner as for baseline measurements, with ROI analyses performed by the same researcher. Finally, the study was performed in obese females only and studies in males might yield different results.

In summary, this study shows that long-term weight loss after RYGB is accompanied by an increase in striatal D_{2/3} R availability, which suggests the reduction of striatal D_{2/3} R availability observed in obesity is reversible. Furthermore, although insulin sensitivity and eating behavior improved after RYGB, neither the changes in QUICKI nor the reductions in food craving and disinhibition correlated with changes in striatal D_{2/3} R availability, challenging an important role for altered basal D_{2/3} R availability in these effects of RYGB. Future studies that measure effects of weight loss on phasic dopamine release are needed to assess whether changes in feeding/food-cue-induced dopamine release might underlie the effects of RYGB on food motivation.

**Acknowledgements:** We would like to thank Michelle Panton, Ruth Versteeg, Mette Stam, Bastiaan Kee, Erik Knaap, Paul Groot, Murat Kilicarslan, Karin Koopman, Shreyas de Jong, and Martine van Vessem-Timmermans for their valuable assistance during the preparation and execution of this study.
References


PART II

OBESITY, METABOLISM AND INFLAMMATION
Hepatic and peripheral insulin sensitivity do not improve 2 weeks after bariatric surgery


Abstract

**Background**: Bariatric surgery has rapid metabolic effects on glucose metabolism before the occurrence of clinically significant weight loss. This suggests an acute effect of the surgery itself, e.g., resulting from bypassing the nutrient flow from the proximal gastrointestinal tract. We aimed to define rapid effects of Roux-en Y gastric bypass surgery (RYGB) on glucose metabolism.

**Methods**: We studied glucose metabolism and total triglyceride hydrolysis in the basal state and during a hyperinsulinemic euglycemic clamp using stable isotopes two weeks before and two weeks after RYGB. We included 18 pre-menopausal women scheduled for RYGB. Two weeks after RYGB median weight loss was 7.8 kg.

**Results**: Basal insulin and glucose levels decreased after surgery. Endogenous glucose production (EGP) was lower after surgery. Also, insulin levels were lower during the clamp after surgery, suggesting enhanced clearance. Hepatic and peripheral insulin sensitivity did not change. Free Fatty Acid (FFA) levels increased after surgery both in the basal state and during the first step of the clamp. Total triglyceride hydrolysis did not change in the basal state and tended to be higher during hyperinsulinemia.

**Conclusions**: Within 2 weeks, RYGB reduces basal EGP as well as insulin and glucose levels without an acute beneficial effect on hepatic or peripheral insulin sensitivity. The latter may be explained by higher rates of lipolysis and exposure to FFA induced by the hypocaloric state.
Introduction

At present, bariatric surgery is the most effective treatment modality to induce sustained weight loss and reversal of the obesity-induced changes in lipid and glucose metabolism. It reduces cardiovascular risk factors and decreases mortality rates (1, 2). Bariatric procedures mainly result in reduced food intake with subsequent weight loss. Roux-en-Y gastric bypass surgery (RYGB) and biliopancreatic diversion are the most effective methods in terms of sustained control of glucose homeostasis (3). Caloric restriction and weight loss are well known mechanisms for improved insulin sensitivity (4). However, recent reports have been published on amelioration of insulin sensitivity within days after bariatric surgery (5, 6). This phenomenon occurred in the absence of significant weight loss (7-9). Traditional glucoregulatory factors, like free fatty acids (FFA) and adiponectin could not explain this early improvement. The beneficial effects on glucose metabolism were accompanied by a change in gut peptide (incretins) secretion (6, 7), i.e., an increased GLP-1 response to an oral glucose load, which could be responsible for an enhanced glucose-induced insulin response and hence lower plasma glucose. Another hypothesis proposes that by bypassing the proximal gastrointestinal tract from the nutrient flow, decreased secretion of substances with an anti-incretin effect is induced, resulting in improvement of glucose metabolism (3). However, this hypothesis has not been validated to date. In addition, it is not clear whether the improvement in glucose metabolism is due to an increase in β-cell function, an increase in hepatic or peripheral insulin sensitivity, or a combination of these factors.

In the present study we report the short-term effects on glucose metabolism and lipolysis in obese women undergoing RYBG. We performed a two-step hyperinsulinemic euglycaemic clamp with stable isotopes to determine hepatic and peripheral insulin sensitivity and lipolysis before and two weeks after surgery. We hypothesized that RYGB increases hepatic and peripheral insulin sensitivity.

Subjects and methods

Subjects

Eighteen obese women scheduled for RYGB surgery were included in this observational intervention study, and served as their own controls. These women were recruited from the outpatient clinics of the Rijnstate Hospital in Arnhem and the Slotervaart Hospital in Amsterdam, from October 2008 until December 2010. The patients were eligible for the study if they met the criteria for bariatric surgery and were scheduled to undergo RYGB surgery, if they had no DSM IV diagnosis, were older than 18 years, understood the objective of the study, and were competent to give informed consent. This competency was evaluated by the investigator and the nursing team and surgeon involved in the treatment of the patient. Exclusion criteria were: insulin dependent DM; a recent history (6 months or less) of substantial alcohol or drug abuse; the use of antipsychotic medication or antidepressant medication; any somatic illness except for obesity-related conditions (hypertension, dyslipidemia and DM
treated with oral anti-diabetics); and no informed consent. Substance (ab)use and physical health were assessed by the team involved in the pre-assessment for surgery. The study was approved by the Medical Ethical Committee of the Academic Medical Center (AMC) of the University of Amsterdam. After a complete description of the study had been given, written informed consent was obtained.

**Surgical procedure**

The surgical procedures were carried out in two medical centers (Rijnstate Hospital, Arnhem and Slotervaart Hospital, Amsterdam) and performed by experienced bariatric surgeons. During surgery, the gastric volume was reduced by stapling off a 30-mL proximal gastric pouch and connecting the antecolic alimentary limb in a gastroenterostomy. The biliopancreatic limb with a length of 45–50 cm from the ligament of Treitz was connected to this alimentary limb at a distance of 100–150 cm as an enteroenterostomy. This procedure resulted in a bypass of the distal stomach, duodenum, and proximal part of the jejunum.

**Hyperinsulinemic euglycemic clamp**

Subjects were admitted to the Metabolic Clinical Research Unit of the AMC and were studied in the supine position. After a 10-h fast from 22:00 PM the day before, a catheter was inserted into the dorsal vein of the hand or distal vein of each arm. One catheter was used for sampling of arterialized blood using a heated hand box (60°C). The other catheter was used for infusion of [6,6-2H2]glucose, [1,1,2,3,3-2H5]glycerol, glucose 20%, and insulin. At 09:00 AM (t= -2), after drawing a blood sample for background enrichment of plasma glucose and glycerol, a primed-continuous infusion of [6,6-2H2]glucose (99% enrichment; Cambridge Isotopes, Andover, MA) and of [1,1,2,3,3-2H5]glycerol (99% enrichment; Cambridge Isotopes, Andover, MA) were started at a rate of 0.11 µmol/kg/min after a priming dose equivalent to 120 min infusion. After 110, 115 and 120 min, blood samples were drawn for determination of glucose and glycerol enrichments, glucoregulatory hormones and FFA. Subsequently, at 11:05 AM (t=0), a continuous infusion of insulin (Actrapid 100U/ml; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2h at a rate of 20 mU/m² body surface area min⁻¹. At t=2, the infusion rate of insulin was increased to 60mU/m² body surface area. Plasma glucose was measured every 10 min and glucose 20% was infused at a variable rate to maintain plasma glucose at 5.0 mmol/liter. [6,6-2H2]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to minimize changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. At t = 2 and t = 4, 5h blood samples with a 5 min interval were drawn to measure glucose and glycerol enrichments and 2 samples were drawn to measure glucoregulatory hormones and FFA. During the study the participants were only allowed to drink water.
Body composition and indirect calorimetry

Body composition was measured using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK). Oxygen consumption \( (V\text{O}_2) \) and \( \text{CO}_2 \) production \( (V\text{CO}_2) \) were measured continuously during the final 20 min of the basal state and the hyperinsulinemic euglycemic clamp by indirect calorimetry using a ventilated hood system (Sensormedics model 2900; Sensormedics, Anaheim, USA) and the final 10 min were used for calculations of the respiratory exchange ratio (RER).

Glucose and lipid metabolism measurements

Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barbleben/Magdeburg, Germany). Plasma FFA concentrations were determined with enzymatic colorimetric method (NEFA-C test kit; Wako Chemicals, Neuss, Germany) with an intra-assay variation of 1%, inter-assay variation of 4-15% and a detection limit of 0.02 mmol/L. \([6,6\text{-}^{2}\text{H}_2]\text{glucose} \) and \([1,1,2,3,3\text{-}^{2}\text{H}_5]\text{glycerol} \) enrichment (tracer-to-tracee ratio) were measured as described earlier (8, 9).

Insulin and cortisol were determined on an Immulite 2000 system (Diagnostic Products, Los Angeles, CA, USA). Insulin was measured with a chemiluminescent immunometric assay with intra-assay variation of 4–5%, inter-assay variation of 5% and detection limit of 15 pmol/l. Cortisol was measured with a chemiluminescent immunoassay with intra-assay variation of 3-6%, inter-assay variation of 5-7% and a detection limit of 50 nmol/l. Glucagon was determined with the Linco 125I RIA (Linco Research, St Charles, MO, USA) with an intra-assay variation of 4-8%, inter-assay variation of 6-11% and detection limit of 15 ng/l. C-peptide was determined with a \(^{125}\text{I} \) radioimmunoassay (Linco Research, Inc, USA). Intra-assay variation 4-8%, inter-assay variation 9-16%, detection limit 50 pmol/L.

Calculations and statistical analyses

Each subject served as its own control. Data were analyzed using non-parametric tests. Comparison of the data before surgery compared to the data obtained two weeks after surgery were analyzed using the wilcoxon signed rank test. SPSS version 16.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Data are presented as median and interquartile range. Comparisons were considered statistically significant if the \( P \) value was <0.05.

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula described previously by Matthews et al. (10). Endogenous glucose production (EGP) and peripheral glucose uptake (rate of disappearance \( [R_d] \) ) were calculated using the modified form of the Steel equation as described previously (11, 12). EGP is expressed as \( \mu\text{mol}(\text{kg FFM})^{-1}\text{min}^{-1} \) (FFM, fat-free mass) and \( R_d \) as \( \mu\text{mol}(\text{kg}^\ast\text{min}^{-1}) \). Insulin clearance was calculated as the rate of insulin infusion (mU [m² body surface area] min⁻¹) divided by the mean plasma insulin concentration during the clamp (13).
Resting energy expenditure (REE), glucose oxidation and fat oxidation rates were calculated from VO₂ and VCO₂ as reported previously (14). Total triglyceride hydrolysis is expressed per REE (μmol/kcal) as suggested by Koutsari et al. (15).

**Results**

**Study participants.**

We included 18 pre-menopausal Caucasian women (median age 40.5 [26-50] yrs, median BMI 42.9 [38.7-61.3] kg/m²) scheduled for RYGB. None of the subjects had type 2 diabetes. Two weeks after RYGB, median weight loss was 7.8kg [2-14 kg], which corresponds to 6.2% (2 – 14 %) weight loss.

**Glucose metabolism.**

The second step of the hyperinsulinemic euglycemic clamp was unsuccessful in one subject before surgery, and in two subjects after surgery. In addition, the first and second step of the hyperinsulinemic clamp were unsuccessful in one subject after surgery, in all cases due to technical failures with the iv-lines. Therefore the paired results shown in the tables represent 18 women in the basal state before and after surgery and 15 women in the hyperinsulinemic state before and after surgery.

HOMA-IR decreased significantly after surgery suggesting enhanced insulin sensitivity (table 1). Basal glucose, insulin and C-peptide levels decreased after surgery. Plasma cortisol was similar, while glugagon increased after surgery (table 1).

Endogenous glucose production (EGP) decreased significantly 2 weeks after the RYGB. Hepatic insulin sensitivity expressed as percentage suppression of EGP by insulin was assessed during the first step of the hyperinsulinemic clamp (insulin before surgery 263 [145-450] pmol/L vs after surgery 189 [130-278] pmol/L, p < 0.001) and showed no significant difference between the preoperative and postoperative condition. In addition, the correlation coefficient between EGP and circulating insulin levels did not differ before versus after surgery (data not shown).

Insulin levels during the second step of the hyperinsulinemic euglycemic clamp were significantly lower after surgery (table 1). Therefore we corrected Rd for circulating insulin levels (Rd/[insulin]). Insulin-mediated peripheral glucose uptake (Rd) showed no significant difference between the preoperative and postoperative condition. (fig 2).

**Lipid metabolism and REE**

REE was significantly lower in the basal state after surgery (table 2). The increase in REE during the hyperinsulinemic clamp was blunted, resulting in lower REE during hyperinsulinemia after surgery. FFA in the basal state increased after surgery and remained higher during the first step of the clamp.

Total triglyceride hydrolysis expressed per REE remained stable in the basal state and tended to be higher during the first step of the clamp after surgery.
HEPATIC AND PERIPHERAL INSULIN SENSITIVITY DO NOT IMPROVE 2 WEEKS AFTER BARIATRIC SURGERY

Table 1. Glucose and lipid metabolism in the basal state and during the hyperinsulinemic euglycemic clamp

<table>
<thead>
<tr>
<th></th>
<th>BASAL STATE (N = 18)</th>
<th>HYPERINSULINEMIC CLAMP (N = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before surgery</td>
<td>After surgery</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.3 (1.6 - 3.9)</td>
<td>1.5 (0.9 – 2.4)</td>
</tr>
<tr>
<td>GLUCOSE (MMOL/L)</td>
<td>5.4 (4.8 – 6.3)</td>
<td>4.8 (4.6 – 5.3)</td>
</tr>
<tr>
<td>EGP (µMOL/KG FFM.MIN)</td>
<td>13 (12.7 – 14.5)</td>
<td>11.4 (10.5 – 12.9)</td>
</tr>
<tr>
<td>SUPRESSION OF EGP (%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CORTISOL (NMOL/LITER)</td>
<td>3.4 (2.8 – 4.7)</td>
<td>3.5 (2.6 – 4.9)</td>
</tr>
<tr>
<td>INSULIN (PMOL/LL)</td>
<td>81 (62.3 – 111)</td>
<td>48 (30 – 67)</td>
</tr>
<tr>
<td>C-PEPTIDE (PMOL/LL)</td>
<td>950 (757 – 1127)</td>
<td>805 (540 – 596)</td>
</tr>
<tr>
<td>GLUCAGON (NG/LITER)</td>
<td>51 (39 – 65)</td>
<td>69 (53 – 78)</td>
</tr>
<tr>
<td>CORTISOL (NMOL/LITER)</td>
<td>250 (182 – 365)</td>
<td>199 (166 – 277)</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range). Endogenous glucose production (EGP) was assessed during the first step of the hyperinsulinemic clamp. It was completely suppressed during the second step of the clamp.

Table 2. Lipid metabolism measurements in the basal state and during the hyperinsulinemic euglycemic clamp.

<table>
<thead>
<tr>
<th></th>
<th>BASAL STATE (N = 18)</th>
<th>HYPERINSULINEMIC CLAMP (N = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before surgery</td>
<td>After surgery</td>
</tr>
<tr>
<td>FFA (MMOL/L)</td>
<td>0.78 (0.72 – 0.84)</td>
<td>0.94 (0.87 – 1.09)</td>
</tr>
<tr>
<td>TTGH (µMOL/KCAL)</td>
<td>291 (243 – 354)</td>
<td>276 (235 – 330)</td>
</tr>
<tr>
<td>REE (KCAL/DAY)</td>
<td>1858 (1682 – 2017)</td>
<td>1731 (1480 – 1915)</td>
</tr>
<tr>
<td>FAT OXIDATION</td>
<td>1.8 (0.25 – 3.1)</td>
<td>0.45 (0 – 1.74)</td>
</tr>
<tr>
<td>GLUCOSE OXIDATION</td>
<td>1.1 (0.9 – 1.2)</td>
<td>0.6 (0.26 – 0.8)</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range). TTGH= total triglyceride hydrolysis.
**Discussion**

We studied the short-term metabolic effects of Roux-en-Y gastric bypass surgery to explore whether the assumed increase in insulin sensitivity could be explained by an increase in hepatic or peripheral insulin sensitivity. We found that within two weeks, bariatric surgery reduces basal EGP, insulin and glucose levels without a beneficial effect on hepatic or peripheral insulin sensitivity. Our results are in line with previous studies (17, 18), which showed no increase in peripheral insulin sensitivity. However, hepatic insulin sensitivity was not assessed in those studies. Surprisingly, we did observe a significant decrease in HOMA-IR and also found that the range in HOMA-IR was smaller after surgery, indicating that the reduction in basal insulin and glucose levels in hypocaloric conditions might be part of a preserved metabolic adaptation in all subjects. Also, HOMA-IR at baseline did not correlate with clamp-derived Rd-rates (data not shown), suggesting that measuring HOMA-IR in morbidly obese women before and after surgery does not reflect true insulin sensitivity. Lower HOMA-IR after surgery might be explained by lower EGP and lower insulin secretion or enhanced insulin clearance rates. The latter is in line with the lower insulin levels during insulin infusion after surgery in our subjects. Although clamp-derived insulin sensitivity was not changed in the short term, earlier studies have shown improvements in glucose tolerance using an oral glucose tolerance tests (19). An enhanced β-cell responsivity or incretin response to an oral glucose load might explain this difference. However, the altered anatomy of the gastrointestinal tract might interfere with glucose absorption and hence lower glucose concentrations after an oral glucose load without frankly changing the β-cell response per se.
Besides lower glucose levels and lower EGP, higher levels of FFA all indicate that our subjects were in a state of prolonged fasting which is known to ameliorate glucose metabolism even in patients with DM2 (20). Also, caloric restriction is known to reduce the incidence of diabetes (21). A very low calorie diet in obese patients with DM2 has been shown to have no effect on hepatic insulin sensitivity in the short term while Markovic et al. (22) did report an increase in hepatic insulin sensitivity 4 days after a hypocaloric diet in obese subjects. These contradictory results might be explained by either a difference in study population or a difference in composition of the hypocaloric diet. Our subjects were mainly on a liquid or pureed diet. Our findings suggest that the major short-term effect of bariatric surgery is inducing a state of prolonged fasting with beneficial effects on basal endogenous glucose production and hence glucose levels.

Higher FFA levels during hyperinsulinemia in our subjects might explain why peripheral and hepatic insulin sensitivity did not change in the short term. FFAs are known to interfere with insulin signaling (23). The increase in FFA in the first weeks after RYGB has been described previously (24) and FFA levels returned to pre-surgery levels after one year, a time span which has been shown to be sufficient to reduce the incidence of diabetes in patients after bariatric surgery (1). Total triglyceride hydrolysis measured with labeled glycerol and expressed in relation to REE tended to be higher during hyperinsulinemia only. The difference between the higher basal FFA concentrations and stable basal tracer-derived lipolytic flux can be explained by either higher incomplete lipolysis (25) or reduced FFA uptake.

REE was lower after surgery and did not increase during hyperinsulinemia. Lowering REE is a general metabolic adaptation to a hypocaloric state (26). The blunted insulin/glucose-mediated increase in REE suggests either a different thermic effect of glucose or different metabolic handling of infused glucose. This warrants further research.

In conclusion, the beneficial early metabolic effects described in morbidly obese adults undergoing bariatric surgery are not caused by an increase in insulin sensitivity. A state of prolonged fasting induced by the RYGB explains lower endogenous glucose production rates with subsequent lower plasma glucose levels. Lower insulin levels in the basal state and during the hyperinsulinemic clamp indicate enhanced hepatic insulin clearance. Therefore lower HOMA-IR in this population does not truly reflect insulin sensitivity. The lack of effect on insulin sensitivity might be explained in part by higher levels of free fatty acids.

Reference list


Influx of macrophages and T cells in visceral and subcutaneous adipose tissue of morbidly obese women is not associated with insulin sensitivity


Submitted
Abstract

Background: Infiltration and activation of adipose tissue immune cells contribute to low grade inflammation in obesity. Whether the expression profiles of inflammatory markers in different adipose tissue compartments contribute to disturbed metabolic fluxes in insulin target tissues in obese humans is unclear.

Methods: mRNA expression profiles of both pro- and anti-inflammatory macrophage and T cell markers as well as mRNA expression of glucose transporter (GLUT) 4 were determined in subcutaneous (SAT) and visceral adipose tissue (VAT) in 20 morbidly obese women undergoing bariatric surgery. Expression profiles were compared to 6 lean controls undergoing elective cholecystectomy. In obese women, insulin sensitivity and insulin-mediated suppression of lipolysis were determined using a hyperinsulinemic euglycemic clamp with stable isotopes.

Results: The obese women were insulin resistant, characterized by lower adipose tissue GLUT 4 expression and reduced peripheral insulin sensitivity. Circulating levels of C-reactive protein (CRP) were increased in the obese subjects. Overall, the expression of both pro- and anti-inflammatory markers were increased in SAT and VAT in the obese compared to the lean controls. SAT displayed a predominant pro-inflammatory phenotype, whereas VAT showed higher expression of anti-inflammatory markers. In addition, obese subjects showed higher influx of T cells in both adipose tissue compartments with higher expression of CD25, a marker of activated T cells, in VAT. Despite these distinct inflammatory phenotypes of adipose tissue in obesity, no correlations were observed between any of the inflammatory markers and insulin sensitivity.

Conclusions: Compared to lean healthy controls, morbidly obese and insulin resistant women show marked inflammation in both SAT and WAT reflected by increased expression of pro- and anti-inflammatory markers. Analysis of adipose tissue extracted CD11b+ macrophages in the obese subjects further revealed higher VAT expression of anti-inflammatory CD163 and mannose receptor and higher SAT expression of CD11c+. This mixed pattern of activated immunity in obesity was not associated with reduced insulin sensitivity of muscle or adipose tissue.
Introduction

Obesity is associated with a state of low-grade inflammation and activation of inflammatory pathways within adipose tissue (AT) is known to interfere with insulin signalling (1, 2, 3, 4). Lean healthy AT displays an anti-inflammatory environment, characterized by high IL4 and IL10 levels (5, 6) while in obesity, AT shows a change towards a more inflammation prone environment. Adipose tissue macrophages (ATM) are crucial mediators of adipose tissue inflammation and have been connected to AT inflammation in obesity over a decade ago (7, 8). In obesity, the number of ATM is positively correlated with BMI and adipocyte size (7, 9) and ATM are predominantly present in so-called crown-like structures, surrounding necrotic adipocytes where they are supposed to scavenge cell debris and free lipids (10). In obese AT, ATM content can increase approximately 4-fold up to 40-50% of total cell numbers (7) and undergo a phenotypic switch from an alternatively activated anti-inflammatory phenotype towards a more classically activated pro-inflammatory phenotype (11). Moreover, obese ATM show an increase in lysosomal biogenesis (8). Inflammation of adipose tissue is associated with insulin resistance in several rodent obesity models and reduction of inflammation either by genetic manipulations in mice or weight loss show reversal of the insulin resistant state (9, 12). More recently, other immune cells besides ATM including T cells, have been connected to the inflammatory AT phenotype as well. Numbers of regulatory T cells (Tregs), which are of an immuno suppressive nature are reduced in adiposity (13, 14). Tregs are CD4+ cells that express CD25+ and FOXP3, a forkhead transcription factor required for their specific development and function (CD4+CD25+FOXP3 regulatory T cells) (15) and secrete the anti-inflammatory cytokine IL10, which inhibits TNF-α production by macrophages, thereby preventing local tissue damage and dampening inflammation. From murine studies it became clear that depletion of Tregs worsens adipose tissue inflammation and thus insulin resistance (16), whereas expansion of the number of Tregs attenuates inflammation and insulin resistance (14). It has been hypothesized that in lean adipose tissue Tregs are keeping chronic inflammation under control, but during adiposity the influx of inflammatory macrophages and other immune cells outnumbers the Tregs, leading to an inflammatory prone environment.

In humans it remains largely unknown whether adipose tissue inflammation in either subcutaneous (SAT) or visceral adipose tissue (VAT) directly contributes to systemic insulin resistance. Therefore, we studied the inflammatory expression profiles of SAT and VAT and of extracted ATM in morbidly obese women undergoing bariatric surgery and in lean controls undergoing elective cholecystectomy. In addition, we assessed insulin sensitivity of muscle (insulin-mediated glucose uptake) and adipose tissue (insulin-mediated suppression of lipolysis) in the obese women using a hyperinsulinemic euglycemic clamp and stable isotope tracers.
Subjects and methods

Subjects

Twenty morbidly obese women scheduled for Roux-en-Y gastric bypass surgery (RYGB) and six matched healthy lean women scheduled for elective cholecystectomy for benign gallbladder disease were included. Subjects were recruited from the outpatient clinics of the Rijnstate Hospital in Arnhem, the Slotervaart Hospital in Amsterdam and the Medical Center Alkmaar in Alkmaar.

The obese patients were eligible for the study if they were scheduled to undergo RYGB surgery, were older than 18 years, understood the objective of the study, and were competent to give informed consent. Exclusion criteria were: childhood onset obesity, insulin dependent DM2, coagulation disorders, a recent history (6 months or less) of substantial alcohol or drug abuse; the use of antipsychotic medication or antidepressant medication; any somatic illness except for obesity-related conditions (hypertension, dyslipidemia and DM2 treated with oral antidiabetics). Inclusion criteria for the lean controls were: BMI < 25 kg/m2, scheduled for elective cholecystectomy for benign gallbladder disease. Exclusion criteria were any somatic disease, coagulation disorders, glucose intolerance and use of medication. Glucose tolerance was assessed during a 75 gr oral glucose tolerance test.

The study was approved by the Medical Ethical Committee of the Academic Medical Center (AMC) of the University of Amsterdam. After a complete description of the study had been given, written informed consent was obtained.

Analytical procedures

Hyperinsulinemic euglycemic clamp

The obese women participated in a study on the short term metabolic effects of bariatric surgery (17). Insulin sensitivity was measured using a two-step hyperinsulinemic euglycemic clamp after an overnight fast as described previously (17). In short, intravenous glycerol and glucose isotope tracers were infused after drawing a blood sample for measurement of background enrichments. After equilibration, blood was drawn for glucose and glycerol enrichments to calculate basal endogenous glucose production (EGP) and lipolysis as well as FFA and insulin. Thereafter insulin was infused at a rate of 20 mU·m−2·min−1 for two hours and a rate of 60 mU·m−2·min−1 for another two hours. After each step of the clamp, blood was drawn for glucose and glycerol enrichments to calculate suppression of basal EGP and lipolysis (step 1) and the rate of glucose disposal (Rd) (step 2) as well as FFA and insulin. To keep euglycemia, exogenous glucose enriched with the glucose isotope tracer was infused simultaneously. The detailed experimental protocol has been published earlier (17).

Body composition

Body composition was measured using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK).
Laboratory analysis

Plasma glucose was measured with a glucose oxidase method (EKF Diagnostics, Barleben / Magedeburg, Germany). Free fatty acids (FFA) were measured by an enzymatic colorimetric method (Nefa-C test kit; Wako Chemicals, Neuss, Germany) with an intra-assay variation of 1%, inter-assay variation of 4-15% and a detection limit of 0.02 mmol/L. \([6,6-^{2}H_2]\)glucose enrichment (tracer-to-tracer ratio) and \([1,1,2,3,3-{^{2}H_5}]\)glycerol enrichment (tracer-to-tracer ratio) were measured as described earlier (18). Insulin was determined on an Immulite 2000 system (Diagnostic Products, Los Angeles, CA, USA) using a chemiluminescent immunometric assay with an intra-assay variation of 4–5%, inter-assay variation of 5% and detection limit of 15 pmol/l. C-reactive protein (CRP, ng/mL) was determined using ELISA (R&D systems Europe, Ltd. Abingdon, UK), according to the manufacturer’s instructions.

Adipose tissue biopsies

The surgical procedures were carried out in three medical centers (Rijnstate Hospital, Arnhem, Slotervaart Hospital, Amsterdam and Medical Center Alkmaar, Alkmaar) and performed by experienced surgeons. Adipose tissue biopsies were taken from two adipose tissue compartments (visceral and abdominal subcutaneous) during RYGB surgery in the obese and during laparoscopic cholecystectomy in the lean controls. Samples were taken from similar tissue locations in all patients and at the same time point during surgery after a comparable overnight fast. Haemostasis was checked directly after the biopsies and at the end of the surgical procedure. A part of the adipose tissue samples was collected in DMEM media (DMEM with glutamine, 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and kept at room temperature for a maximum period of 3 hours. Another part was snapfrozen in liquid nitrogen and stored in -80ºC for subsequent analysis.

Extraction of macrophages from adipose tissue biopsies

Adipose tissue collected in DMEM was thoroughly chopped with a sterile surgical blade and resuspended in 10 ml digestion solution (7 ml Hanks’ Solution, 3 ml 7.5 % BSA, and 20 mg Collagenase type II, Sigma). The digestion was performed at 37 °C using a shaker at 100 rpm for 20 min. After digestion, the adipocyte fraction was passed through a filter: cell strainer 100uM from falcon (ref 352360) and the remaining solution was centrifuged at 1500 rpm, 4 °C for 5 min. This pellet was resuspended in 2 ml of selection buffer (PBS, 2 mM EDTA, 0.5 % BSA). CD11b positive cells were subsequently selected using CD11b micro-beads (Miltenyi Biotec) according to the manufacturer’s instructions. The negative fraction from this isolation was collected and referred to as the stroma vascular fraction (SVF).

RNA extraction

Total RNA was extracted from the biopsies using TRizol reagent (Invitrogen), followed by further extraction using the NucleoSpin RNA II kit according to the manufacturer’s recommendations (Macherey-Nagel GmbH, Duren, Germany). This protocol included a RNase-free DNase step.
RNA concentrations were determined using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was investigated by assessing the RNA integrity number (RIN), using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The mean RIN was 7. Equal amounts of RNA were used to synthesize cDNA, using oligo-(dT)12–18 and random hexamers as primers, and Superscript II reverse transcriptase, according to the manufacturer’s method (Invitrogen). Gene-specific analysis was performed on an iCycler MyiQ single-color real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad Laboratories). Gene expression levels were normalized to acidic ribosomal protein 36B4, also referred to as P0. Specificity of the primers was verified by evaluation of the amplifications with the use of gel electrophoresis and melting curve analysis. The primers used on adipose tissue were CD68, Macrophage Inflammatory Protein (MIP) Il1beta (IL1-b), Mannose receptor (MR), GLUT4 and T cell markers (CD25 and CD4), leptin, adiponectin and PPARy. Primers used on macrophages were IL1b, IL18, MIP1beta, CD11c, MR and CD163. The number of analyzed tissue samples of the obese subjects slightly differ per measurement (between N= 16 and N =20) because of poor quality of some samples.

**Calculations and statistical analyses**

Data were analysed using parametric and non-parametric tests. For the statistical analyses of mRNA expression, the unpaired Mann Whitney U test was used. Correlations were determined using the Spearman’s Rho test. Patient characteristics are presented as mean ± SD. SPSS version 20.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Comparisons were considered statistically significant if the p value was <0.05 and p < 0.1 was considered a trend. Clamp data are presented as median [minimal - maximum]. Endogenous glucose production (EGP) and insulin-mediated peripheral glucose uptake (rate of disappearance [Rd]) were calculated using the modified form of the Steel equation as described previously (19, 20). EGP is expressed as μmol/kg fat-free mass (FFM) min⁻¹ and, Rd as μmol/kg·min⁻¹ and μmol/kg·min. Hepatic and adipose tissue insulin sensitivity were expressed as % insulin-mediated suppression of basal EGP and lipolysis, respectively, during step 1 of the clamp. HOMA-IR was calculated as fasting glucose x fasting insulin divided by 22.5 and quantitative insulin sensitivity check index (QUICKI) as 1/[log(I₀) + log(G₀)].

**Results**

**Study participants**

We included 6 lean and 20 obese women. Their baseline characteristics are shown in Table 1(17). The lean women had a normal fasting glucose and normal HOMA-IR.
INFLUX OF MACROPHAGES AND T CELLS IN VISCERAL AND SUBCUTANEOUS ADIPOSE TISSUE OF MORBIDLY OBESE WOMEN IS NOT ASSOCIATED WITH INSULIN SENSITIVITY

Table 1. Subject characteristics. Data are presented as mean ±SD. BMI=Body mass index.

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>LEAN (N=6)</th>
<th>OBESE (N=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>36 ± 6.6</td>
<td>41 ± 8.5</td>
<td>0.203</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.7 ± 5.0</td>
<td>127.5 ± 21.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.1 ± 1.4</td>
<td>45.3 ± 6.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.0 ± 0.7</td>
<td>2.8 ± 1.2</td>
<td>0.002</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.39 ± 0.09</td>
<td>0.33 ± 0.03</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>31.1 ± 19.8</td>
<td>83.1 ± 32</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0 ± 0.4</td>
<td>5.6 ± 1.1</td>
<td>0.169</td>
</tr>
</tbody>
</table>

Glucose metabolism and lipolysis

As expected, HOMA-IR and QUICKI significantly differed between the lean and obese group showing lower insulin sensitivity in the obese group (table 1). Basal glucose metabolism was assessed in 19 and insulin sensitivity in 17 obese women due to technical difficulties with iv lines. Insulin-mediated peripheral glucose uptake ($R_d$), which is a measure for skeletal muscle glucose uptake (21), was decreased (24.6 (11.5-42.5) μmol kg⁻¹ min⁻¹) in 89% of our subjects (i.e. $R_d$ < 37.3 μmol kg⁻¹ min⁻¹ which is our recently reported cut off for normal insulin sensitivity) (22). Basal endogenous glucose production (EGP) was 13.7 [10.3-18.1] μmol/kg FFM*min. Lipolysis measured with labeled glycerol was 3.01 [1.92-5.05] µmol/kg.min in the fasted state and 1.48 (0.76-3.31) µmol/kg.min during the first step of the hyperinsulinemic euglycemic clamp. The mean suppression of lipolysis, a measure for insulin sensitivity of adipose tissue, was 51.5% (12.8 – 67.1).

Adipose tissue mRNA expression of key metabolic genes associated with metabolic health

Downregulation of adipose GLUT4 in obesity is a hallmark of insulin resistance (23). Indeed, in the obese subjects expression of GLUT4 mRNA in SAT and VAT was significantly lower compared to the lean controls (figure 1a) and correlated with insulin action in muscle SAT ($r$ = 0.58; $p = 0.014$) and VAT ($r$ = 0.576; $p 0.016$). mRNA expression of leptin, a hormone involved in food intake and energy metabolism and known to be higher in obesity in relation to body weight (24) was significantly higher in both adipose compartments in the obese subjects (figure 1b). Next we measured mRNA expression of adiponectin, an insulin sensitizing and anti-inflammatory adipokine that has been shown to be downregulated in insulin resistant obese people (25) and expression of peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor crucial for adipogenesis (26). Adiponectin was significantly lower in SAT in the obese subjects (figure 1c) while expression of PPARγ, was similar in both groups (figure 1d). These data show distinct features associated with obesity and insulin resistance in adipose tissue of the obese subjects.
Inflammatory profiles in SAT and VAT in obese versus lean subjects

First we measured circulating CRP as a measure of whole body low grade inflammation and found CRP to be increased in the obese subjects (obese 9489±6144 ng/mL and lean 1419±521 ng/mL; p < 0.001). To assess the inflammatory phenotype of adipose tissue we measured mRNA expression levels of several pro- and anti-inflammatory markers. Expression of the macrophage marker (CD68) (figure 2a) was significantly increased in SAT in the obese subjects, while the pro-inflammatory chemokine (MIP1beta) (figure 2b), the macrophage marker mannose receptor (MR), which increased expression has been shown on alternative activated macrophages and the anti-inflammatory cytokine IL10 (figures 2c and 2d) were significantly increased in the obese subjects in both SAT and VAT. None of the inflammatory markers correlated with insulin action in muscle or adipose tissue.
INFLUX OF MACROPHAGES AND T CELLS IN VISCERAL AND SUBCUTANEOUS ADIPOSE TISSUE OF MORBIDLY OBESE WOMEN IS NOT ASSOCIATED WITH INSULIN SENSITIVITY

Chapter 6

Figure 2. Relative mRNA expression levels of CD68 (a), MIP1β (b), Mannose Receptor (MR) (c) and IL10 (d) in subcutaneous (SAT) and visceral (VAT) adipose tissue in obese (N=20) and lean (N=6) subjects (* p < 0.05 and # p < 0.001). Data are presented as mean ± SD.

Inflammatory profiles of adipose tissue macrophages (ATM)

To further analyze the inter-compartment differences in inflammatory state of the SAT and VAT resident macrophages in the obese subjects, we isolated CD11b+ macrophages and measured expression levels of M1 and M2 markers. IL1β and IL18 are produced by M1 activated macrophages, but were not differentially expressed between SAT and VAT (figure 3 a and b) while CD11c (figure 3 c) showed a higher and MIP1β (figure 3 d) a lower gene expression in extracted macrophages from SAT. Expression of the anti-inflammatory marker CD163 (figure 3 e) was significantly higher in ATM in VAT, finally the M2 marker MR (figure 3 f) shows a trend towards higher expression in VAT (p=0.071). These data show a more M2-like phenotype in VAT and higher expression of the pro-inflammatory marker CD11c in SAT. ATM expression of M1 and M2 markers were not associated with insulin-mediated peripheral
glucose uptake or suppression of lipolysis, indicative for muscle and adipose tissue insulin sensitivity, respectively.

**Figure 3.** Relative mRNA expression levels of the M1 markers IL1b (a), IL18 (b), CD11c (c) and MIP1beta (d) and the M2 markers Mannose Receptor (MR) (e) and CD 163 (f) in extracted macrophages from subcutaneous (SAT) and visceral (VAT) adipose tissue from 19 obese subjects (* p< 0.05 and # p < 0.001). CD11c in VAT, MR in SAT and CD163 in SAT: N=18. Data are presented as mean ± SD.
**T-cell markers in SAT and VAT of obese and lean subjects**

Since it has been shown recently that T-cells are involved in obesity-associated inflammation, we studied a marker of general T cell influx as well as markers of activated T cells in SAT and VAT by measuring gene expression levels of CD4, CD25 and FOXp3 respectively. CD4 was significantly higher in SAT in the obese subjects compared to the lean controls but it was not differentially expressed between AT compartments (figure 4 a). CD25, a surface marker of activated T cells showed higher expression in both adipose compartments in the obese versus the controls (figure 4 b) and additionally showed higher expression in VAT within the obese group (p=0.001). FOXp3, a marker of regulatory T cells, expression was not different between lean and obese subjects or between compartments in both groups (data not shown). These data show an overall increased influx of CD4 positive T cells in SAT and VAT in obesity as well as an increase of activated T cells, the latter having the highest expression in obese VAT. Importantly, the expression levels of the different T cell markers were not correlated with insulin sensitivity of muscle or adipose tissue.

**Discussion**

In this study we show that in obese and insulin resistant women, subcutaneous and visceral adipose tissue is characterized by lower expression of GLUT4 as well as influx and activation of macrophages and activated T cells. Lower GLUT4 expression in adipose tissue was associated with insulin sensitivity while the inflammatory, T cell and macrophage markers were not correlated with peripheral glucose uptake, insulin-mediated suppression of lipolysis and endogenous glucose production, i.e. insulin action in muscle, adipose tissue and liver respectively. More detailed analyses of the expression levels of pro- and anti-inflammatory markers in CD11b+ enriched ATM isolated from the VAT and SAT compartments revealed that subcutaneous ATM display a predominant pro-inflammatory phenotype, whereas visceral
ATM are of a more anti-inflammatory nature. Finally, we show that the influx of activated T cells in adipose tissue in obese subjects is more pronounced in VAT which is in line with the presence of higher anti-inflammatory markers in that compartment since T cells play a pivotal role in reducing inflammation.

There is robust scientific evidence showing that obesity is associated with a state of low-grade inflammation and that inflammatory changes negatively influence insulin sensitivity and adipose tissue function (1, 8, 27, 28) although most studies showing this direct link have been performed in rodents. In line with other published data (29, for review 30), CRP was markedly elevated in the obese subjects compared to the matched lean controls. CRP is mainly produced by the liver, but adipose tissue also contributes to serum CRP levels (27). Whether circulating CRP has a direct impact on insulin sensitivity is unclear and in our cohort no correlation was present between insulin action and serum CRP (data not shown).

Many rodent studies showed a switch from an anti-inflammatory towards a pro-inflammatory environment in adipose tissue during the course of obesity (31, 32). The underlying mechanism explaining this change in phenotype entails multiple pathways including tissue hypoxia, ER-stress, adipocyte necrosis, altered adipokine secretion, upregulation of MCP-1 and abnormal extracellular matrix remodeling leading to fibrosis of adipose tissue (for review 33). In rodents, these inflammatory changes influence adipose tissue function with enhanced lipolysis leading to increased circulating fatty acids (34, 35). Ectopic uptake and accumulation of these fatty acids and their intermediates in insulin sensitive tissues such as liver and skeletal muscle and increased levels of circulating pro-inflammatory proteins (36) result in whole body insulin resistance. We here show that despite extensive inflammatory changes in both visceral and subcutaneous adipose tissue in obese compared to lean subjects no correlation with lipolysis or insulin sensitivity was present. It has been shown that insulin resistance in obesity is mainly predicted by the amount of visceral fat mass (37) and therefore inflammatory changes in that adipose tissue compartment could contribute to this association (38, 39). In addition, in upper body obese women lipolysis rates are higher compared to lower body obese women, suggesting an independent effect of adipose tissue compartment on insulin action in adipose tissue (40).

Furthermore, it has been shown in mice that intra-abdominal transplantation of subcutaneous adipose tissue reversed high fat diet induced inflammation and glucose intolerance, suggesting that subcutaneous adipose tissue protects from metabolic deterioration in the setting of obesity by reducing inflammation (41). Surprisingly, the obese subjects in our study showed a more anti-inflammatory profile with more M2 markers and higher expression of T cell markers in visceral adipose tissue suggesting that as long as inflammation in visceral adipose tissue is predominantly anti-inflammatory in nature, no effect on adipose tissue or muscle insulin sensitivity occurs. On the other hand, lipolysis was assessed as whole body total triglyceride lipolysis and which makes it impossible to distinguish between subcutaneous and visceral adipose tissue lipolysis and, therefore, we cannot exclude a
direct effect of visceral adipose tissue inflammation on visceral adipose tissue lipolysis. Insulin sensitivity of skeletal muscle, reflected as insulin-mediated glucose uptake, was not correlated to any of the inflammatory markers assessed in adipose tissue. This is in line with a more recent study showing that the presence of crown-like structures, i.e. macrophages surrounding necrotic adipocytes in inflamed adipose tissue, in mesenteric but not omental or subcutaneous adipose tissue predicted insulin resistance. However these were elderly men with vascular disease and insulin sensitivity was measured using HOMA index (42).

In the obese subjects, both adipose tissue compartments showed influx of macrophages and activated T cells suggesting that attraction of immune cells into adipose tissue in obesity is not adipose tissue depot-specific. However, activation of the invaded immune cells showed a depot specific difference. Surprisingly, visceral adipose tissue showed less pro-inflammatory features compared to the subcutaneous compartment. Studies on differences in inflammation between VAT and SAT show conflicting results. Secretome analyses in pre-adipocytes from VAT compared to SAT from obese subjects revealed VAT pre-adipocytes having higher chemo-attractant properties (43) but the intracellular protein differences in between compartments were less prominent prominent suggesting an overlap in expression of intracellular inflammatory proteins between AT compartments. Another recent study in elderly men with aortic aneurysm and a broader range in BMI, showed that CD68 was lowest in SAT and that expression of many inflammatory proteins was similar for omental and subcutaneous adipose tissue except for IL-18 and MIF which were higher in omental AT (42). However the current study subjects were female, younger on average and without vascular disease. The more prominent pro-inflammatory profile in SAT in the obese subjects could be explained by lower adiponectin since it has been shown that adiponectin has anti-inflammatory properties (44) and adipose tissue expression levels are correlated to circulating CRP (45). Finally the higher expression levels of markers of activated T cells in VAT might explain the anti-inflammatory phenotype in that compartment because T cells exert anti-inflammatory actions (46). We did not perform FACS analyses, which would have allowed us to quantify the activated T cell regulatory fraction of CD4 positive cells. Future studies are needed to study the potential protective role of T regulatory cells in adipose tissue inflammation and insulin resistance. Furthermore, a future therapy might be directed towards blunting the M1 response without affecting M2 polarization by targeting specific molecules. Additional translational studies from rodents to humans are needed since their inflammatory responses may be substantially different. Moreover, it has yet to be established whether inhibition of the inflammatory response contributes to improvements in glucose homeostasis in humans.

Since our data were analyzed in a cross-sectional manner, we cannot draw conclusions on how changes in degree and type of inflammation over time during further weight gain or weight loss would affect metabolic health. Thus, follow up studies are needed to elucidate whether modulation of specific inflammatory markers or profiles over time predict changes in metabolic health.
We conclude that SAT and VAT of morbidly obese and insulin resistant women is characterized by influx of macrophages and T cells and that VAT is less pro-inflammatory compared to SAT. The latter might be explained by lower expression of adiponectin in SAT and the presence of higher anti-inflammatory T cells in VAT. Despite the presence of inflamed adipose tissue in these women, none of the inflammatory markers in adipose tissue correlated with insulin action in muscle, liver or adipose tissue.

Acknowledgements

We thank Unga Unmehopa for her assistance with the PCR’s and Edo Aarts for his help in recruiting patients.

References


Supplemental data

The following primers were used: **CD68** forward primer 5’-GCTGGCTGTGCTTTTCTCG-3’, reverse primer 5’-GTCACCGTGAAGGCA-3’ (NM_001251; 197–307); **MR** forward primer 5’-TGCAGAAGCAAAACCACCT-3’, reverse primer 5’-CAGGCTTTAAAGCCACGAAACT-3’, **MIP-1β/CCL4** forward primer 5’-GCGTGAATG-TCTGCTCTCC-3’, reverse primer 5’-AACATGGTGAAACCGCGTA-3’. Human **CD4** forward primer 5’-CAGATCAAGAGACTCTCTAGTGAG AA-3’; reverse primer 5’-GCCTCTGGCTCAATGG-3’; **humanCD25** forward primer 5’-CCTGGAAACCAATGTAAT-3’, reverse primer TTCTCAGGTGGTGTCAGTTTT-3’; human **IL1β** forward primer 5’-GCCTCTGGCTCAATGG-3’, reverse primer 5’-TCTCCAGAAAGGACTCTTTTA-3’ (NM_000572; 197–277); **CD163** forward primer 5’-ACATAGATCATGCATCTGTCATTG-3’, reverse primer 5’-ATTCTCAGTGAAGACTCTTTTA-3’; Human **PPARγ** forward primer 5’-GCTGTGCTGAGGAGATCACAGA-3’, reverse primer 5’-GGGCTCATAAAGTCACCAA-3’; human **adiponectin** forward primer 5’-CTGGAAACCTACGGCTCATTGC-3’, reverse primer 5’-AAGTTGACCGATGTGATGG-3’; human **leptin** forward primer 5’-AGAGTACGGGCTGAGATGC-3’, reverse primer 5’-CTTGCTGCTAACGCTCC-3’; **IL-1β** forward primer 5’-AGCTGAGGCTGATACTGCTT-3’, reverse primer 5’-GGACATGGGAGACTCTTTT-3’. **IL18** forward primer 5’-CCAGGAAATCGGCTCTATT-3’, reverse primer 5’-CTTCACAGGATAGTTACAGCCAC-3’.
Serum Retinol Binding Protein–4 Is Inversely Associated with Insulin Action in Adipose Tissue, Skeletal Muscle and Liver in Obese Women


† * These authors contributed equally to this work.
Abstract

**Background:** Adipose-tissue expression of retinol binding protein 4 (RBP4) is associated with obesity-induced insulin resistance and impairs insulin action in skeletal muscle, but less is known about its effects on insulin sensitivity in adipose tissue and liver in humans. We aimed to explore the relation between serum and tissue RBP4 and peripheral and hepatic insulin sensitivity as well as insulin sensitivity of adipose tissue in obese humans.

**Methods:** We performed a 2-step hyperinsulinemic-euglycemic clamp using a stable glucose and a stable glycerol isotope tracer to assess hepatic and peripheral as well as adipose-tissue insulin sensitivity respectively in 20 morbidly obese women undergoing Roux-en-Y gastric bypass surgery. To assess RBP4 expression levels, tissue biopsies were obtained during surgery from liver, subcutaneous and visceral adipose tissue in the obese women as well as in 6 lean healthy controls undergoing elective laparoscopic cholecystectomy.

**Results:** RBP4 expression was increased in both liver and subcutaneous and visceral adipose tissue in the obese subjects. Serum RBP4, but not tissue RBP4 expression, inversely correlated with peripheral glucose uptake and insulin-mediated suppression of lipolysis, free fatty acids and endogenous glucose production.

**Conclusions:** Gene expression of RBP4 is increased in liver and adipose tissue of morbidly obese subjects compared to lean controls. While tissue RBP4 expression does not correlate with insulin sensitivity, serum RBP4 correlates inversely with insulin action in adipose tissue, skeletal muscle and liver. These data indicate that in obesity RBP4 signals as a hormone affecting insulin sensitivity of multiple metabolic pathways.
Chapter 7

SERUM RETINOL BINDING PROTEIN-4 IS INVERSELY ASSOCIATED WITH INSULIN ACTION IN ADIPOSE TISSUE, SKELETAL MUSCLE AND LIVER IN OBESE WOMEN

Introduction

The last several decades the prevalence of obesity has increased worldwide. Obesity increases the risk for, among others, type 2 diabetes mellitus (T2DM), dyslipidemia and cardiovascular disease. As a result, obesity is associated with a higher mortality risk (1). The major underlying cause of the metabolic complications of obesity is the development of insulin resistance, which is defined as impaired insulin action in insulin-sensitive tissues. Insulin resistance in skeletal muscle results in reduced glucose uptake whereas in adipose tissue reduced insulin action results both in reduced glucose uptake and reduced suppression of lipolysis. Finally insulin resistance in the liver leads to reduced suppression of endogenous glucose production (EGP) (2). Obesity-induced insulin resistance is characterized by a downregulation of the glucose transporter-4 (GLUT4) in adipocytes (3), leading to whole-body insulin resistance (4) and elevated expression of retinol binding protein 4 (RBP4) (5, 6). RBP4 increases the expression of the major gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) in liver and impairs insulin signaling in skeletal muscle (5). In obese and T2DM subjects, adipose expression and serum levels of RBP4 are increased and are associated with insulin resistance in most, but not all studies (7). RBP4 increases basal EGP and reduces insulin-mediated EGP suppression in rodents (5), but whether serum RBP4 is associated with impaired hepatic insulin sensitivity in humans is unknown. The liver is the main production site of RBP4 (8, 9) and while hepatic RBP4 expression is increased in obese rodents (10), the effect of obesity on hepatic RBP4 expression in humans is unclear. Next to disturbed glucose handling in states of insulin resistance, dysfunctional adipose tissue in obesity is characterized by increased release of free fatty acids (FFA), resulting in whole-body insulin resistance (15). Whether RBP4 interacts with the lipolytic pathway is unknown.

We studied the relation between circulating and tissue RBP4 and insulin action in adipose tissue, skeletal muscle and liver and show that adipose-tissue and hepatic RBP4 expression are both increased in obesity and that serum RBP4, but not tissue RBP4, is inversely correlated with insulin action in adipose tissue, skeletal muscle and liver.

Methods

Subjects

Twenty morbidly obese female subjects scheduled for laparoscopic Roux-en-Y gastric bypass (RYGB) surgery were included (16). Six lean healthy women undergoing elective laparoscopic cholecystectomy served as control subjects for the measurement of tissue RBP4 only. The control subjects were eligible for the study if they were older than 18 years, understood the objective of the study, were competent to give informed consent, had a body mass index (BMI) between 20-25 kg/m² with a stable weight and had a normal glucose tolerance test according to the criteria of the ADA (17). Exclusion criteria were performance of vigorous exercise, family history of T2DM, a recent history (6 months or less) of substantial alcohol or drug abuse and the use of any medication. The study was approved by the Medical Ethical...
Committee of the Academic Medical Center (AMC) of the University of Amsterdam. After a complete description of the study had been given, written informed consent was obtained.

**2-step hyperinsulinemic euglycemic clamp**

Glucose metabolism and lipolysis were assessed in the obese subjects only (16). The subjects were admitted to the Metabolic Clinical Research Unit of the AMC at 08:00 a.m. after an overnight fast. A catheter was inserted into an antecubital vein for infusion of [6,6-²H₂]glucose, [1,1,2,3,3-²H₅]glycerol (>99% enriched; Cambridge Isotopes, Andover, MA, USA), glucose 20%, and insulin. Another catheter was inserted into a contralateral hand vein and kept in a thermoregulated (60°C) plexiglas box for sampling of arterialized venous blood. Saline was infused as NaCl 0.9% at a rate of 50 ml/h to sustain catheter patency. [6,6-²H₂]-glucose and [1,1,2,3,3-²H₅]glycerol were infused as tracers to study glucose kinetics and lipolysis (total triacylglycerol hydrolysis), respectively. At 09:00 a.m. (T = -2h), after drawing a blood sample for background enrichment of plasma glucose and glycerol, a primed-continuous infusion of [6,6-²H₂]glucose and of [1,1,2,3,3-²H₅]glycerol were started at a rate of 0.11 µmol/kg/min after a priming dose equivalent to 120 min infusion. After 110, 115 and 120 min, blood samples were drawn for determination of glucose and glycerol enrichments, glucoregulatory hormones and FFA. Subsequently, at 11:05 a.m. (T = 0), a continuous infusion of insulin (Actrapid 100U/ml; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2h at a rate of 20 mU/m² body surface area/min. At T = 2h, the infusion rate of insulin was increased to 60mU/m² body surface area. Plasma glucose was measured every 10 min and glucose 20% was infused at a variable rate to maintain plasma glucose at 5.0 mmol/L. [6,6-²H₂]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to minimize changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. At T = 2h and T = 4h, 5 blood samples with a 5 min interval were drawn to measure glucose and glycerol enrichments and 2 samples were drawn to measure glucoregulatory hormones and FFA. During the study, the participants were allowed to drink water only.

**Biopsies**

RYGB surgery was performed by experienced bariatric surgeons in two medical centers (Rijnstate Hospital, Arnhem and Slotervaart Hospital, Amsterdam, the Netherlands). The laparoscopic cholecystectomies were performed by an experienced surgeon in one medical center (Alkmaar Medical Centre, Alkmaar, the Netherlands). Before starting the Roux-en-Y procedure, tissue biopsies were taken from the liver and visceral and subcutaneous abdominal adipose tissue. Local hemostasis was checked directly after the biopsies and at the end of the surgical procedure. Tissue samples were obtained after a comparable fasting period (10-12 hours). The samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analyses.
Gene expression

Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands), followed by the NucleoSpin II extraction kit (Macherey & Nagel GmbH, Duren, Germany) according to the manufacturer’s recommendations. Briefly, 1 ml of TRIzol and glass beads (Biospec Products Inc., Bartlesville, OK, USA) were added to the tissue. After vigorous shaking using a Fast Prep-24 machine for 20 sec. at 4.5 m/s (MP Biomedicals, Santa Ana, CA, USA), the homogenate was centrifuged (10 min at 12,000 x g at 4 °C). The non-lipid containing fraction was transferred and 200 μl of chloroform was added. The mixture was subsequently vortexed and centrifuged (15 min. at 16,100 x g at 4 °C). The aqueous phase was transferred to a new tube and an equal volume of 70% ethanol was added. Afterwards, we continued with the NucleoSpin II extraction kit according to the manufacturer’s instructions. RNA concentrations were measured using the Nanodrop Spectrophotometer 1 (Nanodrop Technologies, Wilmington, NC, USA). cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer’s instructions. After synthesis, the cDNA was diluted 20 times. Expression of RBP4 and GLUT4 were normalized to the housekeeping gene Acidic Ribosomal Phosphoprotein P0 (RPLP0 or 36B4).

Protein isolation

Cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris–HCl pH 7.4, 2mM EDTA, 0.5% deoxycholaat, 1mM Na3VO4, 20mM NaF, 0.5% Triton X-100), supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF.

RBP4 protein

Serum was diluted 30 times in a standard lysis buffer. Adipose tissue was lysed in standard lysis buffer. 1 μl equivalent of human serum and 50 μg equivalent of human adipose tissue were separated by 18% SDS-PAGE and transferred to nitrocellulose membranes. Human RBP4 proteins were detected using anti-human RBP4 polyclonal antisera (Dako, Glostrup, Denmark, catalog #A0040).

Analytical procedures

Glucose was measured using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/ Magdeburg, Germany). Insulin was determined using an Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, CA, USA), with a chemiluminiscent immunometric assay (intra-assay variation 4-5%; inter-assay variation 5%; detection limit 15 pmol/l). FFA were measured using an enzymatic method (NEFA-Cc, Wako Chemicals, Neuss, Germany; intra-assay variation 1%; inter-assay variation 4-15%; detection limit 0.02 mmol/l). [6,6-2H2]glucose and [1,1,2,3,3-2H5]glycerol enrichment were measured as described previously (16).
Body composition
Fat-free mass was assessed using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK).

Calculations
EGP, lipolysis rate and peripheral glucose uptake (rate of disappearance [R_d]) were calculated using modified versions of the Steele equations as described previously (18, 19). EGP is expressed as μmol/(kg fat-free mass)/min. Lipolysis and R_d are expressed as μmol/kg/min. Insulin-mediated suppression of lipolysis, FFA and EGP are expressed as the % suppression of the basal values and was assessed during the first step of the hyperinsulinemic-euglycemic clamp. Insulin-mediated suppression of lipolysis and FFA represent a measure of adipose tissue insulin sensitivity. As approximately 80% of insulin-mediated glucose uptake occurs in skeletal muscle, Rd represents a measure of skeletal-muscle insulin sensitivity. Finally insulin-mediated suppression of EGP is a measure of hepatic insulin sensitivity. The quantitative insulin sensitivity check index (QUICKI) was used as an indirect measure of insulin sensitivity to compare the lean versus obese subjects and was calculated as described previously (20).

Statistical analysis
Data are presented as mean and range. Due to the small sample size and non-normal distribution of study parameters, nonparametric tests were used. Between-group differences were tested using the Mann-Whitney U test. Correlations were calculated using the Spearman correlation test. All statistical analyses were run on IBM SPSS version 21 (SPSS, Chicago, IL, USA). A p-value < 0.1 was considered a trend and a p-value < 0.05 was considered statistically significant.

Results
Demographic and metabolic characteristics
Table 1 summarizes demographic and metabolic characteristics. The subjects were all female and similar in age. The obese subjects were insulin resistant, reflected in a lower QUICKI than the control group. Since an adipose-tissue specific decreased GLUT4 expression is a central feature of whole-body insulin resistance (3), we measured GLUT4 expression in the adipose tissue compartments of both groups. GLUT4 expression was lower in the obese subjects in both the subcutaneous (P < 0.001) and visceral (P = 0.019) adipose tissue compartments (fig. 1a). Both, subcutaneous and visceral adipose GLUT4 expression, were inversely correlated with BMI ($r_s = -0.601$, $P = 0.001$; $r_s = -0.502$, $P = 0.011$, resp.). Subcutaneous but not visceral adipose GLUT4 expression correlated with QUICKI ($r_s = 0.551$, $P = 0.004$ and $r_s = 0.307$, $P = 0.136$ respectively). Similarly, in the obese subjects subcutaneous GLUT4 expression correlated with R_d ($r_s = 0.591$, $P = 0.013$) while visceral GLUT4 did not ($r_s = 0.397$, $P = 0.115$) (fig. 1b).
**Serum RBP4 is inversely correlated with insulin sensitivity**

We performed flux measurements in the obese in order to distinguish the relation of RBP4 and insulin action in different tissues. Because circulating RBP4 interferes with insulin signaling (5), we hypothesized that elevated circulating RBP4 would be inversely associated with insulin action in insulin-sensitive tissues. Indeed, serum RBP4 correlated inversely with R_d, which primarily reflects insulin action in skeletal muscle ($r_s = -0.551$, $P = 0.022$) and correlated strongly inversely with insulin-mediated suppression of lipolysis ($r_s = -0.635$, $P = 0.008$) and FFA ($r_s = -0.672$, $P = 0.03$), reflective of insulin action in adipose tissue. In addition, serum RBP4 correlated inversely with insulin-mediated suppression of EGP, indicating that in obesity RBP4 interacts with insulin action in liver ($r_s = -0.637$, $P = 0.006$) (fig. 2). Adipose and hepatic RBP4 expression did not correlate with any of the metabolic fluxes.

**Adipose and hepatic RBP4 is increased in obese women**

Subcutaneous adipose RBP4 expression was increased in the obese subjects compared to the lean controls ($P = 0.015$) (fig. 3a). Visceral-adipose RBP4 expression showed a trend toward an increase ($P = 0.062$) (fig. 2b), therefore we measured RBP4 protein in that adipose compartment and show VAT RBP4 protein levels were significantly increased in the obese ($P = 0.009$) (fig. 2c). Hepatic RBP4 expression was also significantly increased in the obese subjects ($P < 0.001$) (fig. 2d). In obese subjects, neither hepatic nor SAT or VAT expression of RBP4 correlated significantly with serum levels.

**Table 1. Demographic and metabolic characteristics**

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>LEAN (N=6)</th>
<th>OBESE (N=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE (YEARS)</td>
<td>36 [26-45]</td>
<td>41 [26-58]</td>
<td>0.120</td>
</tr>
<tr>
<td>BMI (KG/M^2)</td>
<td>22.1 [20-24.3]</td>
<td>45.3 [38.7-61.3]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FASTING GLUCOSE (MMOL/L)</td>
<td>5.0 [4.5-5.6]</td>
<td>5.7 [4.4-8.8]</td>
<td>0.200</td>
</tr>
<tr>
<td>FASTING INSULIN (PMOL/L)</td>
<td>31.1 [14-63]</td>
<td>83.1 [20-142]</td>
<td>0.002</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.39 [0.34-0.44]</td>
<td>0.33 [0.30-0.41]</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data are presented as mean [range]; QUICKI: quantitative insulin sensitivity check index with a higher index indicating higher insulin sensitivity.
Figure 1. Relative GLUT4 mRNA expression in subcutaneous and visceral adipose tissue. Data presented as mean ± standard error. Adipose GLUT4 mRNA is increased in obese subjects versus lean controls (* p < 0.001 and # p = 0.019) (A). Subcutaneous adipose GLUT4 expression is correlated with glucose disappearance rate ($R_d$) in obese subjects (B). SAT = subcutaneous adipose tissue, VAT = visceral adipose tissue.
SERUM RETINOL BINDING PROTEIN-4 IS INVERSELY ASSOCIATED WITH INSULIN ACTION IN ADIPOSE TISSUE, SKELETAL MUSCLE AND LIVER IN OBESE WOMEN

Figure 2. Serum RBP4 is inversely correlated with glucose disappearance rate ($R_d$) and insulin-mediated suppression of basal lipolysis, free fatty acids and endogenous glucose production (EGP).
Figure 3. Adipose and liver RBP4 expression. Data presented as mean ± standard error (# p < 0.05 and * p < 0.01). A. Subcutaneous RBP4 mRNA expression is increased in obese subjects versus lean controls. B. RBP4 mRNA expression in visceral adipose tissue in obese subjects and lean controls. C. Visceral RBP4 protein levels are increased in obese subjects versus lean controls. D. Hepatic RBP4 mRNA expression is increased in obese subjects versus lean controls.

Discussion

As shown previously (5, 7), expression of RBP4 was increased while GLUT4 was decreased in subcutaneous and visceral adipose tissue in obese subjects. In addition to adipose tissue, we show that hepatic RBP4 expression was increased in our obese subjects compared to the lean controls. The regulation of tissue RBP4 is only partly understood. In liver, RBP4 is synthesized primarily in hepatocytes (22) and serves as the main carrier of vitamin A that is stored as retinyl esters in hepatic stellate cells (21). Retinyl esters can be hydrolyzed by retinyl ester hydrolases and transferred to hepatocytes where they are bound to RBP4 and released into the circulation (23). In addition to retinol itself (23, 24), other factors can also induce RBP4 expression, such as glucagon (25).

While many studies have shown a relation between circulating RBP4 and glucose infusion rates, indicative of whole body insulin sensitivity, we aimed to further elucidate which insulin target tissues are affected by increased levels of circulating and tissue RBP4. In obese subjects, tissue RBP4 did not correlate with any of the insulin-sensitive fluxes while serum
SERUM RETINOL BINDING PROTEIN-4 IS INVERSELY ASSOCIATED WITH INSULIN ACTION IN ADIPOSE TISSUE, SKELETAL MUSCLE AND LIVER IN OBESE WOMEN

Chapter 7

RBP4 showed a strong inverse correlation with insulin sensitivity of adipose tissue, reflected in suppression of lipolysis and FFA, skeletal muscle, reflected in glucose uptake, and liver, reflected in suppression of EGP.

Whether there is a specific role for RBP4 in insulin-mediated suppression of lipolysis is unknown. Intracellular lipolysis within adipose tissue is inhibited by insulin through modulation of the major lipases adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (26). Activated HSL-induced hydrolysis of retinyl esters increases cytosolic retinol (27, 28), which could induce RBP4 expression (23, 24), thereby providing a link between lipolysis and retinol metabolism in adipocytes. Conversely, RBP4 could impair insulin-mediated lipolysis suppression in adipose tissue. Previous studies have shown that RBP4 activates antigen-presenting cells resulting in activation of T cells and macrophages within adipose tissue. This further induces a proinflammatory phenotype with production of cytokines such as TNFα and is associated with impaired insulin signaling in adipocytes (11, 12). As a result, systemic insulin resistance and glucose intolerance develop (12). Next to its effects on glucose handling, TNFα also stimulates lipolysis (29) and therefore RBP4 may indirectly inhibit lipolysis suppression by activating proinflammatory immune cells. However, further studies are needed to elucidate the role of RBP4 in insulin-mediated suppression of adipose-tissue lipolysis.

In addition, in the obese subjects serum RBP4 correlated inversely with insulin-mediated peripheral glucose uptake, indicating an effect of circulating RBP4 on insulin action primarily in skeletal muscle, since the majority of infused glucose is disposed in muscle under hyperinsulinemic conditions (30). The mechanism of RBP4-induced skeletal muscle insulin resistance is partly understood, e.g. it might act through binding to its high affinity receptor STRA6 and activating pathways that attenuate insulin signaling, such as SOCS3 (13), which inhibits insulin-mediated tyrosine phosphorylation of insulin receptor substrate 1 (31), ultimately blocking downstream insulin signaling. Alternatively, data from twin studies suggested that RBP4 might contribute to insulin resistance in a secondary manner (ref 32).

In our obese subjects, serum RBP4 was inversely correlated with insulin-mediated suppression of EGP, consistent with previous findings in rodent and in-vitro studies (5) showing that RBP4 induces hepatic expression of PEPCK, the major gluconeogenic enzyme and directly impairs insulin-mediated suppression of glucose production by hepatocytes (5, 10). RBP4 might also act by a retinol-dependent mechanism as retinoids induce the expression of gluconeogenic enzymes such as PEPCK and glucose-6-phosphatase in a forkhead box protein 1-dependent manner (33).

In conclusion, although hepatic and visceral and subcutaneous adipose RBP4 expressions all are increased in morbidly obese, circulating but not tissue RBP4 is inversely correlated to insulin sensitive metabolic fluxes, suggesting that RBP4 also signals as a circulating hormone. We conclude that in obesity, serum RBP4 is inversely correlated with insulin action in adipose tissue, liver and muscle.
References

Chapter 7
SERUM RETINOL BINDING PROTEIN-4 IS INVERSELY ASSOCIATED WITH INSULIN ACTION IN ADIPOSE TISSUE, SKELETAL MUSCLE AND LIVER IN OBESE WOMEN

Morbid obesity is associated with hepatic inflammation independent of liver fat content and insulin sensitivity
Abstract

Background: Obesity is associated with hepatic steatosis, hepatic insulin resistance and low-grade inflammation. Liver inflammatory markers, insulin resistance and liver fat were shown to be associated in obese animal models of NAFLD. Whether inflammation and liver fat contribute to hepatic insulin resistance in obese humans is controversial.

Methods: We studied insulin sensitivity during a two-step hyperinsulinemic euglycemic clamp with a stable glucose isotope tracer in 20 obese women (41 [26-58] yrs.; body mass index (BMI) 45.3 [39-61] kg/m2) undergoing bariatric surgery. Gene expression of inflammatory markers in liver biopsies were compared to expression levels in liver biopsies from 6 lean glucose tolerant matched controls (36 [26-45] yrs.; BMI 22 [20-24] kg/m2) scheduled for elective cholecystectomy. Intrahepatic triglycerides (IHTG) were assessed in the obese women using 1H-MR Spectroscopy (1H-MRS) and liver biopsies were scored for steatosis and non-alcoholic fatty liver disease (NAFLD) activity.

Results: Mean IHTG was 7.7 ± 9.0% [range 0-28.8%) and one of the subjects showed histological signs of non-alcoholic steatohepatitis (NASH). Liver expression levels of pro-and anti-inflammatory markers along with CD68 were markedly increased in the obese patients compared to the controls. Within the obese group with liver steatosis, IL10 inversely correlated with liver fat content. None of the other inflammatory markers predicted liver fat content, basal endogenous glucose production (EGP) or hepatic and peripheral insulin sensitivity. IHTG did not correlate with hepatic or peripheral insulin resistance.

Conclusions: In conclusion, in morbidly obese women, the liver is inflamed independent of intrahepatic triglycerides and hepatic insulin sensitivity. This suggests that stored triglycerides do not promote inflammation or insulin resistance per se and that liver inflammatory changes in the absence of NASH do not contribute to insulin resistance.
Introduction

Obesity is associated with hepatic steatosis, hepatic insulin resistance and low-grade inflammation (1, 2). Growing evidence shows that these metabolic conditions are intertwined and contribute to the development of type 2 diabetes mellitus (DM2) and non-alcoholic fatty liver disease (NAFLD) (3). NAFLD represents a state of excessive triglyceride accumulation in the liver, which may progress to non-alcoholic steatohepatitis (NASH) and finally hepatic cirrhosis and end stage liver disease (4). The etiology of hepatic steatosis in obesity includes increased portal delivery of free fatty acids (FFA), reduced beta-oxidation and increased de novo lipogenesis (5, 6). In obese patients with DM2 liver fat content is increased and is accompanied by impaired insulin clearance and hepatic insulin resistance (7). Although the relation between liver fat and insulin resistance has been demonstrated in rodents and humans, the pathophysiological mechanism is only partly elucidated. We previously showed that insulin sensitivity in subjects with familial hypobetalipoproteinaemia and severe liver steatosis was comparable to matched controls without hepatic steatosis (8) suggesting that intrahepatic triglycerides per se do not cause hepatic insulin resistance. Instead, a major role for diacylglycerol with subsequent activation of protein kinase C (PKC) has been described (9, 10).

Another characteristic of obesity-associated insulin resistance is inflammation. Whether liver inflammation in the setting of obesity contributes to hepatic steatosis or hepatic insulin resistance is currently unknown. Inflammatory changes in NAFLD have been attributed to a reduction in peroxisome proliferator-activated receptor γ (PPARγ) expression, lower circulating adiponectin, exposure to cytokines derived from adipose tissue as well as lipotoxicity induced by fatty acid overload (11, 12). Moreover lipid-induced endoplasmic reticulum (ER) stress has been associated with insulin resistance in mice (13) and recently heme oxygenase-1 has been proposed as a pro-inflammatory signal linking inflammation to insulin resistance in mice on a high fat diet (14). Finally, Kupffer cells (the resident macrophages of the liver) decrease in rodents on a high fat diet while recruited myeloid cells invade the liver through a mechanism involving the C-C chemokine receptor type 2 (CCR2). Subsequently the invaded immune cells trigger hepatic fat accumulation which is dependent on chemokine (C-C motif) ligand 2 (CCL2)/CCR2 (15). In support, short term infusion of oleate increases hepatic recruitment of myeloid cells, suggesting that increased portal delivery of FFA both induces inflammation and triglyceride (TG) accumulation through induction of chemotaxis (15). These findings in animals suggest a complex interplay between diet and obesity- associated inflammation, hepatic fat accumulation and insulin resistance. To study the relation between liver inflammation, hepatic fat content and hepatic insulin sensitivity in humans, we studied morbidly obese women with a wide range in hepatic fat content and assessed glucose metabolism, intrahepatic triglycerides (IHTG) and expression profiles of inflammatory markers in liver biopsies. The expression profiles were compared to matched lean and glucose tolerant controls.
Subjects and methods

Subjects

Twenty morbidly obese women scheduled for Roux-en-Y gastric bypass surgery (RYGB) and six healthy lean women scheduled for elective cholecystectomy for benign gallbladder disease were included. Subjects were recruited from the outpatient clinics of the Rijnstate Hospital in Arnhem, the Slotervaart Hospital in Amsterdam and the Medical Center Alkmaar in Alkmaar.

The obese patients were eligible to participate if they were scheduled to undergo RYGB surgery, were older than 18 years, understood the objective of the study, and were competent to give informed consent. Exclusion criteria were: childhood onset obesity, insulin dependent DM2, coagulation disorders, a recent history (6 months or less) of alcohol or drug abuse; the use of antipsychotic medication or antidepressant medication; any somatic illness except for obesity-related conditions (hypertension, dyslipidemia and DM2 treated with oral anti-diabetics); Inclusion criteria for the lean controls were: body mass index (BMI) < 25 kg/m2, scheduled for elective cholecystectomy for benign gallbladder disease. Exclusion criteria were any other medical condition, coagulation disorders, glucose intolerance and use of any medication. Glucose tolerance was assessed during a 75 g oral glucose tolerance test in the lean controls.

The study was approved by the Medical Ethical Committee of the Academic Medical Center (AMC) of the University of Amsterdam. After a complete description of the study had been given, written informed consent was obtained.

Hyperinsulinemic euglycemic clamp

The obese women participated in a study on the short-term metabolic effects of bariatric surgery (16). Two weeks before surgery, they were admitted to the Metabolic Clinical Research Unit of the AMC after an overnight fast and were studied in the supine position. A catheter was inserted into the dorsal vein of the hand or distal vein of each arm. One catheter was used for sampling of arterialized blood using a heated hand box (60°C). The other catheter was used for infusion of [6,6-2H2]glucose, glucose 20%, and insulin. At T=09:00 h (T= -2h), after drawing a blood sample for background enrichment of plasma glucose, a continuous infusion of [6,6-2H2]glucose (99% enrichment; Cambridge Isotopes, Andover, MA) was started at a rate of 0.11 µmol/kg*min after a priming dose equivalent to 120 minutes infusion. After 110, 115 and 120 min, blood samples were drawn for determination of glucose enrichments, glucoregulatory hormones and FFA. Subsequently, at T=11:05 h (T=0:05), a continuous infusion of insulin (Actrapid 100U/ml; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2h at a rate of 20 mU/m2 body surface area min⁻¹. At T=2h, the infusion rate of insulin was increased to 60mU/m² body surface area min⁻¹. Plasma glucose was measured every 10 min and glucose 20% was infused at a variable rate to maintain plasma glucose at 5.0 mmol/L. [6,6-2H2]glucose was added to the 20% glucose solution to achieve glucose enrichments.
of 1% to minimize changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. At T=2h and T=4h, 5 blood samples with a 5 minutes interval were drawn to measure glucose enrichments and 2 samples were drawn to measure glucoregulatory hormones and FFA. During the study the participants were allowed to drink water only.

**Analytical procedures**

Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/Magdeburg, Germany). Plasma FFA concentrations were determined with an enzymatic colorimetric method (NEFA-C test kit; Wako Chemicals, Neuss, Germany) with an intra-assay variation of 1%, inter-assay variation of 4-15% and a detection limit of 0.02 mmol/L. [6,6-2H2]glucose enrichment (tracer-to-tracee ratio) was measured as described earlier (17). Insulin and cortisol were determined on an Immulite 2000 system (Diagnostic Products, Los Angeles, CA, USA). Insulin was measured with a chemiluminescent immunometric assay with intra-assay variation of 4–5%, inter-assay variation of 5% and detection limit of 15 pmol/L. Cortisol was measured with a chemiluminescent immunoassay with an intra-assay variation of 3-6%, inter-assay variation of 5-7% and a detection limit of 50 nmol/L. Glucagon was determined with the Linco 125I RIA (Linco Research, St Charles, MO, USA) with an intra-assay variation of 4-8%, inter-assay variation of 6-11% and detection limit of 15 ng/L.

C-reactive protein (CRP) was determined using ELISA (R&D systems Europe, Ltd. Abingdon, UK), according to the manufacturer’s instructions.

**Intrahepatic triglycerides (1H– Magnetic Resonance Spectroscopy (1H–MRS))**

In the obese subjects, 1H-MRS measurements were performed two weeks before surgery on an open 1.0 Tesla Magnetic Resonance (MR) scanner with a 160 cm-wide patient aperture and a height of 45 cm (Panorama HFO, Philips Healthcare, Best, The Netherlands) using the body coil in supine position (total scan time including localizers 30 minutes). A 20 x 20 x 20 mm voxel was positioned in the right liver lobe and a voxel was positioned left, avoiding overlap with extrahepatic structures and intrahepatic vascular and biliary structures. IHTG was determined from the mean of these two voxels.

Spectra were acquired using first order iterative shimming, a point resolved spectroscopic sequence (PRESS) with TE/TR=35/2000 ms and 64 signal acquisitions. Data was processed using jMRUI software (18). Signal resonances from water and fat located at 4.65 and 1.3 ppm respectively were analyzed. Prior knowledge was used for peak localization by using soft constraints. Signal resonances were fitted using Lorentzian line shapes. Phase variation was allowed (40 degrees) around a manually selected optimum. Relative fat content was expressed as a ratio of the fat peak area over the cumulative water and fat peak areas (1.3 ppm / (1.3 ppm + 4.65 ppm)). No correction for T1 relaxation was performed, as T1-weighting is negligible at 1.0 Tesla using a TR of 2000 ms. Calculated peak areas of water and fat were corrected for T2 relaxation. T2 measurements were performed in all patients by using a
multi echo PRESS (TE= 40, 70, 100, 130 and 160 ms). The T2 measurements were used for calculating the weight percentage IHTG according to Szczepaniak et al (19).

**Body composition**

Body composition was measured using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK).

**Surgical procedure and tissue biopsies.**

The surgical procedures were carried out in three medical centers (Rijnstate Hospital, Arnhem, Slotervaart Hospital, Amsterdam and the Medical Center Alkmaar, Alkmaar) and performed by experienced surgeons. During surgery, liver biopsies were taken at the beginning of the surgical procedure. Hemostasis was checked directly after the biopsies and at the end of the surgical procedure. Samples were taken from similar locations in all patients of segment 3 of the liver, and at the same time point during surgery. Part of the tissue was snapfrozen in liquid nitrogen, and thereafter stored in -80ºC for subsequent analysis. Another part of the tissue was fixed in buffered formalin and subsequently paraffin embedded.

Total RNA was extracted from the frozen biopsies using TRIzol reagent (Invitrogen), followed by further extraction using the NucleoSpin RNA II kit according to the manufacturer’s recommendations (Macherey-Nagel GmbH, Duren, Germany). This protocol included a RNase-free DNase step. RNA concentrations were determined using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was investigated by assessing the RNA integrity number (RIN), using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany); mean RIN numbers were 7.

Equal amounts of RNA were used to synthesize cDNA, using oligo-(dT)$_{12-18}$ and random hexamers as primers, and Superscript II reverse transcriptase, according to the manufacturer’s method (Invitrogen). Gene-specific analysis was performed on an iCycler MyiQ single-color real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad Laboratories). Gene expression levels were normalized to the acidic ribosomal protein 36B4, also referred to as P0. The following primers were used: CD68, CD163, Mannose receptor (MR), CCL2 (the primary ligand of CCR2), Tumor necrosis factor alpha (TNFα), macrophage inflammatory protein (MIP1β), and interleukin 10 (IL10). The primer sequences are shown in a supplemental table. Specificity of the primers was verified by evaluation of the amplifications with the use of gel electrophoresis and melting curve analysis.

**Histopathology liver**

Liver specimens were routinely formalin-fixed and paraffin-embedded. For scoring, a hematoxylin and eosin and a Sirius Red stain were available. Sections were scored by an experienced hepatopathologist, who was blinded to the study results. Sections were scored for macrovesicular steatosis grade, inflammation, fibrosis, and ballooning according to the NASH Clinical Research Network scoring system defined by Kleiner et al. 2005 (20).
Percentage of steatosis was graded as follows: none (0–5%), mild (5–33%), moderate (33–66%), and severe (> 66%). To assess the severity of NAFLD/NASH, the NAFLD activity score (NAS) was calculated [Kleiner et al, 2005]. The NAFLD activity score is a histologic scoring system that represents the sum of scores for steatosis, lobular inflammation, and ballooning and ranges from 0 to 8. NAFLD activity scores of 0–2 are considered not diagnostic of NASH; 3 and 4 borderline for NASH and 5–8 diagnostic of NASH.

**Calculations and statistical analyses**

Data were analysed using parametric and non-parametric tests. For the statistical analyses of mRNA expression, the unpaired Kruskal Wallis was used. Correlations were done using the Spearman’s Rho test. Patient characteristics are presented as mean ± SD. SPSS version 20.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Comparisons were considered statistically significant if the p value was <0.05 and p < 0.1 was considered a trend. Clamp data are presented as median [minimal - maximum]. Endogenous glucose production (EGP) and insulin-mediated peripheral glucose uptake (rate of disappearance [Rd]) were calculated using the modified form of the Steel equation as described previously (21, 22). EGP is expressed as μmol/kg fat-free mass (FFM) min⁻¹ and Rd as μmol/kg*min⁻¹. HOMA-IR was calculated as fasting glucose x fasting insulin divided by 22.5 and quantitative insulin sensitivity check index (QUICKI) as 1/[log(I₀) + log(G₀)].

**Results**

**Study participants**

Twenty morbidly obese women scheduled for RYGB surgery and six healthy lean normal glucose tolerant women, scheduled for elective cholecystectomy for benign gallbladder disease, were included (table 1).
Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>LEAN (N=6)</th>
<th>OBESE (N=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE (YEARS)</td>
<td>36 ± 6.6</td>
<td>41 ± 8.5</td>
<td>0.203</td>
</tr>
<tr>
<td>WEIGHT (KG)</td>
<td>69.7 ± 5.0</td>
<td>127.5 ± 21.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (KG/M²)</td>
<td>22.1 ± 1.4</td>
<td>45.3 ± 6.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.0 ± 0.7</td>
<td>2.8 ± 1.2</td>
<td>0.002</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.6 ± 0.09</td>
<td>0.3 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>INSULIN (PMOL/L)</td>
<td>31.1 ± 19.8</td>
<td>88 ± 32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GLUCOSE (MMOL/L)</td>
<td>5.0 ± 0.4</td>
<td>5.6 ± 1.1</td>
<td>0.169</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>16.5 ± 2.9</td>
<td>22 ± 14.7</td>
<td>0.77</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>22.2 ± 9.1</td>
<td>17.5 ± 25.7</td>
<td>0.91</td>
</tr>
<tr>
<td>Γ-ΓT (U/L)</td>
<td>27.5 ± 14.3</td>
<td>24.5 ± 43.7</td>
<td>0.60</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>67.8 ± 34.2</td>
<td>53 ± 18.9</td>
<td>0.38</td>
</tr>
<tr>
<td>TRIGLYCERIDES (MMOL/L)</td>
<td>1.3 ± 0.7</td>
<td>1.0 ± 0.7</td>
<td>0.77</td>
</tr>
<tr>
<td>HDL-CHOLESTEROL (MMOL/L)</td>
<td>1.5 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-CHOLESTEROL (MMOL/L)</td>
<td>3.6 ± 0.7</td>
<td>2.8 ± 0.6</td>
<td>0.008</td>
</tr>
<tr>
<td>TOTAL CHOLESTEROL (MMOL/L)</td>
<td>5.8 ± 0.9</td>
<td>4.3 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean and SD. BMI=body mass index, AST=aspartate aminotransferase, ALT=alanine aminotransferase, γ-ΓT = γ-glutamyltranspeptidase, ALP=alkaline phosphatase.

Glucose Metabolism

HOMA-IR and QUICKI significantly differed between the lean and obese group (table 1). Basal glucose metabolism was assessed in 19 and insulin sensitivity in 17 obese women due to technical problems related to iv access. EGP was 13.7 [10.3-18.1] µmol/kgFFM*min and Rd 24.6 [11.5 – 42.5] µmol/kg/min. Hepatic insulin sensitivity, expressed as insulin-mediated suppression of EGP, was assessed during the first step of the hyperinsulinemic clamp and was 78 [55-99]% (16).

IHTG

We analysed 18 obese subjects because 1H-MRS measurements were not successful in two patients. One subject was claustrophobic and one scan failed due to a technical error. The interval between the 1H-MRS assessment and surgery was two weeks. Mean IHTG was 7.7±9.0% [range 0-28.8%] (fig. 1). There was no correlation between IHTG and BMI (p = 0.184, r = -0.328) or insulin-mediated EGP suppression (fig. 2) while basal EGP tended to be positively correlated to IHTG (p = 0.073, r = 0.782).
Liver Histopathology

Histopathology assessment of liver specimens (n = 19) showed no hepatitis steatosis in 7 subjects, 9 subjects had mild macrovesicular steatosis, 1 had moderate steatosis, and 2 had severe steatosis. Hepatic steatosis assessed in the liver biopsies correlated significantly with IHTG measured with MRS (p = 0.03, r = 0.52). No lobular inflammation foci were found in 7 subjects, < 2 foci in 11 subjects and one subject showed a few ballooning cells. The NAFLD activity score was <3 in 16 subjects, 3-4 in 2 subjects and 5 in one subject.

Liver pro- and anti-inflammatory markers in lean and obese subjects

In 2 of the obese subjects the obtained frozen material was of poor quality and could not be used for further analyses. Obese subjects (n = 18) demonstrated overall increased gene expression levels of pro- and anti-inflammatory markers. Expression of the pro-inflammatory markers CD68, TNFα, CCL2 and MIP1beta and the anti-inflammatory markers IL10, MR and the scavenger receptor CD163 were significantly higher in the obese compared to the lean controls (fig. 3). In the lean group there was no correlation between QUICKI and expression of any of the inflammatory genes. In the obese subjects, the expression levels of the pro- and anti-inflammatory markers did not correlate with basal EGP or hepatic and peripheral insulin sensitivity.
Inflammatory markers in subjects with and without hepatic steatosis

Hepatic fat content did not correlate with expression of liver inflammatory markers within the obese group. We next analysed the subjects with (n = 5) and without (n = 11) hepatic steatosis (cut off point 5.7% (23) separately. Interestingly, in the subjects with hepatic steatosis, a strong inverse correlation between the expression of IL-10 and IHTG was found (p = 0.004 ; r = 0.953; fig. 4) while MIP1beta showed a trend for an inverse correlation with IHTG (p = 0.059; r = 0.746). The other inflammatory markers did not correlate with IHTG in both groups. There was no significant difference in liver IL-10 expression between the subjects with or without hepatic steatosis.

Inflammatory markers in subjects with and without hepatic insulin resistance

Within the obese group, hepatic insulin sensitivity did not correlate with gene expression levels of any of the inflammatory markers. We next studied whether the inflammatory
markers were differentially expressed when insulin-mediated suppression of EGP was below or above the median for the whole group (>79% EGP suppression). There were no significant correlations between expression of analysed inflammatory markers in liver tissue and subjects with higher or lower hepatic insulin sensitivity.

**C-reactive protein**

Plasma CRP was significantly higher in the obese group (obese 9489 ng/ml ± 6144 (1221-21238) vs lean 1419 ng/ml ± 521 (784-2196); p < 0.001). There was neither a significant correlation between IHTG and circulating CRP (p = 0.50; r = 0.17) nor between CRP and hepatic insulin sensitivity (p = 0.11; r = 0.39) in the obese group. These results did not change when analysing the hepatic insulin resistant or hepatic steatosis groups separately (data not shown).

**Discussion**

We studied gene expression of pro- and anti-inflammatory markers in liver tissue of morbidly obese women and matched lean controls and show that pro-inflammatory as well as anti-inflammatory markers are markedly increased in the obese group. Next we studied the association between liver fat content, hepatic and peripheral insulin sensitivity and expression of the inflammatory markers in the obese women and showed that overall, morbidly obese women are characterized by an inflamed liver independent of liver fat content or insulin sensitivity. Within the obese subgroup with hepatic steatosis, expression of the anti-inflammatory cytokine IL10 was inversely correlated with IHTG.

Obesity and high fat diets are associated with the production of cytokines in liver through an activated NFkB pathway (24). In experimental models, this inflammatory response can be triggered by excessive uptake of fatty acids leading to lipotoxicity, ER stress and reactive oxygen species (ROS) formation (25). Besides, gut-derived endotoxins, dietary derived lipids and fructose might activate Kupffer cells resulting in a pro-inflammatory phenotype. (26, 27, 28, 29)

Although it has been shown that hepatic inflammation can induce hepatic and system insulin resistance in rodents (24), no correlation was present between inflammatory markers and hepatic and peripheral insulin sensitivity in our obese subjects. In theory, this discrepancy can be explained by a species-specific phenomenon, by a difference in the magnitude of inflammatory changes or by the presence of a counterbalanced anti-inflammatory response as observed in our subjects. The origin of the increase in inflammatory proteins cannot be deducted from the current study, but in rodents it has been shown that active recruitment of monocyte-like myeloid cells expressing the CCR2 receptor occurs in response to a high fat diet resulting in obesity (15). Additionally, a high fat choline deficient diet, resulting in hepatic steatosis, increases the number of hepatic natural killer cells and activated T-cells that in turn can activate Kupffer cells and induce a proinflammatory phenotype (30). Further studies in
humans are needed to characterize the immune cells involved in obesity-associated hepatic inflammation. Overall, our subjects did not show a correlation between gene expression of inflammatory proteins in liver and liver triglyceride content, while several earlier studies suggested that lipid accumulation in liver can trigger an inflammatory response and that liver inflammation per se can trigger hepatic steatosis through upregulation of Sterol Regulatory Element-Binding Proteins 1c (SREBP1c) and lipogenic enzymes (11, 31, 32). In line, TNFα-knock out mice on a high fat diet are protected from hepatic steatosis through reduced lipid uptake and triglyceride synthesis (33). The broad range in hepatic fat content (from none to severe steatosis) in our subjects might in part explain the lack of a significant association. One of the included subjects had a NAS score of 5 suggesting the presence of NASH. Interestingly peripheral insulin sensitivity was lowest and basal EGP was highest in this subject. This is in line with an earlier report on the association between NASH and insulin resistance (34). Unfortunately, assessment of IHTG with 1H-MRS failed in this patient due to claustrophobia. Studying the groups with and without hepatic steatosis separately showed a significant inverse correlation between IL-10 and IHTG. Recently it has been shown that IL-10 secreted by M2 polarized Kupffer cells in liver induces apoptosis of pro-inflammatory M1 polarized Kupffer cells (35), indicating that the M2/M1 balance in hepatic steatosis contributes to the overall inflammatory phenotype. This suggests that in patients with hepatic steatosis, IL10 serves to dampen the inflammatory response and might contribute to reducing further accumulation of liver fat since hepatic inflammation has been shown to increase de novo lipogenesis as described above. Finally, no correlation was found between IHTG and systemic and hepatic insulin resistance. This is in line with an earlier study showing that increased hepatic triglycerides per se do not cause insulin resistance (8) and other studies showing that diacylglycerol and activated PKC are more important in the induction of hepatic insulin resistance (9, 10). In conclusion, morbid obesity is associated with hepatic inflammation independent of liver fat content and insulin sensitivity. In hepatic steatosis, IL10 might play a pivotal role in controlling the magnitude of the pro-inflammatory phenotype and further increment of liver fat accumulation. Further studies are needed to characterize the different types of immune cells present in the liver of obese subjects.

References


2. Anty R, Bekri S, Luciani N, et al. The inflammatory C-reactive protein is increased in both liver and adipose tissue in severely obese patients independently from metabolic syndrome, Type 2 diabetes, and NASH. Am J Gastroenterol 2006; 101(8):1824–33.
Chapter 8

MORBID OBESITY IS ASSOCIATED WITH HEPATIC INFLAMMATION INDEPENDENT OF LIVER FAT CONTENT AND INSULIN SENSITIVITY


Supplemental table

Primers used:
CD68 forward primer 5’-GCTGGCTGTGCTTTTCTCG-3’, reverse primer 5’-GTCACCGTGAGGATGGCA-3’ (NM_001251; 197–307); TNFα forward primer 5’-GGCGTGGAGCTGAGGATA-3’, reverse primer 5’-CAGCCTTGGCCCTTGAAGA-3’ (NM_000594; 515–603); IL10 forward primer 5’-TGCTTTCAGCAGAGTGAAGACTT-3’, reverse primer 5’-TCCTTCAGCAAGGACTCCTTTA-3’ (NM_000572; 197–277). MCP-1/CCL2 forward primer 5’-CCTAGCTTTCCCAGACACC-3’, reverse primer 5’-CCCAGGGTGAAGACG-3’; MR forward primer 5’-TGCAGAAGGAACACCCT-3’, reverse primer 5’-CAGGCCTTAAGCCACGAAC-3’; MIP-1β/CCL4 forward primer 5’-GGTGACTGTCCTGCCTCTCC-3’, reverse primer 5’-ACCACAAAGTGGCAGGAAGC-3’; CD163 forward primer 5’-ACATAGCATGATCTGTATTGG-3’, reverse primer 5’-ATTCTCTTGGAATCCTCATTCTCTA-3’
Summary and General Discussion
Summary

This thesis addresses three topics in obesity research: a. alterations in the obese brain and b. insulin resistance and inflammation. The studies described in this thesis are performed in humans with the major aim to translate findings from rodents and explore new pathways that might be targeted for future medical treatment.

PART I Obesity and dopaminergic signaling in the brain

The brain has gained interest in human obesity and metabolism research because of its role as master regulator of energy metabolism. Previous studies have shown lower striatal dopamine receptor availability (D<sub>2/3</sub>R) in obese subjects compared to lean controls. It is unknown whether this is a cause or consequence of obesity. We first reproduced these findings (chapter 2) and next we studied whether striatal dopamine receptor availability (D<sub>2/3</sub>R) changes after bariatric surgery induced weight loss. To this end, we determined striatal D<sub>2/3</sub>R availability in the brain using ([123 I]IBZM SPECT two weeks before and six weeks after Roux-en-Y gastric bypass (RYGB) surgery in morbidly obese women. We found no increase in striatal D<sub>2/3</sub>R availability despite clinically significant weight loss, suggesting that a short-term hypocaloric period, i.e. a negative energy balance per se, does not induce major alterations in striatal dopaminergic neurotransmission. Moreover, striatal D<sub>2/3</sub>R availability did not correlate with measures of insulin sensitivity (chapter 3). To determine the effects of long-term weight loss on striatal D<sub>2/3</sub>R binding we repeated ([123 I]IBZM SPECT to assess D<sub>2/3</sub>R availability at least 2 years after RYGB and the first ([123 I]IBZM SPECT. We also assessed metabolic parameters and eating behavior. We found an increase, but not normalization, in D<sub>2/3</sub>R availability compared with the first assessment, indicating that reduced striatal D<sub>2/3</sub>R availability in obesity is reversible to some extent, suggesting that it is merely a cause of obesity. To our knowledge we are the first to show intra-individual changes in striatal D<sub>2/3</sub>R availability after long-term surgery induced weight loss. Interestingly, the improvements in insulin sensitivity and food craving after RYGB did not correlate with changes in striatal D<sub>2/3</sub>R availability, challenging an important role for dopamine receptor availability (D<sub>2/3</sub>R) in these RYGB induced effects (chapter 4).

PART II Obesity, metabolism and inflammation

To further unravel the association between obesity and glucose metabolism, we studied metabolic parameters in morbidly obese women before and two weeks after bariatric surgery (chapter 5). We assessed insulin sensitivity during a two-step hyperinsulinemic euglycemic clamp with a stable glucose isotope tracer. We found a decrease in endogenous glucose production (EGP), lower plasma insulin and glucose levels and increased free fatty acids (FFA) two weeks after bariatric surgery. There was no effect on peripheral or hepatic insulin sensitivity. Thus, the early beneficial metabolic effects reported in morbidly obese adults after RYGB surgery do not include increased insulin sensitivity. Lower EGP shortly after
RYGB probably is a physiological adaptation to the semi-starvation postoperative period and does not reflect improved insulin action. Most studies use HOMA-IR as a surrogate index of insulin sensitivity and the reduced postoperative insulin and glucose levels inevitably led to a lower HOMA-IR. However, using a hyperinsulinemic euglycemic clamp, which is considered the gold standard for determining insulin sensitivity, we did not detect any improvement in insulin sensitivity, suggesting that HOMA-IR does not reflect true insulin sensitivity in this setting. The lack of effect on insulin sensitivity may be explained by higher plasma levels of FFA, which are known to impair insulin signaling. Since tapering down of insulin treatment shortly after RYGB has been reported repeatedly, other factor besides insulin sensitivity must account for that effect. Gut related factors, like gut peptides and the gut-brain axis are likely candidates. Indeed it has been shown that meal-induced elevations in GLP-1 are increased shortly after RYGB.

Next we focused on the role of inflammation in adipose tissue on insulin action in skeletal muscle and adipose tissue, measured as insulin-mediated glucose uptake and suppression of lipolysis respectively. In chapter 6 we show extensive inflammatory changes in adipose tissue in insulin resistant obese women compared to healthy lean controls. Insulin resistance was confirmed by low peripheral insulin sensitivity ($R_d$), high basal insulin levels, low QUICKI and high HOMA-IR, and additionally by low GLUT 4 mRNA expression in adipose tissue. The latter correlated positively with $R_d$. A state of low grade systemic inflammation was shown by an increase of C-reactive protein (CRP) and adipose tissue was characterized by an increased influx of macrophages and T cells. Subcutaneous adipose tissue (SAT) displayed a predominant pro-inflammatory phenotype, whereas visceral adipose tissue (VAT) showed higher expression levels of anti-inflammatory markers. Finally, we show that the influx of activated T cells was higher in visceral fat, which might account for the higher expression of anti-inflammatory markers since T cells dampen local inflammation. We did not find any correlations between adipose tissue inflammatory markers and insulin sensitivity of adipose tissue or muscle. In conclusion, inflammation in adipose tissue of insulin resistant, morbidly obese women is characterized by increased influx of macrophages and activated T cells, but this is not associated with insulin action. Further long-term follow up studies are needed to elucidate the role of inflammation in insulin resistance in humans.

We then focused on the recently identified adipokine retinol-binding protein 4 (RBP4). RBP4 is a transport protein, mainly synthesized by hepatocytes and adipose tissue, and its main function is delivering retinol to tissues. Recent studies revealed that RBP4 is increased in obesity and that overexpression of RBP4 induces insulin resistance. In chapter 7 we show that in morbidly obese women, besides increased expression of RBP4 in adipose tissue, liver tissue is characterized by increased RBP4. While tissue levels did not correlate with insulin sensitivity, serum RBP4 inversely correlated with hepatic, adipose tissue and skeletal muscle insulin sensitivity. These results suggest that RBP4 acts as a circulating hormone on insulin sensitive metabolic pathways. Lowering serum RBP4 might be an attractive treatment strategy in reducing insulin resistance.
Finally, in chapter 8 we studied whether hepatic inflammation and accumulation of hepatic fat contribute to hepatic insulin resistance in morbidly obese subjects. Intrahepatic triglycerides (IHTG) were assessed using $^1$H-MR Spectroscopy ($^1$H-MRS) and liver biopsies were scored for steatosis and criteria for non-alcoholic fatty liver disease (NAFLD). Gene expression of pro- and anti-inflammatory markers and CD68 (macrophage marker) in liver tissue were markedly increased in obese subjects compared to lean controls. In the obese group with liver steatosis IL10, an anti-inflammatory cytokine, showed an inverse correlation with IHTG. Thus, IL10 may play a role in the interaction between liver steatosis and inflammation. This is in line with studies showing that influx of immune cells in mice on a high fat diet worsen hepatic steatosis. Interestingly, we found no correlation between IHTG, inflammation and hepatic or peripheral insulin resistance. In conclusion, morbid obesity is associated with hepatic inflammation independently of liver fat content and insulin sensitivity. Further studies are needed to determine the pathogenesis of hepatic inflammation in obesity and to explore the role of specific inflammatory pathways in hepatic glucose and lipid metabolism.

General Discussion

The obesity epidemic poses a threat for individuals and society because of its devastating consequences for our health in general. Treatment of obesity is difficult and many diet intervention studies showed regain of the lost weight after longer term follow up. Maintenance of health despite the presence of obesity might be a more realistic goal and therefore it is important to study determinants of metabolic health by dissecting the multiple pathways that are involved in development of the metabolic syndrome. Despite the overall modest effects of hypocaloric diets on long-term weight loss, the ultimate goal in the treatment and prevention of obesity would be to achieve a state of chronically reduced food intake. This could be accomplished in the future by targeting brain areas involved in body weight regulation since accumulating evidence in rodents and humans shows that many brain circuits involved in food intake and energy expenditure are altered in the obese state. Since many studies on causes and consequences of obesity are performed in rodents, we aimed to perform translational studies and focused on central (striatal dopaminergic system) and peripheral (inflammation, insulin sensitivity) changes in the obese state.

PART I Obesity and the brain

Previous studies showed that striatal dopamine receptor 2 and 3 (D$_{2/3}$R) availability is lower in obese humans (1, 2) and although we reproduced these findings (chapter 2) and showed that striatal D$_{2/3}$R availability is lower in obese women compared to lean controls, D$_{2/3}$R availability did not correlate with BMI per se. This means that excessive adipose tissue itself is not directly related to lower striatal D$_{2/3}$R. Other direct factors that might be involved are (excessive use of) nutrients and eating habits besides genetically determined expression or functionality of striatal dopamine receptors. In other words it is currently unknown whether obesity induces lowering of striatal D$_{2/3}$R or whether lower striatal dopamine receptors
SUMMARY AND GENERAL DISCUSSION

predispose to obesity in an obesogenic environment. As discussed in chapter 3, it has been hypothesized that overeating in subjects susceptible to obesity constitutes a compensatory response to make up for decreased dopaminergic signaling in the reward circuitry caused by reduced expression of dopamine receptors due to genetic factors (3, 4). In contrast, downregulation of striatal D2/3 R occurring after the onset of obesity in animal studies suggests changes in the striatal dopaminergic system to be a consequence of a persistent increase in palatable food consumption, positive energy balance and/or fat mass (5, 6). To study whether lower striatal D2/3 R availability in obesity is reversible and thus a consequence of obesity, we studied striatal D2/3 R availability before, 6 weeks and >2 years after surgery-induced weight loss. While D2/3 R availability does not change 6 weeks after RYGB surgery despite clinically significant weight loss (chapter 3), we demonstrated that striatal D2/3 R availability increases >2 years after RYGB surgery (chapter 4). Surprisingly, the change in striatal D2/3 R availability did not correlate with the change in body weight, body fat mass or BMI, again pointing to a body weight independent relation. It remains to be established if peripheral factors (and if so, which ones) modulate striatal D2/3 R availability. Furthermore, striatal D2/3 R availability did not normalize, i.e. increase to the levels of lean controls, which might be explained by the fact, that despite massive weight loss, BMI did not return to normal, i.e. < 25 kg/m2. Also at present it is unknown whether an increase in striatal D2/3 R availability represents an increase in dopamine receptors or a decrease in synaptic striatal dopamine in humans. Studying food cues or amphetamine induced dopamine release before and after weight loss could shed light on these questions. Moreover, it would be informative to repeat the SPECT study once the participants reach a BMI < 25 kg/m2. In addition, it would be of interest to study whether diet-induced weight loss or different types of diets as well as changes in eating patterns would differentially affect striatal D2/3 R availability. Finally, studying the changes in striatal D2/3 R availability upon nutrient excess and weight gain would clarify the change over time and its relation to either specific nutrients or a critical increase in body weight. Further studies in rodents and humans are needed to elucidate whether and how striatal dopamine and striatal D2/3 R receptors contribute to the obese state.

Body weight is determined by energy intake and energy expenditure. Energy, or food, intake is a result of a complex interplay between hedonic and homeostatic brain circuits. When obese subjects consume food or imagine consuming food, they show less striatal activation compared to lean individuals (4, 7). This might be explained by hypo-responsiveness of the reward (hedonic) circuitry. This is in line with the lower striatal D2/3 R availability observed in obese versus lean individuals and rats (chapter 2, 1, 6, 8) and lower basal dopamine levels in obesity-prone rats (9). Upon surgery-induced weight loss, our study participants showed less craving for food and disinhibition but these changes were not related to changes in D2/3 R availability, nor to the amount of body weight loss (chapter 4). Because we did not study food cue induced striatal dopamine release, we are unable to address the question whether reduced craving and disinhibition after weight loss are related to dynamic changes in striatal dopamine release. Correlating specific aspects of eating behavior, eating patterns
and food preferences in relation to the striatal dopaminergic system in addition to studying the changes occurring during weight gain and weight loss might unravel the role of striatal dopamine/receptors in food intake and thus body weight regulation. Although lower striatal D$_{2/3}$R availability is a consistent finding in obesity, it is still unknown whether this represents a hypodopaminergic or hyperdopaminergic state. While children at high risk for obesity show increased responsiveness to consumption of palatable food (10) obese (adult) individuals show low responsivity (4). The propagated reward deficiency might therefore be a consequence of overconsumption/obesity itself rather than a cause of obesity. This is in line with the contradictory results on the relation between a dopamine receptor polymorphism leading to lower receptor expression and BMI (11) and with rodent studies showing that eating high fat diets or diets with an increased fat/carbohydrate ratio decreases striatal D$_{2/3}$R availability not explained by obesity (6, 12, 13). However it was recently shown that lentivirus-mediated knockdown of striatal D$_{2/3}$R rapidly accelerated the development of addiction-like reward deficits and the onset of compulsive-like food seeking in rats (14). Therefore more studies in rodents and humans showing that striatal dopaminergic signaling or receptors contribute significantly to body weight regulation are needed, before designing novel drugs, or other therapies, targeting the striatal dopaminergic system.

In conclusion, the striatal dopaminergic system is altered in obese rodents and humans with lower receptor availability and less striatal activity. Although these changes are partially reversible after weight loss, it remains to be determined whether these alterations are a cause or a consequence of obesity and whether they represent a hyper- or hypodopaminergic state. Further insight is needed before development of treatments targeted at striatal dopaminergic signaling in obese humans.

**PART II Obesity, metabolism and inflammation**

**a. Bariatric surgery and insulin resistance**

A better understanding of the pathogenesis of obesity-induced insulin resistance is a vital step in developing new paradigms for a) maintenance of metabolic health, specifically insulin sensitivity and b) treatment of insulin resistance and diabetes mellitus type 2 (T2DM). Bariatric surgery, in particular Roux-en-Y gastric bypass (RYGB) surgery has proven to be the most effective treatment for obesity, leading to sustained weight loss and improved glucose metabolism. RYGB has rapid clinical effects but whether these are related to the bariatric intervention itself, or to the semi-starvation period after surgery remains matter of debate. An improvement in insulin sensitivity would suggest that creating a small gastric pouch, bypassing the first part of the proximal gut has body weight loss-independent effects on glucose metabolism while a reduction in basal glucose metabolism only would imply an effect of food restriction because it has been shown that starvation reduces glucose output (15). Dissecting these effects is of importance because if the former is true, more research should be directed towards gut factors or the gut-brain axis in modulation of glucose metabolism. This could lead to new treatments in the battle against obesity-induced insulin
resistance and T2DM. We found that in the first two weeks following RYGB, basal endogenous glucose production, as well as fasting insulin and glucose levels decrease, without a significant effect on peripheral or hepatic insulin sensitivity (Chapter 5). This suggests that the beneficial rapid metabolic effect of RYGB is merely an adaptation to fasting. The observed higher FFA levels in our subjects support this since fasting strongly induces lipolysis and our results are in line with an earlier report (16). Increased FFA might explain the lack of improvement in insulin sensitivity in our study since FFA are known to impair insulin signaling (17). Although we show, using golden standard techniques, that there is no improvement in insulin sensitivity, clinical studies reported on rapid tapering down of insulin requirements and lower postprandial blood glucose. Therefore other mechanisms, besides pure effects on insulin sensitivity, must play a role. RYGB comprises both a restriction and a malabsorption component. The restrictive component follows from the creation of a small gastric pouch, resulting in reduction of caloric intake while malabsorption is caused by bypassing the duodenum and the proximal jejunum. Although this has been the classical view for years, it has been shown recently that malabsorption could not account for the effects on weight loss and other factors should be considered to be responsible for the decline in body weight (for review 18). Therefore the question arises whether restriction, i.e. reduced food intake, alone could explain the major beneficial effects of RYGB. Several studies in patients with T2DM and/or obesity reported a rapid decrease in insulin and glucose levels after a calorie restricted diet (19, 20). However, Foo et al showed that with comparable weight loss RYGB has a more pronounced effect on glucose metabolism than a very low calorie diet alone (21). Alternatively, reduced postoperative levels of the stomach derived hunger hormone ghrelin might contribute to the reduction in food intake and therefore account for the effects on glucose metabolism as well, but ghrelin levels in the longer term tend to increase despite sustained weight loss (22). This indicates that the additional effect of RYGB surgery is related to the second aspect of the procedure, which is rerouting of food directly from the small gastric pouch into the jejunum bypassing the duodenum and the first part of the jejunum (150 cm). Several mechanisms have been proposed to contribute independently to the metabolic improvements after bariatric surgery, including a) an increase in gastrointestinal hormones like GLP-1 leading to an enhanced β-cell responsivity and insulin release, b) remodeling of the cell structure of the intestine, c) changes in bile acid metabolism accompanied by a change in the gut microbiome, d) altered communication between the gut and the brain and finally in the longer term e) the decrease in fat mass leading to a reduction in low grade inflammation. The latter requires clinically significant weight loss and is unlikely to be a specific effect of the bariatric intervention itself as it can also be achieved by low calorie diets. Our data as well as reports in the literature indicate that the relation between improved insulin sensitivity and RYGB is less straightforward than previously suggested.

In conclusion, we primarily aimed to explore whether the reported early improvements in glucose metabolism are explained by an increase in insulin action and conclude that in the short term, i.e. 2 weeks after RYGB, insulin-mediated suppression of endogenous glucose
production, representing hepatic insulin sensitivity and insulin-mediated glucose uptake, indicative of skeletal muscle insulin sensitivity, are not increased while basal endogenous glucose production, glucose and insulin are reduced. The latter probably represents the metabolic adaptation to the semi-starvation postoperative period. Lower postprandial glucose and insulin requirements in T2D patients in the early postoperative phase can be explained by higher insulin secretion either induced by higher nutrient-induced GLP-1 secretion or other gut or gut-brain axis related factors. This requires further studies in humans using study designs including a very low calorie diet control group.

b. Obesity and inflammation

Obesity is associated with systemic low grade inflammation and invasion of various immune cells into adipose tissue has been described (23, 24, 25). Many studies in rodents show that inflammation in adipose tissue affects adipose function and insulin sensitivity resulting in insulin resistance/glucose intolerance. During the development of obesity the number of macrophages in adipose tissue can increase up to four fold (26, 27). Chemotaxis induced by MCP-1 secreted from enlarged adipocytes attracts circulating monocytes that differentiate into macrophages within adipose tissue (28, 29). Depending on the local environment and only partly understood, adipose tissue macrophages (ATM) can be classically (M1) or alternatively (M2) activated and produce either predominantly pro- or anti-inflammatory cytokines, respectively. Rodent and human studies found that in the lean state, most macrophages show a M2 phenotype but when adipose tissue expands they switch towards a pro-inflammatory profile (30, 31, 32). Rodent studies show that increased lipolysis induced by the inflammatory state as well as release of cytokines into the systemic circulation both can hamper insulin action resulting in insulin resistance or T2DM. Conversely, reducing or preventing inflammation restores insulin sensitivity in different rodent models (33, 34). Although human studies confirm the presence of inflammation in adipose tissue in obesity, less is known on the effects of these inflammatory changes on insulin sensitivity in humans. We show extensive pro- and anti-inflammatory changes in adipose tissue of morbidly obese subjects compared to lean controls (chapter 6) but surprisingly no direct correlation was demonstrated between any of these markers and insulin action in adipose tissue, liver and skeletal muscle. Notably, different inflammatory profiles were present between subcutaneous (SAT) and visceral adipose tissue (VAT) compartments with subcutaneous adipose tissue showing a more pro-inflammatory state. This is in line with an earlier published paper (35). Since VAT is associated with insulin resistance (for review 36) we hypothesized that pro-inflammatory changes in that compartment might contribute to insulin resistance independently of the inflammatory state in SAT and as long as the overall VAT phenotype is shifted towards a more anti-inflammatory pattern, no clinically significant effect on insulin sensitivity might be expected. Our study showed no correlation between inflammation in either SAT and VAT and insulin action in adipose tissue but since this was measured on the whole body level we cannot exclude that inflammation in VAT affected local
lipolysis specifically in that compartment. Study designs including the use of micro dialysis, metabolic tracers, AV differences and portal blood analyses could shed more light on these questions.

Finally, activation of macrophages in adipose tissue attracts circulating T cells to dampen the inflammatory response (37). Regulatory T cells (Tregs), which are of an immunosuppressive nature are reduced in adiposity (37, 38). Tregs are CD4+ cells that express CD25+ and FOXp3, a forkhead transcription factor required for their specific development and function (CD4+CD25+FOXp3 regulatory T cells) (39). Tregs secrete the anti-inflammatory cytokine IL10, which inhibits TNF-α production by macrophages, preventing local tissue damage and counteracting inflammation. We show that T-cell markers are increased in both adipose tissue compartments in the obese subjects compared to the lean controls. Higher expression of CD4 suggests increased influx of T cells in general and higher expression of CD25 shows increased levels of activated T-cells. FOXP3, a marker of regulatory T cells, was not significantly different between lean and obese or within compartments but it has been shown in rodents and humans that they play an important role in counteracting the pro-inflammatory macrophages (40). Furthermore, the higher expression levels of activated T cells in VAT might be an explanation for the predominantly anti-inflammatory environment in that compartment. Studies using FACS analysis are needed to further characterize the subpopulations of T-cells and future rodent studies should be aimed at studying their role in obesity-induced inflammation and insulin sensitivity by modulating expression of different T-cell populations. In humans, additional longer term follow up studies are needed to get insight in T-cell dynamics during the development of obesity and insulin resistance. Also, it remains to be studied whether weight loss reduces T-cell influx and activation. If regulatory T cells play an important role in maintaining adipose tissue homeostasis by counterbalancing the pro-inflammatory response induced by obesity, treatment strategies aimed at increasing Tregs might be a promising option in the treatment of obesity-associated metabolic disturbances.

Besides low grade inflammation and reduced insulin action in adipose tissue and skeletal muscle, obesity is also associated with non-alcoholic fatty liver disease (NAFLD) and hepatic insulin resistance. Hepatic steatosis occurs in states of increased de novo lipogenesis, reduced fatty acid oxidation or a combination of both, but its contribution to obesity-associated hepatic insulin resistance is uncertain. In our cohort, no correlation between IHTG and hepatic and peripheral insulin sensitivity was present (chapter 8) and we recently confirmed this lack of a correlation between IHTG and insulin resistance in a large obese cohort (Ter Horst et al, unpublished data) which is in line with others (41). In addition, we showed earlier that severe hepatic steatosis in subjects with a genetic lipid disorder was not associated with peripheral or hepatic insulin resistance (42). In contrast, a number of other studies in humans and rodents have reported that increased IHTG is associated with hepatic and adipose tissue insulin resistance (43, 44, 45) but whether these subjects had uncomplicated NAFLD
or progressed to non-alcoholic steatohepatitis is unknown. In addition, several studies in humans and animal models of NAFLD have revealed that increases in hepatic diacylglycerol (DAG) leads to activation of protein kinase C (PKC), which in turn attenuates insulin signalling (46), and this mechanism is thought to be a major determinant of hepatic insulin resistance. We did not measure PKC and DAG in our tissue samples and therefore we cannot rule out an effect of steatosis on these parameters but since the obese subjects did not show a correlation between IHTG and insulin resistance, this is less likely. Conflicting results on correlations between IHTG and insulin sensitivity may further be due to the various methods used to measure insulin sensitivity. We used the two-step euglycemic hyperinsulinemic clamp and a stable glucose isotope tracer, which is the gold standard for measuring insulin sensitivity, but not all investigators use this method.

The role of hepatic inflammation in hepatic insulin sensitivity in humans remains unclear. Inflammatory changes associated with hepatic steatosis have been described (47) but it is unclear what the mutual underlying mechanism is. Human data are limited and we here show that gene expression of pro- and anti-inflammatory markers in liver tissue of morbidly obese women are markedly increased compared to matched lean controls. However, inflammation of liver tissue did not correlate with intrahepatic triglyceride (IHTG) accumulation or insulin sensitivity (chapter 8). Only one person in our cohort showed signs of NASH, which might have affected our results since it has been shown that steatosis and inflammation are related in the presence of NASH (48). However only a smaller percentage of subjects with NAFLD progress to NASH meaning that other mechanisms besides liver fat must contribute to hepatic inflammation in the setting of obesity. Excessive (dietary) fatty acids trigger an inflammatory response leading to lipotoxicity, ER stress and reactive oxygen species (ROS) formation (49), and fructose and dietary derived lipids might provoke a hepatic stress response by activating the NFkB pathway (50), in turn leading to the production of pro-inflammatory cytokines by activated Kupffer cells (51). Obstfeld et al (47) showed that systemic immune cells invade the liver in mice on a high fat diet and that they promote hepatic steatosis in a chemokine receptor 2 dependent manner. Finally, we did find that hepatic expression of IL-10, an anti-inflammatory cytokine, inversely correlates with hepatic steatosis and recently it has been shown that IL10 secreted by M2 activated Kupffer cells selectively promotes apoptosis of pro-inflammatory M1 polarized Kupffer cells (52). This shows a complex interplay between diet, inflammation and liver fat. More detailed studies on the role of specific cytokines and immune cells involved in hepatic steatosis (or vice versa) and hepatic insulin resistance in rodents and humans are needed to develop treatment strategies aimed at reducing steatosis, inflammation or both.

Furthermore, studies in rodents consistently show that hepatic steatosis and hepatic insulin resistance can be induced before the onset of obesity in response to a high fat diet (HFD) (53). We did not have information on the dietary fat or carbohydrate/fructose content in our subjects neither on meal frequency, which are known contributors to hepatic steatosis in a hypercaloric setting. We recently showed that hypercaloric diets (high-fat-high-sugar; HFHS)
with increased meal frequency increases both IHTG and abdominal fat in lean men, whereas similar diets with increased meal size do not (54), suggesting that snacking independently contributes to IHTG accumulation. In addition fructose intake in excess of caloric need has been shown to trigger hepatic steatosis through an increase in de novo lipogenesis (for review 55). Taken together, both meal composition and meal frequency can contribute independently to the development of hepatic steatosis and inflammation. The range in IHTG was broad and did not correlate with BMI in the obese subjects. This might be related to genetic factors, as several genes have been described in rodent models and in humans that predispose for the development of NAFLD (56, 57, 58, 59).

In conclusion, our results show that morbidly obese women are characterized by an inflamed liver independent of liver fat and hepatic insulin resistance. This suggests that hepatic inflammation per se does not trigger hepatic insulin resistance in obesity. Studies on changes in inflammatory phenotype during the course of obesity with emphasis on different immune cells are needed to explore whether, how and when inflammation affects hepatic glucose metabolism. In addition, no correlation between liver fat and either inflammatory markers or hepatic insulin sensitivity was found. More detailed studies in rodents and humans are needed to clarify the interaction between disturbed hepatic lipid and glucose metabolism in obesity and inflammation.

Clinical implications and further studies

Current obesity management strategies include lifestyle changes to induce weight loss. Unfortunately this strategy shows a high failure rate with weight regain in the long term. Bariatric surgery is associated with significant long-term weight loss and improved metabolic health, but this treatment is not available for all obese subjects. A third option is medical pharmacotherapy in addition to increased physical activity and dietary changes. Lower striatal D2/3 R availability in obesity (chapter 2) and the observed increase >2 years after RYGB surgery (chapter 4) suggests that the dopaminergic system might be a potential target in the treatment of obesity with the aim to increase dopamine release, receptor expression and/or signaling. Several anti-obesity drugs that act directly in the central nervous system are already available. Drugs like lorcaserin, a serotonin 2C receptor agonist, and phentermine/topiramate, a sympathomimetic amine with anorectic effect, affect body weight by reducing appetite (60). However, these drugs are only approved for short term use and as an adjunct to a reduced-calorie diet and increased physical activity. A number of other anti-obesity drugs have been removed from the market due to serious side effects. Therefore pharmacological targeting of the dopaminergic system may be a promising alternative strategy. Alternatively, the dopaminergic system can be influenced by deep brain stimulation (DBS). This method has already been successfully used to treat severe resistant depression, obsessive compulsive disorder (OCD) and addiction (61, 62, 63). In rats it has been shown that DBS of the nucleus accumbens (Nac) modulates feeding behavior (64, 65) as well as glucose metabolism in an intensity dependent matter (66). The nucleus accumbens (NAc) is involved in food related
reward (67). Thus, DBS of the NAc could be a potential target for the treatment of refractory morbid obesity (68).

Obesity is characterized by low grade systemic and tissue inflammation. In our studies this did not correlate with insulin sensitivity but since these studies were cross-sectional we cannot rule out that modulation of inflammation will affect insulin sensitivity. In chapters 7 and 8 we show that morbid obesity is associated with invasion of macrophages and T cells in adipose tissue leading to a mixed inflammatory phenotype in both adipose tissue compartments. Moreover we show an inflamed liver in these obese women. Since rodent studies show protective effects of reducing inflammation on the development of insulin resistance and diabetes, it might be of interest to develop compounds that interact with local immunity to prevent metabolic derangements in obesity. Also, the potential protective role of T cells in adipose tissue needs to be further elucidated. Of interest, therapies are now being developed to specifically target M1-activated macrophages using hybrid nanoparticles (69), without affecting anti-inflammatory macrophages (M2). These techniques are not yet applicable in the clinical setting and more research is needed to further develop this approach.

References


55. Samuel VT. Fructose induced lipogenesis: from sugar to fat to insulin resistance. Trends Endocrinol Metab. 2011; 22(2):60-5


Appendix

Nederlandse samenvatting

Author Affiliations

Dankwoord

PhD portfolio

About the author
Nederlandse samenvatting

De studies in dit proefschrift beschrijven drie onderwerpen van onderzoek naar obesitas: a) veranderingen in de hersenen, b) insulineresistentie en c) inflammatie. De studies die worden beschreven in dit proefschrift zijn uitgevoerd bij mensen als belangrijkste doelen om te kijken of bevindingen die zijn gedaan in studies met knaagdieren vertaald kunnen worden naar de mens en om nieuwe paden te onderzoeken die eventueel onderdeel zouden kunnen worden van een toekomstige medische behandeling voor obesitas.

DEEL I Obesitas en de hersenen

De hersenen zijn een belangrijke onderdeel geworden in de studies naar humane obesitas en onderzoek naar metabolisme omdat ze een belangrijk rol spelen in de regulatie van energie metabolisme. Eerdere studies hebben aangetoond dat de beschikbaarheid van de dopamine receptor (D2/3R) in het dorsale striatum van obese vrouwen lager is vergeleken met slanke personen. Het is niet bekend of dit een oorzaak of een gevolg is van obesitas. Als eerste hebben we deze bevindingen gereproduceerd (hoofdstuk 2), en vervolgens hebben we bestudeerd of de beschikbaarheid van de D2/3R toeneemt als mensen gewicht verliezen ten gevolge van bariatrische chirurgie. Om dit te onderzoeken hebben we de beschikbaarheid van de D2/3R bepaald in de hersenen met behulp van een ([123I]IBZM SPECT scan twee weken voor en zes weken na een maag verkleiningsoperatie van het type Roux-en-Y gastric bypass (RYGB), in morbide obese vrouwen. We zagen geen toename van de beschikbaarheid van de D2/3R ondanks het feit dat deze mensen significant veel waren afgevallen. Dit suggereert dat er na een korte periode van een negatieve energie balans geen veranderingen optreden in striatale dopaminerge neurotransmissie. Ook zagen we geen correlatie tussen D2/3R en de mate van insuline gevoeligheid (hoofdstuk 3). Om te bepalen of gewichtsverlies op de lange termijn wel leidt tot normalisatie of toename van D2/3R in het dorsale striatum hebben we de ([123I] IBZM SPECT scan op zijn minst twee jaar na de RYGB operatie herhaald, en daarnaast hebben we gekeken naar metabole parameters en eetgedrag. Voor zo ver we weten zijn we de eersten die laten zien dat er dan een verhoging optreedt in de beschikbaarheid van de dopamine receptor (D2/3R) wat suggereert dat de verminderde beschikbaarheid van de dopamine receptor (D2/3R) reversibel is. We zagen echter geen relatie tussen de toename van deze receptoren enerzijds en de verbetering van de insuline gevoeligheid of zin in eten anderzijds. Deze effecten dus lijken direct het gevolg te zijn van de RYGB operatie en niet zozeer van de toename van de D2/3R (hoofdstuk 4).

DEEL II Obesitas, inflammatie en insuline resistentie

Om de link tussen obesitas en glucose metabolisme te onderzoeken hebben we de snelle effecten van RYGB chirurgie bestudeerd in morbide obese vrouwen, twee weken voor en twee weken nadat ze geopereerd werden (hoofdstuk 5). We hebben de insuline gevoeligheid bestudeerd met behulp van een twee-staps hyperinsulinemische euglycemische clamp
met stabiele glucose istopen. We zagen dat endogene glucose productie van de lever verminderde, dat de insuline- en glucosespiegels lager waren en dat vrije vetzuren toenamen zonder dat de insuline gevoeligheid van de perifere weefsels en de lever veranderde. We concludeerden hieruit dat de vroege metabole effecten die worden beschreven in morbide obese volwassenen na RYGB chirurgie niet het gevolg zijn van toegenomen insuline gevoeligheid. Door het vellen na de operatie gaat de productie van insuline door de lever omlaag wat tot gevolg heeft dat de glucose levels in het bloed lager zijn. De lagere insuline spiegels lijken het gevolg te zijn van betere insuline klaring door de lever. Deze lagere insuline en glucose waarden leiden vervolgens tot een lagere HOMA-IR, ook al hebben we geen verbetering in insuline gevoeligheid waargenomen met behulp van de gouden standaard meting voor insuline gevoeligheid, de twee-staps hyperinsuline euglycemische clamp. Dit suggereert dat de HOMA-IR geen juiste methode is om de insuline gevoeligheid aan te geven in deze situatie. Het gegeven dat we geen effect zien op de insuline gevoeligheid kan mogelijk worden verklaard door de hogere spiegels van vrije vetzuren, want hiervan weten we dat ze de insuline signalering beïnvloeden. Er zijn veel onderzoeken beschikbaar, die laten zien dat kort na RYGB de behandeling met insuline kan worden afgebouwd. Aangezien we geen verbetering zagen van de insuline gevoeligheid moeten er andere factoren zijn die bijdrage aan dit effect. Mogelijke kandidaten zijn eiwitten die worden gemaakt door de darmen en de neurale connectie tussen de darmen en de hersenen. Zo is al aangetoond dat GLP-1 spiegels verhoogd zijn na het nuttigen van een maaltijd kort na een RYGB.

Vervolgens hebben we ons gericht op de rol die ontsteking in vetweefsel speelt met betrekking tot de werking van insuline in skeletspieren en vetweefsel. We hebben dit gemeten aan de hand van insuline gemedieerde opname van glucose en onderdrukking van lipolyse. In hoofdstuk 6 laten we zien dat er meer ontsteking is in vetweefsel van insulin resistente obese vrouwen vergeleken met slanke controle personen. Insuline resistentie in de obese groep werd bevestigd door een lagere perifere insuline gevoeligheid (Rd), hoge basale insuline levels, een lage QUICKC en een hoge HOMA-IR, en tenslotte door een lage expressie van mRNA van GLUT4 in vetweefsel. GLUT4 liet tevens een positieve correlatie zien met Rd. Dat er sprake was van chronische ontsteking konden we bevestigen met verhoogde waarden van het acutr fase eiwit CRP in het bloed van de obese vrouwen. Daarnaast zagen we in het vetweefsel van de obese vrouwen een verhoogde influx van macrofagen en T cellen. Het subcutane vetweefsel liet met name een pro-inflammatoire fenotype zien, in tegenstelling tot het viscerale vet weefsel wat juist een toename liet zien van anti-inflammatoire markers. Tenslotte zagen we dat geactiveerde T cellen met name aanwezig zijn in het viscerale vetweefsel van de obese vrouwen, en dit kan een verklaring zijn waarom we hier hogere expressie zien van anti-inflammatoire markers. Het is namelijk bekend dat T cellen de ontstekingsreactie onderdrukken. We vonden geen correlatie tussen de inflammatoire markers in het vetweefsel enerzijds en insuline gevoeligheid van het vetweefsel of in de skeletspieren anderzijds. Hieruit concludeerden we dat er bij obese insuline resistente vrouwen sprake is van laaggradige ontsteking en dat deze geconstateerd wordt door
een verhoogde influx van macrofagen en geactiveerde T cellen, maar dit fenomeen niet is geassocieerd met de werking van insuline. Er zijn meer lange termijn studies nodig om de rol van ontsteking met betrekking tot insuline resistentie in mensen te ontdekken. Hierna hebben we ons gericht op het recent geïdentificeerde retinol bindende eiwit 4 (RBP4). RBP4 is een transport eiwit dat met name geproduceerd wordt door lever en vetcellen. De belangrijkste functie is het afleveren van retinol aan de weefsels. Recente studies hebben laten zien dat RBP4 levels verhoogd zijn bij mensen met obesitas en dat overexpressie van RBP4 leidt tot insuline resistentie. In hoofdstuk 7 laten we zien dat, naast de verhoogde expressie van RBP4 in vetweefsel, de genexpressie van RBP4 ook verhoogd is in leverweefsel. De RBP4 concentraties in het weefsel correleerden niet met insuline gevoeligheid, dit in tegenstelling tot RBP4 in het bloed, wat een omgekeerde correlatie liet zien met insuline gevoeligheid van de lever, vetweefsel en skelet spieren. Deze resultaten suggereren dat RBP4, werkt als een circulerend hormoon dat insuline gevoelige paden beïnvloedt. Het verlagen van serum waarden van RBP4 kan derhalve een aantrekkelijke strategie zijn in de behandeling van insuline resistentie.

Tenslotte hebben we in hoofdstuk 8 bekeken of ontsteking en vetophoping in de lever bijdragen aan insuline resistentie van de lever in morbide obese vrouwen. De mate van triglyceriden in de lever werd vastgesteld met behulp van een MRI van de lever (1H-MRS) en leverbiopsieën die werden gescoord op de mate van steatose en non-alcoholische lever ziekte activiteit (NAFLD). Genexpressie van pro- en anti-inflammatoire markers en CD68 (een macrofaag marker) waren verhoogd in leverweefsel van de obese personen vergeleken met de slanke controles. Binnen de groep obesen met leversteatose zagen we dat IL10, een anti-inflammatoire cytokine, negatief correleerde met de hoeveelheid triglyceriden in de lever. Dit geeft aan dat IL10 de ontstekingsreactie mogelijk probeert af te remmen om op die manier verdere vetopslag in de lever te voorkomen. Dit sluit aan bij bevindingen die zijn gedaan door anderen, er zijn studies die laten zien dat influx van immuun cellen in muizen die een hoog vet dieet krijgen, de mate van hepatieke steatose vererger. Opmerkelijk genoeg vonden we geen correlatie tussen ophoping van triglyceriden en ontstekingsparameters in de lever en hepatieke of perifere insuline resistentie. In het kort kunnen we concluderen dat morbide obesitas kan worden geassocieerd met ontsteking van de lever onafhankelijk van de mate van vetopslag en insuline gevoeligheid. In de toekomst zijn er meer studies nodig om te bepalen wat de pathogenese is van inflammatie van de lever bij obesitas en welke rol specifieke inflammatoire paden, met betrekking tot glucose en vet metabolisme van de lever, hierbij spelen.
AUTHOR AFFILIATIONS

Dr. Edo Aarts
Department of Surgery, Rijnstate Hospital, Arnhem, the Netherlands.

Dr. IR. Mariëtte Ackermans
Department of Clinical Chemistry, laboratory of Endocrinology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Thérèse A. van Amelsvoort
department of Psychiatry, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands.

Dr. Frits J. Berends
Department of Surgery, Rijnstate Hospital, Arnhem, the Netherlands.

Prof. Dr. Jan Booij
Department of Nuclear Medicine, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Erik Boot
Ipse de Bruggen, Centre for People with Intellectual Disability, Zwammerdam, the Netherlands. department of Psychiatry, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands.

Dr. Breg Braak
department of Gastroenterology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands

Drs. Hamit Cakir
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Marco C. van Eijk
Department of Biochemistry, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Prof. Dr. Eric Fliers
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.
Dr. Susanne E. la Fleur  
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Theo B. Geijtenbeek  
Department of Experimental Immunology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Elsmarieke van de Giessen  
Department of Nuclear Medicine, Academic Medical Center, Amsterdam, The Netherlands.

Drs. Kasper W. ter Horst  
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Lex P. Houdijk  
Department of Surgery, Alkmaar Medical Center, Alkmaar, the Netherlands.

Drs. Ignace Janssen  
Department of Surgery, Rijnstate Hospital, Arnhem, the Netherlands.

Prof. Dr. Karin Kaasjager  
Department of Surgery and Department of Internal Medicine, Rijnstate Hospital, Arnhem, the Netherlands.

Dr. Barbara B. Kahn  
Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School.

Drs. Murat Klicarslan  
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Drs. Arnold van de Laar  
Department of Surgery, Slotervaart Hospital, Amsterdam, the Netherlands.

Dr. Ir. Aart J. Nederveen  
Department of Radiology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.
Prof. Dr. Hans A. Romijn
Department of Internal Medicine, University of Amsterdam, The Netherlands.

Dr. Mireille J. Serlie
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Joanna Verheij
Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

Dr. Toni Vidal-Puig
Department of Clinical Biochemistry, Institute of Metabolic Science, Metabolic Research Laboratories, Addenbrooke’s Hospital, University of Cambridge, Cambridge, UK.

Dr. Sam Virtue
Department of Clinical Biochemistry, Institute of Metabolic Science, Metabolic Research Laboratories, Addenbrooke’s Hospital, University of Cambridge, Cambridge, UK.

Drs. Barbara A. de Weijer
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Esther M. van der Zwaal
Department of Nuclear Medicine, Academic Medical Center, Amsterdam, The Netherlands.
Dankwoord

Mijn promotie was een lang en intensief traject, gekenmerkt door ups en downs, maar bovenal was het een unieke ervaring. Dit traject heb ik niet alleen doorlopen, daarom wil ik een aantal mensen in het bijzonder bedanken.

Allereerst wil ik de proefpersonen die hebben deelgenomen aan de studies ontzettend bedanken voor hun deelname, zonder hen was dit boekje immers niet tot stand gekomen. Jullie waren heel geïnteresseerd, enthousiast en betrokken, ondanks de pittige proeven bleven jullie opgewekt en vriendelijk, daarom kijk ik met veel plezier terug op de dagen dat jullie er waren.

Mijn dank gaat ook uit naar mijn promotor, prof. Dr. E. Fliers, beste Eric, je bent heel toegankelijk en altijd beschikbaar voor een luisterend oor en het geven van advies. Het is bewonderingswaardig hoe snel en compleet je promovendi verder kunt helpen bij het schrijven van protocollen, manuscripten en presentaties. Je wist me altijd weer gerust te stellen als ik het even niet meer zag zitten, mede daardoor heb ik dit proefschrift tot een goed einde kunnen brengen.

Mijn co-promotoren, Dr. M. J. Serlie en Dr. M.C. van Eijk, wil ik bedanken voor jullie begeleiding bij verschillende onderdelen van mijn promotie. Beste Mireille, na mijn eerste gesprek bij jou kon ik vrijwel meteen beginnen, voor mijn onderzoek moesten nog samenwerkingsprojecten met andere ziekenhuizen worden opgezet, nieuwe technieken worden geleerd, zoals het maken van MRI's, SPECT scans, clampen en het isoleren van macrofagen voor dit laatste werd zelfs samenwerking met Cambridge aangegaan, dit bood meteen voldoende uitdaging. Naast het doen van de intensieve experimenten, waren er onder andere ook de leuke en leerzame congressen met de traditionele etentjes, achtbaanritjes in Orlando, en de bezoeken aan Cambridge, die voor afwisseling zorgden. Ik heb bewondering voor de manier waarop jij de afgelopen jaren een mooie onderzoeksgroep hebt opgebouwd. Jij laat promovendi los zodat ze leren om zichzelf te ontwikkelen als een zelfstandig onderzoeker, maar je bewaakt de kwaliteit van ons werk door onze manuscripten zeer nauwkeurig en kritisch te redigeren om ze op die manier naar een hoger niveau te tillen. En je houdt je promovendi scherp door te stimuleren om ons eigen werk overal ter wereld te presenteren en zo het onderste uit de kan te halen. Ik heb veel van je geleerd, niet alleen over wetenschap, maar ook op veel andere vlakken waar ik de rest van mijn loopbaan nog profijt van zal hebben.

Beste Marco, mijn terugkeer op het laboratorium was even wennen, maar omdat jij altijd beschikbaar was voor begeleiding en overleg, verliep dit toch soepel en het werd een plezier om weer terug te keren naar de wereld van qPCR’s en pipetten. We hebben gemerkt dat humaan vet niet gemakkelijk is om mee te werken, maar ik denk dat we het maximale uit de samples hebben gehaald, bedankt voor je begeleiding.
De leden van mijn promotie commissie Prof. dr. E. Lutgens, Prof. dr. T. van der Poll, Prof. dr. H. Pijl, Dr. L.M. de Brauw en Prof. Dr. C.J.J. Tack en Prof. Dr. U.H.W. Beuers wil ik bedanken voor uw bereidheid om zitting te nemen in mijn promotiecommissie het kritisch beoordelen van mijn proefschrift.

Omdat er nog veel nieuwe samenwerkingsverbanden moesten worden gesmeed tussen de onderzoeksgroep en andere ziekenhuizen heb ik gedurende mijn promotietraject op meerdere locaties gewerkt. Daarom wil ik de mensen en met name de chirurgen in het Rijnstate ziekenhuis, het Slotervaart ziekenhuis, het Rode Kruis ziekenhuis en het Lucas Andreas ziekenhuis bedanken voor de prettige samenwerking en hun bereidwilligheid om mij altijd te helpen bij de uitvoer van mijn onderzoek.

I would also like to thank Sam Virtue and Toni Vidal for the collaboration. Sam, it was great to work with you, I admire your scientific knowledge and skills, thank you for the work you did on the macrophages.

Daarnaast zijn er diverse andere mensen met wie ik heb samengewerkt. Jan Booij, je bent altijd heel geïnteresseerd en betrokken geweest bij het onderdeel van de SPECT sans, ik heb onze samenwerking als heel prettig ervaren. Aart Nederveen, jammer genoeg hebben niet alle onderzoeken met de MRI dit proefschrift gehaald, wel was het een bijzondere ervaring om zelf MRI’s te mogen maken en ik heb veel geleerd van jouw kritische blik op de resultaten. Mariette Ackermans, bedankt voor de isotopen bepalingen en alle andere analyses. Susanne la Fleur bedankt voor het kritisch lezen van verschillende manuscripten en alle gezellige momenten.

En dan zijn er natuurlijk de mensen met wie je dagelijks op de werkvloer staat, met wie je lief en leed deelt, over pieken klimt en door dalen gaat, dat zijn de mensen die als geen ander weten wat je meemaakt en doorstaat, dat zijn de directe collega’s. Ik wil eerst het woord richten tot mijn paranimfen. Nicolette, jij was al op F5 toen ik kwam, en jij was degene die mij heeft leren clampen, een vaardigheid die ik uiteraard altijd zal koesteren. We hebben veel onvergetelijke momenten meegemaakt op congressen binnen en buiten Europa, maar ook in het AMC en in onze vrije tijd. Vergeet ook vooral de zelfontspanner momenten niet! We hebben samen zenuwen gedeeld voordat we een presentatie moesten houden, maar ook de nodige momenten van ontspanning gepakt nadien. Met jou kon ik vanaf het begin eindeloos kletsen, lachen en relativeren, bedankt dat je naast me wilt staan om de laatste keer de zenuwen ook te delen. Karin, het is zover, eindelijk doen we het in omgekeerde setting over! Jij kent inderdaad de hoogte en diepte punten van mijn promotie, gelukkig zijn de ontmoetingen met bier, zoals in de gele keet naast het AMC, niet opgehouden toen ik weg was uit het AMC en jij zelfs al gepromoveerd was, wel fijn dat we nu op luxere locaties afspreken! Jij vloog door jouw promotietraject heen, uiteindelijk heb jij mij voor een groot
 CHAPTER 10

deel kunnen motiveren om de laatste maanden van mijn promotietraject hard te blijven werken en niet op te geven als ik het even niet meer zag zitten, dank daarvoor! **Anke,** we begonnen tegelijk en we gingen tegelijk weg, we deden een heel ander soort onderzoek en we hadden andere begeleiding, maar we zijn lange tijd roomies geweest, waardoor we toch vaak samen mooie momenten en frustraties konden delen, en vooral ook veel konden kletsen, het was gezellig! **Esther,** bedankt voor je hulp bij de follow up studie met de SPECT scan, jij bent heel georganiseerd en secuur, het was heel prettig om met je samen te werken, hoofdstuk 3 is mooi geworden. **Elsmarieke,** zonder jouw hulp was de interpretatie van de hersen MRI’s en de SEPCT scans niet zo goed gegaan, bedankt voor je aandeel daarin. **Murat,** succes met de databank en het afronden van je promotie, gewoon volhouden dan gaat het lukken! **Charlene, Merel, Dirk-Jan, Arvid, Hamid, Laura, Linda, Marieke, Bouwien, Eveline, Joelle, Myrte, Ruth, Annegreet, Sam, Pim, Kasper, Leslie, Emmely, Jacqueline, Unga, Dennis, Jose** en alle mensen die ik nog ben vergeten op F2 en F5, de mensen van de vasculaire op G2 en F4, en de mensen van de biochemie, bedankt voor de samenwerking, de besprekingen, de biertjes op de congressen, de wintersportvakanties, de onderzoekerslunch, de koffies en de gesprekken, kortom alle gezellige, leerzame en leuke momenten.

**Birgit,** je bent een held, zonder jouw hulp zou het afmaken van dit proefschrift een chaos zijn geworden. De high five die je me gaf toen we de mappen naar de commissie stuurde zal ik nooit vergeten. Nog steeds als ik op de afdeling kom is het leuk om je even te spreken, ook al is het altijd afwachten waar het secretariaat dan weer is. Bedankt voor al je hulp en gezelligheid. **Martine,** bedankt voor je hulp met name bij het prikken van de infusen als het niemand anders lukte en jouw ondersteuning bij de proeven.

Collega’s uit Tergooi, gedurende mijn periode in Tergooi achtervolgde mijn proefschrift mij overal, gelukkig was ik niet de enige met een nog lopend promotietraject en kon ik mijn frustraties met jullie delen, het was niet gemakkelijk, maar nu is het ons uiteindelijk allemaal gelukt!

Ik heb ook weer tijd voor gezellig dingen nu het af is. **Kerensa,** vanaf de eerste blabo was het gezellig, laten we nooit ophouden met de vrimibo. Jij hoorde op het terras continu mijn verhalen over successen en frustraties van het promoveren aan, toch ben je de uitdaging zelf ook aangegaan, de komende jaren zal ik jouw klankbord zijn! **Frank,** die database krijg je wel klein! **Arjan,** binnenkort kom ik op de fiets naar Utrecht. **Karen, Floor** en **Sandra,** ik kijk er naar uit dat jullie ook naar het AMC komen. **Weena,** wat goed dat we bijna tegelijk promoveren, het is gelukt. **Christa,** succes met de laatste loodjes, daarna kunnen we op zoek naar andere gespreksonderwerpen voor tijdens de etentjes. **Rinse** hou vol, ooit komt het af! **Annemarie** en **Saskia,** nu hoor ik ook bij de club! Op naar de korte vakanties en nieuwe projecten!

Suzanne, ik kan altijd (even) bij jou en Jasper langskomen, als we samen weggaan hebben we onvergetelijke momenten en moeten we altijd rennen om de laatste trein te halen. Bedankt dat je zo’n lieve zus bent, daar bof ik enorm mee. Merel, we gaan snel een keer samen naar de dierentuin kunnen we weer even bijkletsen! Esmee, Fleur en Reinout jullie worden zo snel groot, ik kom weer wat vaker langs, beloofd.

Lieve pappa en mamma, mijn laatste woorden zijn voor jullie, bedankt voor jullie onvoorwaardelijke liefde en steun bij alles wat ik doe, jullie geloven in mij en mij aanmoedigen om altijd door te gaan en het onderste uit de kan te halen. Jullie hebben geen idee hoe belangrijk jullie voor me zijn. Dit boekje is voor jullie!

Barbara
**Name PhD student: Barbara Anna Maria de Weijer**  
PhD period: Juli 2007 – May 2012  
Name PhD supervisors: Prof. dr. E. Fliers  
Name PhD co-supervisors: Dr. M.J.M. Serlie & Dr. M.C. van Eijk

<table>
<thead>
<tr>
<th>PhD training</th>
<th>Year</th>
<th>Workload (Hrs/ECTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General courses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Basic course Law and Organization for clinical researchers (BROK)</td>
<td>2009</td>
<td>0.9</td>
</tr>
<tr>
<td>- Mass spectrometry, proteomics and protein research</td>
<td>2009</td>
<td>0.5</td>
</tr>
<tr>
<td>- Practical biostatistics</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Specific courses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Radiation Protection 5B</td>
<td>2010</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Seminars, workshops and master classes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Weekly department seminars Endocrinology &amp; Metabolism</td>
<td>2007-2012</td>
<td>3</td>
</tr>
<tr>
<td>- Weekly department seminars Clinical Diabetology</td>
<td>2007-2012</td>
<td>1</td>
</tr>
<tr>
<td>- Weekly department seminars Vascular Medicine</td>
<td>2007-2012</td>
<td>3</td>
</tr>
<tr>
<td>- Ruysch Lectures</td>
<td>2007-2012</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>National Presentations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Striatal dopamine receptor binding in obese women compared to non-obese women and before and after gastric bypass surgery, NVDO Annual Meeting.</td>
<td>2010</td>
<td>0.5</td>
</tr>
<tr>
<td>- Short term effects of bariatric surgery on glucose and fatty acid metabolism, NVDO Annual Meeting.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- Striatal dopamine receptor binding in obese women before and after gastric bypass surgery, NVDO Annual Meeting.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- Retinol Binding Protein 4 (RBP4) expression in adipose tissue and liver in relation to metabolic fluxes in obese women, NASO Annual meeting.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- Obese women display inflammatory changes in liver which are not correlated to hepatic insulin sensitivity or liver fat content, NASO Annual meeting.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- The expression of pro- and anti-inflammatory markers in adipose tissue in relation to metabolic fluxes in obese women, NASO Annual meeting.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- Striatal dopamine receptor binding in obese women compared to non-obese women and before and after gastric bypass surgery, NMB.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>- Obese women display inflammatory changes in liver which are not correlated to hepatic insulin sensitivity or liver fat content, AMGRO Annual Meeting.</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>- Short term effects of bariatric surgery on glucose and fatty acid metabolism, Annual Meeting Dutch Endocrine Society (NVE).</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>- Striatal dopamine receptor binding in obese women compared to non-obese women and before and after gastric bypass surgery, Annual Meeting Dutch Endocrine Society (NVE).</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>- Short term effects of bariatric surgery on glucose and fatty acid metabolism, Annual Meeting Dutch Society of Metabolic and Bariatric Surgery (DSMBS).</td>
<td>2012</td>
<td>0.5</td>
</tr>
</tbody>
</table>
**International Presentations**

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Year</th>
<th>Impact Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo measurements of central regulation of glucose metabolism in humans, Summerschool AMC.</td>
<td>2010</td>
<td>0.5</td>
</tr>
<tr>
<td>Striatal dopamine receptor binding in obese women compared to non-obese women and before and after gastric bypass surgery, XVI IFSO.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>Striatal dopamine receptor binding in obese women compared to non-obese women and before and after gastric bypass surgery, 71th ADA Scientific Sessions.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>Basal endogenous glucose production and insulin levels are reduced 2 weeks after bariatric surgery with no effect on hepatic and peripheral insulin sensitivity, 71th ADA Scientific Sessions.</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>Retinol Binding Protein 4 (RBP4) expression in adipose tissue depots and liver in relation to metabolic fluxes in obese women, 72nd ADA Scientific Sessions.</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>The expression of pro- and anti-inflammatory markers in adipose tissue depots in relation to metabolic fluxes in obese women, 72nd ADA Scientific Sessions.</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>Obese women display inflammatory changes in liver which are not correlated to hepatic insulin sensitivity or liver fat content, 72nd ADA Scientific Sessions.</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>Obese women display inflammatory changes in liver which are not correlated to hepatic insulin sensitivity or liver fat content, Nutrition, Metabolism and the Brain (NMB) Meeting.</td>
<td>2012</td>
<td>0.5</td>
</tr>
<tr>
<td>Persistent reduction in striatal D_{2/3} receptor binding after Roux-en-Y gastric bypass surgery- induced weight loss, 74th ADA Scientific Sessions.</td>
<td>2014</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**(Inter)national conferences**

<table>
<thead>
<tr>
<th>Conference</th>
<th>Year</th>
<th>Impact Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th Dutch Endo Neuro Pschyco Meeting, Doorwerth</td>
<td>2009</td>
<td>0.25</td>
</tr>
<tr>
<td>12th Dutch Atherosclerosis Society, Annual Meeting (DAS), Ede</td>
<td>2009</td>
<td>0.25</td>
</tr>
<tr>
<td>XV International Symposium on Atherosclerosis (ISA), Boston</td>
<td>2009</td>
<td>1</td>
</tr>
<tr>
<td>45th EASD Annual Meeting, Vienna</td>
<td>2009</td>
<td>1</td>
</tr>
<tr>
<td>ADDRM/NVDO Annual Meeting</td>
<td>2009</td>
<td>0.25</td>
</tr>
<tr>
<td>70th ADA Scientific Sessions, Orlando</td>
<td>2010</td>
<td>1</td>
</tr>
<tr>
<td>ADDRM/NVDO Annual Meeting</td>
<td>2010</td>
<td>0.25</td>
</tr>
<tr>
<td>71st ADA Scientific Sessions, San Diego</td>
<td>2011</td>
<td>1</td>
</tr>
<tr>
<td>XVI Congress of the international federation for the surgery of obesity and metabolic disorders (IFSO), Hamburg</td>
<td>2011</td>
<td>1</td>
</tr>
<tr>
<td>ADDRM/NVDO Annual Meeting</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>NASO Annual Meeting</td>
<td>2011</td>
<td>0.25</td>
</tr>
<tr>
<td>NMB Annual Meeting</td>
<td>2011</td>
<td>0.25</td>
</tr>
<tr>
<td>72nd ADA Scientific Sessions, Philadelphia</td>
<td>2012</td>
<td>1</td>
</tr>
<tr>
<td>Annual Meeting Dutch Society of Metabolic and Bariatric Surgery (DSMBS)</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>Dutch Endocrine Society (NVE) Annual Meeting</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>ADDRM/NVDO Annual Meeting</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>NASO Annual Meeting</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>AMGRO Annual Meeting</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>NVE Annual Meeting</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>DSMBS</td>
<td>2012</td>
<td>0.25</td>
</tr>
<tr>
<td>74th ADA Scientific Sessions, San Francisco</td>
<td>2014</td>
<td>1</td>
</tr>
</tbody>
</table>

**Teaching**

<table>
<thead>
<tr>
<th>Topic</th>
<th>Year</th>
</tr>
</thead>
</table>
### Supervising

| - P. Rootjes, master thesis medicine. 'Nocturnal free fatty acids Measurements in obese and lean subjects and the effect of β-blockage on pulsatile release'. |
|---|---|
| | 2011 | 1 |

### Masterclass Lecture

| - Blood pressure and cardiac output before and after bariatric surgery. Masterclass Metabolism and Obesity, AMC. |
|---|---|
| | 2012 | 0.5 |

NB. 1 ECTS = 28 hours, based on the European Credit Transfer System.

### Peer reviewed Publications


Over de auteur