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ADIPOSE MARKERS OF METABOLIC OUTCOME AFTER WEIGHT LOSS

Daniel Eriksson Hogling

Karolinska Institutet

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Adipose Markers of Metabolic Outcome after Weight Loss

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som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Föreläsningssalen C1:87, plan 8, Karolinska Universitetssjukhuset i Huddinge.

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Till Matilda och Agnes

POPULÄRVETENSKAPLIG SAMMANFATTNING

Fetma är kopplat till störningar i kroppens ämnesomsättning och kan ge upphov till sjukdomar som typ 2 diabetes. Men det är inte bara mängden fett som avgör om man blir sjuk av sin fetma, utan även vilka egenskaper fettväven har. Den effektivaste behandlingen av fetma är fetmakirurgi, till exempel så kallad gastrisk bypass. I denna avhandling studeras kopplingen mellan olika egenskaper hos fettväven och störningar i ämnesomsättningen, samt hur detta förändras efter viktnedgång.

Studie I. Vid samma fettmängd i kroppen kan fettväven bestå av många små fettceller eller få men stora fettceller. Det senare har visat sig förknippat med försämrad känslighet för kroppens insulin, vilket är ett förstadium till typ 2 diabetes. I denna studie undersöktes om fettcellernas storlek i förhållande till fettmängden i kroppen kan förutsäga förbättrad känslighet för kroppens insulin efter viktnedgång (genom kostförändring eller gastrisk bypass). Studien visade att de med störst fettceller i förhållande till fettmängd förbättrade sin insulinkänslighet mest efter viktnedgång, vilket inte kunde förutsägas genom enklare mått som body mass index (BMI) eller förhållandet mellan midje- och höftomfång (midje-höft kvot).

Studie II. I denna studie jämfördes ämnesomsättning och fettväv hos patienter som gått ned i vikt genom gastrisk bypass med patienter som inte gått ned i vikt men hade samma BMI, kön, ålder och fettmängd. Studien visade att patienter som gått ned i vikt har bättre insulinkänslighet och blodfetter än patienter med samma BMI som inte gått ned i vikt. Dessutom hade försökspersonerna som gått ned i vikt en bättre fettväv med fler men mindre fettceller, mindre mängd bukfett och en mer gynnsam utsöndring av proteiner ur fettväven.

Studie III. Fettväven utsöndrar många olika proteiner som kan påverka kroppen och ibland störa ämnesomsättningen. I studie III undersöktes om ett visst protein, som kallas ”CCL18”, utsöndras från fettväven och om det är förknippat med störd ämnesomsättning. Studien visade att CCL18 utsöndras från fettväven och att denna utsöndring och nivåerna i blodet var kopplat till störningar i ämnesomsättningen. Några tydliga effekter av CCL18 på olika celltyper i fettväven kunde inte påvisas.

Studie IV. Här undersöktes om fettmängd och fettfördelning i kroppen kan förutsäga viktnedgång eller förbättrad ämnesomsättning efter gastrisk bypass. Kroppens fettmängd och fettfördelning mättes med en röntgenundersökning, så kallad DXA, innan operationen och uppskattades även med enklare mått som BMI och midje-höft kvot. Efter operationen förbättrades alla värden och fettet omfördelades i kroppen. Fettfördelningen mellan midja och höfter innan operationen kunde förutsäga hur mycket känsligheten för insulin förbättrades efter viktnedgång, och andelen kroppsfett kunde förutsäga hur mycket man gick ned i vikt. De enklare måtten kunde förutsäga utfallen ungefär lika bra som röntgenundersökningen, och värdet av röntgenundersökningen innan operation verkar därför vara begränsat.

ABSTRACT

Adipose tissue is closely linked to metabolic disturbances in obesity. Bariatric surgery such as roux-en-Y gastric bypass (RYGB) remains the most effective treatment of obesity and obesity-related disease. More factors than fat mass *per se* determine the metabolic complications of obesity, including alterations in fat cell size, body fat distribution, adipose protein release, inflammation and lipolysis. The aim of this thesis was to further characterize the relationship between adipose tissue characteristics and metabolic parameters before and after weight loss, and to investigate if adipose phenotype can predict metabolic improvement after weight loss.

Study I. At any given fat mass, adipose tissue may constitute of many small fat cells (hyperplasia) or few but large fat cells (hypertrophy). This latter morphology is associated with worse metabolic profile. Study I examined if adipose morphology, i.e. hyperplasia or hypertrophy, could predict improved insulin sensitivity after weight loss. Abdominal subcutaneous adipose biopsies were performed before weight loss by diet or RYGB. Body fat mass was measured by dual-energy x-ray absorptiometry (DXA) or bioimpedance and insulin sensitivity assessed by homeostasis model assessment of insulin resistance (HOMA-IR). Results showed a higher improvement in HOMA-IR in patients with hypertrophy.

Study II. The degree of improvement in metabolic profile and adipose tissue phenotype after weight loss is in relation to weight stable controls is not fully understood. In this study, women that had undergone RYGB were compared with a weight stable matched control group. Subjects that had undergone RYGB had lower HOMA-IR, better lipid profile and higher adiponectin levels and their adipose tissue was characterized by smaller fat cells, less visceral fat and lower secretion of tumor necrosis factor α (TNF-α) than controls.

Study III. Herein, the CC chemokine ligand 18 (CCL18) was examined in adipose tissue. CCL18 was found to be released from adipose tissue in a time dependent manner. M2 macrophages were the primary source of CCL18. Serum- and adipose secreted levels of CCL18 correlated with metabolic risk factors in women. We could not demonstrate effects of CCL18 on adipocyte expression of inflammatory or extracellular matrix proteins in vitro.

Study IV. The aim of this study was to investigate if body fat mass distribution measured by DXA or simple anthropometric measures could predict improved metabolic profile or weight loss after RYGB. Android/gynoid fat mass ratio and waist-to hip ratio could predict improved HOMA-IR, and BMI and body fat percentage could predict weight loss. DXA measures and simple anthropometric measures performed equally well, indicating a limited value for DXA to predict metabolic outcome after RYGB.

Conclusions: Adipose tissue morphology and body fat distribution can predict improved insulin sensitivity following weight loss. Metabolic and adipose phenotype improves beyond the control state after RYGB. CCL18 is released from M2 macrophages in adipose tissue, and adipose released and circulating levels correlate with metabolic risk markers in women.

LIST OF SCIENTIFIC PAPERS

I. **Eriksson Hogling D**, Andersson DP, Bäckdahl J, Hoffstedt J, Rössner S, Thorell A, Arner E, Arner P, Rydén M. Adipose tissue morphology predicts improved insulin sensitivity following moderate or pronounced weight loss. Int J Obes. 2015 Jun;39(6):893-8. [Epub 2014 April 23]

II. Hoffstedt J, Andersson DP, **Eriksson Hogling D**, Theorell J, Näslund E, Thorell A, Ehrlund A, Rydén M, Arner P. Long-term Protective Changes in Adipose Tissue After Gastric Bypass. Diabetes Care. 2017 Jan;40(1):77-84. [Epub 2016 Nov 16]

III. **Eriksson Hogling D**, Petrus P, Gao H, Bäckdahl J, Dahlman I, Laurencikiene J, Acosta J, Ehrlund A, Näslund E, Kulyte A, Mejhert N, Andersson DP, Arner P, Rydén M. Adipose and circulating CCL18 levels associate with metabolic risk factors in women. J Clin Endocrinol Metab. 2016 Nov;101(11):4021-4029. [Epub 2016 Jul 26]

IV. **Eriksson Hogling D**, Rydén M, Bäckdahl J, Thorell A, Arner P, Andersson DP. Body fat mass and distribution as predictors of metabolic outcome and weight loss after Roux-en-Y gastric bypass. Surg Obes Relat Dis. 2018 Jul;14(7):936-942. [Epub 2018 Mar 17]

CONTENTS

LIST OF ABBREVIATIONS

AG-ratio	Android-to-gynoid ratio
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
ATP III	Adult treatment panel III
BMI	Body mass index
CCL18	CC chemokine ligand 18
CT	Computed tomography
DXA	Dual-energy X-ray absorptiometry
DAG	Diacylglycerols
EGIR	European group for the study of insulin resistance
ELISA	Enzyme-linked immunosorbent assay
ESAT	Estimated subcutaneous adipose tissue
EVAT	Estimated visceral adipose tissue
FFA	Free fatty acids
GDR	Glucose disposal rate
FSIVGTT	Frequently sampled intravenous glucose tolerance test
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HSL	Hormone sensitive lipase
IDF	International diabetes federation
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
MCP-1	Monocyte chemoattractant protein 1

MRI	Magnetic resonance imaging
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PPAR-γ	Peroxisome proliferator-activated receptor-γ
QUICKI	Quantitative insulin sensitivity check index
RYGB	Roux-en-Y gastric bypass
T2D	Type 2 diabetes mellitus
TAG	Triacylglycerides
TNF-α	Tumor necrosis factor-α
UCP-1	Uncoupling protein 1
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
WHO	World health organization
WHR	Waist-to-hip ratio

1 BACKGROUND

1.1 OBESITY

1.1.1 Epidemiology

Obesity is a growing health concern associated with extensive comorbidity and mortality[1]. The World Health Organization (WHO)[2] defines overweight as a body mass index (BMI) ≥ 25 kg/m^2 and obesity as (BMI) ≥ 30 kg/m^2. Since 1975, the world-wide prevalence of obesity has almost tripled. In 2016, 39 % of the adult population of the world had overweight, whereof 13 % had obesity[2]. The corresponding prevalence in the Swedish adult population was 51 % and 15 % respectively in 2016[3].

1.1.2 Metabolic syndrome

Obesity is linked to development of metabolic complications such as type 2 diabetes mellitus (T2D), hypertension and dyslipidemia, which together with visceral obesity are clustered into the metabolic syndrome. The latter is associated with a marked increase in cardiovascular disease[4-6] and can be defined in several ways based on different clinical parameters and cut-off values. The most commonly used definitions are defined by WHO (1999)[7], the Adult Treatment Panel III (ATP III) in 2002[4] and the International Diabetes Federation (IDF) 2006[6] (Table 1). For some values, such as waist circumference and high-density lipoprotein (HDL) cholesterol, cut-off values differ between men and women. WHO included microalbuminuria as a criterion, while the IDF uses ethnic specific cut-off values for waist circumference. The European Group for the Study of Insulin Resistance (EGIR) suggested that insulin resistance or hyperinsulinemia (defined as the highest quartile in a non-diabetic population) should be a mandatory criterion[8]. In an attempt to unify the different criteria, a harmonizing definition[9] was proposed in 2009 after a joint meeting. Although the metabolic syndrome is well recognized and frequently used in clinical and research settings, it has been questioned whether the clustering of the individual risk factors is meaningful[10].

1.1.3 Treatment of obesity

Identification of obesity complications including metabolic disturbances is important to identify patients that would benefit the most from weight loss, and to select proper treatment after adequate risk-benefit analyses in patients with obesity[11]. Treatment strategies in obesity include lifestyle interventions, medical treatment and bariatric surgery (surgery for weight loss). Five percent weight loss is usually considered clinically meaningful and associated with improved metabolic risk factors, although glucose metabolism may improve even after less weight loss and further improvements are expected after more pronounced weight loss[12]. Bariatric surgery is by far the most effective treatment, both in terms of weight reduction and metabolic improvement, as shown in comparative studies with conventional medical therapy[13-15].

Table 1. Definitions of the metabolic syndrome by the World Health Organization (WHO), Adult Treatment Panel III (ATP III), International Diabetes Federation (IDF) and the joint Harmonizing definition

	Abdominal obesity	Blood lipids	Blood pressure	Blood glucose	Additional criteria
WHO[7] Impaired glucose regulation plus two other criteria	WHR >0.90 (men); >0.85 (women)	Triglycerides ≥1.7 mM and/or HDL cholesterol <0.9mM (men) <1.0 mM (women)	≥140/90 mmHg	Impaired glucose tolerance, insulin resistance or type 2 diabetes[a]	Microalbuminuria (urinary albumin excretion rate ≥20 µg/min or albumin:creatinine ratio ≥ 30 mg/g
ATP III[4] 3 or more criteria	>102 cm (men)[b] >88 cm (women)	Triglycerides ≥1.7mM HDL cholesterol <1.0mM (men) <1.3 mM (women)	≥130/85 mmHg	Fasting glucose ≥6.1 mM	
IDF[6] Abdominal obesity plus two additional criteria	Ethnic specific: ≥85-94 cm (men) ≥80-90 cm (women)	Triglycerides ≥1.7mM HDL cholesterol <1.0 mM (men) <1.3mM (women)	Systolic: ≥130 mmHg or Diastolic: ≥ 85 mmHg	Fasting glucose ≥ 5.6 mM or previously diagnosed type 2 diabetes	
Harmonizing criteria[9] Three of five criteria	Ethnic specific: ≥85-102 cm (men) ≥80-90 cm (women)	Triglycerides ≥1.7 mM [c] HDL cholesterol <1.0 mM (men) <1.3 mM (women)	Systolic ≥130 and/or Diastolic ≥85 mm Hg[c]	Fasting glucose ≥ 5.6 mM[c]	

[a] As defined by WHO. [b] "Some male persons can develop multiple metabolic risk factors when the waist circumference is only marginally increased, e.g. 94–102 cm"[4]. [c] Or corresponding drug treatment as alternative criterion. ATP III, Adult treatment panel III; HDL, high-density lipoprotein; IDF, International diabetes federation; WHO, World health organization; WHR, waist-to-hip ratio.

1.1.3.1 Lifestyle interventions

Lifestyle interventions with focus on increased physical activity, diet changes and weight loss induce an average 5-10 % decrease in body weight and in the frame of more intense programs also some metabolic improvement[16, 17]. Interventions with hypocaloric diets have been shown to induce more pronounced weight loss than exercise training[18, 19], and a combination of diet and exercise is probably most effective[17]. There is no clear evidence demonstrating superior effects of any specific low-calorie diet, although Mediterranean diet may have more

beneficial effects including decreased risk of cardiovascular disease and very low-calorie diet may induce faster weight loss[20]. However, most patients regain body weight and sustained pronounced weight loss is difficult to achieve by lifestyle changes alone[17, 18].

1.1.3.2 Anti-obesity drugs

Anti-obesity drugs have been reported to induce an average weight loss of 3-11.5 % depending on drug and dose, but have yet not been shown to reduce cardiovascular morbidity or mortality in patients with obesity without diabetes[21, 22]. The development of anti-obesity drugs has been limited by severe adverse effects for several substances as reviewed[23, 24]. The central nervous stimulant amphetamine was introduced for weight reduction during the 1930s, but withdrawn after a couple of decades due to adverse effects including cardiovascular concerns and substance abuse. More recently, the norepinephrine and serotonin reuptake inhibitor sibutramin was withdrawn due to cardiovascular adverse effects, and the cannabinoid receptor antagonist rimonabant was withdrawn because of psychiatric adverse effects including depression and suicide[23, 24]. The gastrointestinal lipase inhibitor orlistat and the gut hormone (i.e. incretin) glucagon-like peptide 1 (GLP-1) analogues are some of the few currently accessible alternatives for weight loss[21, 23]. In the USA, the two combination drugs phentermine/topiramate and naltrexone/bupropion are also available, the first suppressing appetite and the mechanism of the latter not fully understood[25]. The GLP-1 analogue liraglutide has been shown to induce an average 5.6 kg weight loss[26]. More recently, studies of the GLP-1 analogue semaglutide demonstrated a dose-dependent weight loss up to 11.5 % in comparison to placebo after one year[22]. A dual GLP-1 and glucose-dependent insulinotropic peptide (GIP) receptor agonist[27] has been reported to induce a weight loss of more than 10% in up to 39 % of participants versus 9 % with the GLP-1 analogue comparator in patients with T2D and an average BMI of 32.6 kg/m^2. Thus, the incretin analogues represent a novel approach in the pharmacological treatment of obesity, demonstrating promising effects that may change the field if not troubled by emerging major adverse effects.

1.1.3.3 Bariatric surgery

Bariatric surgery has been shown to induce substantial weight loss and decreased comorbidity and mortality[28], making it the most effective treatment of obesity and associated comorbidity to date. Indications for bariatric surgery have been basically unchanged since the early 1990s, briefly constituting BMI > 40 kg/m^2 or > 35 kg/m^2 in combination with obesity associated comorbidity[29, 30]. According to a recent report[31], 5400 patients underwent bariatric surgery in Sweden 2017. The most common bariatric surgery procedure was Roux-en-Y Gastric Bypass (RYGB) constituting 54.4 % of primary surgery, although sleeve gastrectomy increases (44.6 %). Laparoscopic technique was used almost exclusively, and perioperative severe complications rare (around 3 %) and 90-days mortality rates very low (0.4 ‰). Higher rates of severe adverse events have been reported in other studies with longer follow-up time[32]. The RYGB approach is illustrated in Figure 1. In sleeve gastrectomy, a part of the ventricle is removed. Pure restrictive procedures such as gastric banding have not been shown as effective[28], and are now infrequently used. Degree of weight loss after surgery differs

between surgical methods[28, 32]. RYGB and sleeve gastrectomy result in similar weight loss, on average around 30 % in the weight stable phase after two years[13, 28]. This is usually followed by weight regain during the following years, although not returning to the initial weight[28].

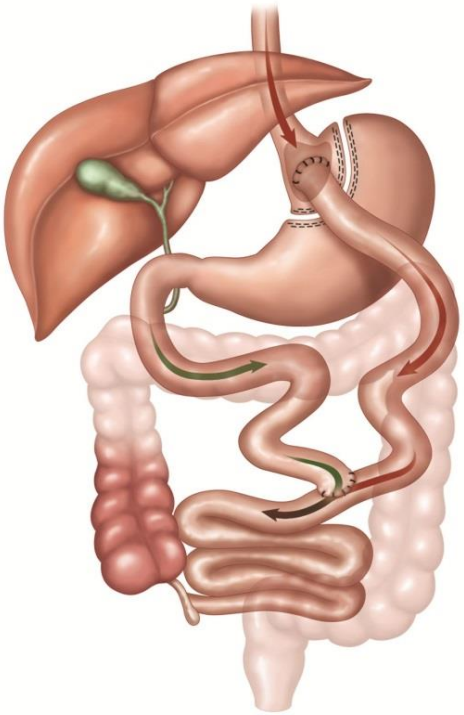


Figure 1. Roux-en-Y gastric bypass. A small pouch of the ventricle is created and attached to the distal jejunum, and the proximal "secretory limb" consisting of the remaining ventricle and duodenum is anastomosed back to the small bowel. Published with permission from the rights holder.

In patients with obesity and T2D, there is a high degree of diabetes remission after bariatric surgery (Table 2). One meta-analysis reports improved diabetes control in ~90% of patients and diabetes remission in ~ 65 %[33], although 19-53 % of the patients displayed diabetes relapse after 5-10 years[14, 34-37]. Comparative studies have shown RYGB to be a much more effective treatment of T2D than conventional medical treatment[13, 14, 38], and bariatric surgery remains the most effective treatment of obesity and associated comorbidity to date.

Bariatric surgery results in malabsorptive and restrictive effects. However, the favorable metabolic effects are also mediated by mechanisms beyond weight loss, including hormonal changes. Diabetes remission is often observed within the first post-operative week, i.e. before substantial weight loss has been achieved. Changes in the secretion of gastrointestinal hormones such as the incretins GLP-1 and GIP are thought to play a role in the early improvement of glycemic control[39-41]. Independent of weight loss, the GLP-1 response to oral glucose intake increases markedly after gastric bypass[41, 42]. Accelerated nutrient flow in the intestines enhances incretin release from the ileum, in turn increasing insulin release and inhibiting gastric emptying[39].

1.1.3.4 Prediction of metabolic outcome and weight loss after bariatric surgery

Given the increasing prevalence of obesity and its complication and that not all patients with obesity can undergo bariatric surgery, identification of patients who have the most to gain

from weight loss becomes a matter of great importance. To perform proper risk-benefit analyses before selecting treatment option, predictive factors and models are necessary.

Most studies concerning prediction of metabolic improvement after weight loss focus on prediction of T2D remission after bariatric surgery. Less seem to be studied about predictors of improved insulin sensitivity or T2D incidence after weight loss in patients with prediabetes or without diabetes. Factors reported to predict T2D remission include markers of insulin production and sensitivity, BMI and age (Table 2). High age, long diabetes duration and insulin dependence are associated with a less degree of diabetes remission after weight loss. Different scoring systems for clinicians have been suggested. One of these suggested scoring system is DiaRem[43] based on age, Hemoglobin A1c (HbA1c) concentration, use of sulfonylurea or other insulin sensitizing agent or insulin, that in one comparative analysis has been shown to have the best prediction error[44]. A more recent study has found similar predictors in larger cohorts with longer follow-up time[45]. Among adipose factors, adipocyte size has been shown to predict resolution of T2D or diabetes risk after weight loss[46].

Table 2. Remission rates of type 2 diabetes (T2D) after bariatric surgery and predictors of T2D remission

Author, year	Follow-up time (months)	T2D remission rate	Preoperative predictors of T2D remission
Hayes et al 2011[47]	12	84 %	HbA1c, fasting glucose, insulin treatment, BMI, hypertension
Robert et al 2013[48]	12	62.8 %	BMI, diabetes duration, HbA1c, fasting glucose, oral antidiabetic treatment without insulin
Lee et al 2013[49]	12	65.3 %	"ABCD score": age, BMI, C-peptide, T2D duration
Dixon et al 2013[50]	12	30.1 % (HbA1c < 42 mmol/mol)	BMI, T2D duration
Ugale et al 2014[51]	Mean 12.7 and 30.2 depending on procedure	46 % and 72 % depending on procedure/follow-up time	Age, BMI, T2D duration, insulin treatment, stimulated C-peptide, micro and macrovascular complications
Still et al 2014[43]	14	63 %	"DiaRem score": age, HbA1c, type of T2D medication, insulin treatment
Aminian et al 2017[45]	84	49 % (gastric bypass) and 28 % (sleeve gastrectomy)	T2D duration, number of T2D medications, insulin use, HbA1c
Pucci et al[52] 2018	24	68.6 %	"DiaBetter score": HbA1c, T2D duration, type of T2D medication

Partly adapted from Zhang et al[53]. BMI, body mass index; HbA1c, hemoglobin A1c; T2D, type 2 diabetes mellitus.

Few studies follow patients for more than ten years after bariatric surgery, and a limitation of many studies is low follow-up rates. There is limited data concerning the durability of diabetes remission after bariatric surgery[54, 55]. In the Swedish Obese Subjects study[28], 2010 obese subjects undergoing bariatric surgery and a similar number of matched controls receiving standard care have been followed up to 20 years. In the control group, the weight remained within 3 % around baseline values during follow-up, whereas a 13-27 % decrease was observed in the surgery group depending on procedure. Mortality rates were almost 30 % lower in the surgery group after 16 years, and despite a 50 % relapse after initial diabetes remission at the 10 years follow-up, the cumulative T2D incidence was at least 75 % lower in the surgery group[28].

1.2 ADIPOSE TISSUE

There are two main forms of adipose tissue in mammals, white adipose tissue (WAT) and brown adipose tissue. WAT constitutes the great majority of human adipose tissue. Brown adipocytes are specialized in transforming stored fatty acids into heat by a "uncoupling" function enabling heat formation instead of adenosine triphosphate (ATP) generation by the action of uncoupling protein 1 (UCP-1)[56]. Results in recent years have also shown that there is also an intermediate form of fat that displays both white and brown phenotypes. This is sometimes referred to as "beige" adipose tissue. The metabolic impact of brown adipose tissue is debated in man, and brown and beige adipose tissue will not be discussed further in this thesis.

Adipose tissue is composed of different cell types (Figure 2). While fat cells (adipocytes) make up to 90 % of the mass they only constitute 40-50 % of adipose tissue cell number. The rest of WAT is referred to as the stromal vascular fraction and includes mesenchymal stem cells, adipocyte precursor cells, endothelial cells, fibroblasts and immune cells such as macrophages and other leukocytes[57].

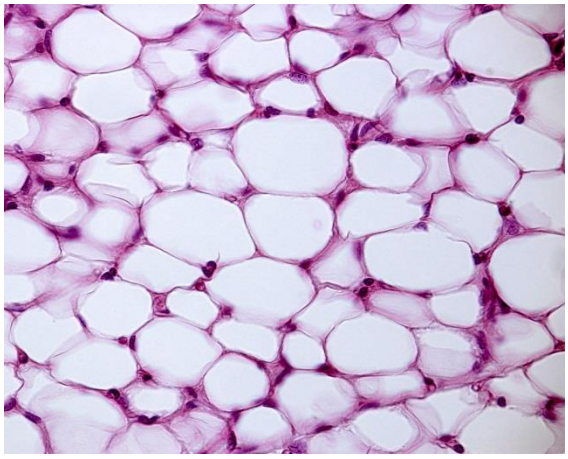


Figure 2. Histological section of white adipose tissue stained with hematoxylin and eosin. Adipose tissue consists primarily of adipocytes. The adipocytes store lipids in a large lipid droplet which constitutes most of the cell and pushes the nucleus and other organelles to the periphery. Between the adipocytes, other cell types including leukocytes, mesenchymal stem cells and preadipocytes are found in the stromal vascular fraction. Published with permission from Shutterstock.com.

From previously being mainly regarded as an energy depot, WAT is now known to be an important organ contributing to the development of metabolic complications of obesity. Several observations illustrate that factors beyond a mere increase in fat mass per se are involved in the metabolic disturbances associated with obesity. For instance, surgical removal of fat does not induce favorable metabolic effects. Removing subcutaneous fat by liposuction does not improve metabolic profile[58], and neither does removal of visceral WAT (i.e. the greater omentum) alone[59] or in addition to RYGB surgery[60]. The absence of a functional adipose tissue is also detrimental, as illustrated by the condition lipodystrophy in which functional fat mass is lacking and insulin resistance observed[61]. Furthermore, the term metabolically healthy obesity has been used to reflect the fact that up to 30 % of obese patients display relative intact insulin sensitivity and lack of other metabolic complications[62]. However, the notion of metabolically healthy obesity has been questioned as there appears to be an increased risk of cardiovascular disease in this group compared to healthy non-obese, although lower than in "metabolically unhealthy" obesity[63-65]. Nevertheless, WAT mass expansion leads to a more pernicious phenotype, although these changes may be more or less pronounced as illustrated in Figure 3. A number of different adipose tissue factors contribute to the negative effects of expanding fat mass. These include alterations in distribution of body fat, adipocyte size and number, lipolysis, polypeptide (adipokine) secretion and inflammation as described in the following sections.

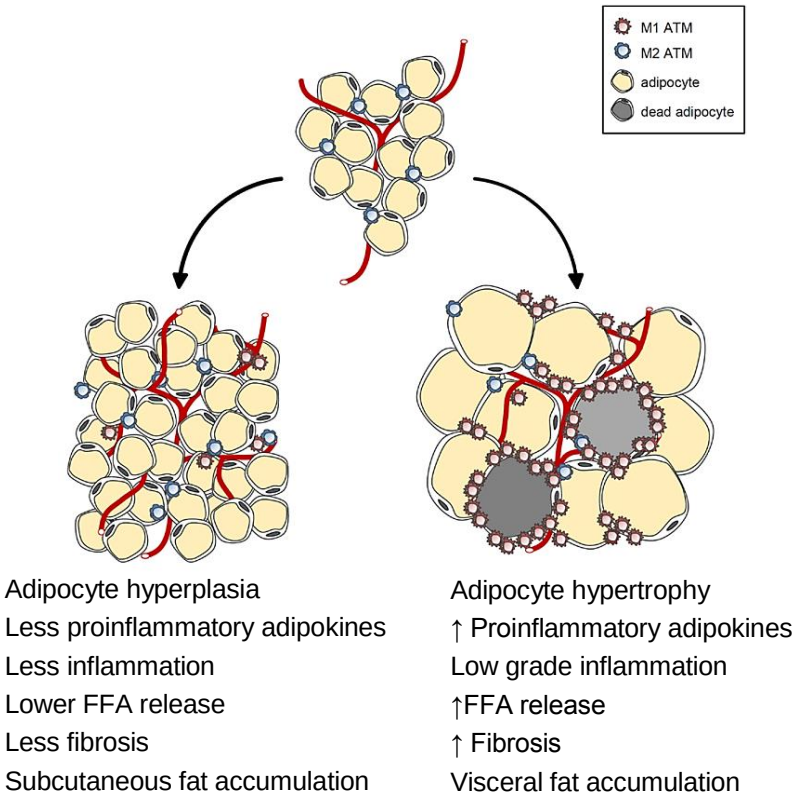


Figure 3. Benign versus pernicious expansion of adipose tissue. Pernicious expansion of adipose tissue (right) is to a higher degree characterized by adipocyte hypertrophy, low grade inflammation including more abundant proinflammatory M1 adipose tissue macrophages than anti-inflammatory M2 macrophages, disturbed lipolysis with a higher release of free fatty acids (FFA) and development of fibrosis. "Crown-like structures" of M1 macrophages surrounds the dead adipocytes. Figure from Choe SS et al[66], slightly modified and published with permission. ATM, adipose tissue macrophages; FFA, free fatty acids.

1.2.1 Body fat distribution

Body fat distribution can be described in different ways. The most common classification of WAT based on its location is subcutaneous versus visceral adipose tissue. Subcutaneous adipose tissue constitutes more than 80 % of total body fat[67]. Body fat distribution can also be described as central versus peripheral, upper body versus lower body, abdominal versus gluteofemoral, or as android versus gynoid[68]. This latter terminology originates from the observation that men usually are more prone to abdominal fat accumulation whereas women in general have a tendency to gluteofemoral fat accumulation. The physiology behind these sex differences in body fat distribution includes estrogen effects, and a tendency towards more android fat accumulation is observed in women after menopause[69]. Intraabdominal visceral adipose tissue can be further divided into mesenteric and omental fat.

Abdominal, and in particular visceral, WAT is highly associated with metabolic disturbances. As previously mentioned, abdominal obesity is one of the cornerstones of the metabolic syndrome and it associates with increased mortality independent of BMI[70, 71]. Whether abdominal obesity is causally linked to metabolic disturbances or rather a consequence or a marker of ectopic fat accumulation is not fully understood[72]. However, the potential pernicious properties of visceral fat accumulation may include several factors. The anatomic location of intraabdominal fat enables direct action of released adipokines and free fatty acids (FFA) on the liver through the portal vein, inducing hepatic insulin resistance and resulting in increased hepatic gluconeogenesis and very-low-density lipoprotein (VLDL) release and reduced insulin clearance, as formulated by Björntorp in the "portal theory"[73]. Basal lipolysis and catecholamine stimulated lipolysis is higher in visceral adipose tissue, the latter due to a dominance of β-adrenergic receptors[74], and this has been correlated to metabolic disturbance[75]. Several further differences between visceral and subcutaneous adipose tissue are reported. Subcutaneous adipogenic capacity is higher than visceral[72]. Large inter-depot variations in macrophage numbers may contribute to differences in inflammation[67, 72].

In contrast to android fat accumulation, gynoid fat is considered protective[68]. The protective properties of WAT in the gynoid region may be mediated by its capacity to harbor excess fat and thus preventing ectopic fat accumulation and its negative metabolic consequences[67].

Body fat mass and distribution can be estimated or measured in several different ways. Simple methods such as waist circumferences, waist-to-hip ratio (WHR) and formulas based on age, gender and BMI[76] can be used as indirect estimations. Bioelectrical impedance can be used to estimate total fat mass and fat free mass. Body fat mass and distribution can also be measured using magnetic resonance imaging (MRI) or x-ray based methods such as computed tomography (CT) and dual-energy x-ray absorptiometry (DXA).

1.2.2 Adipocyte size and number

As described above, adipocytes constitute less than half of the cell number in WAT. In weight stable adults, the number of fat cells is unaltered in adulthood although on average 10 % of adipocytes are turned over, i.e. die and are renewed, each year[77]. With increasing fat

mass, adipocyte size increases. However, there is a limit for adipocyte size which implies that at some point, the number of adipocytes needs to increase as well. This is demonstrated by the curvilinear relationship between fat mass and fat cell size (Figure 4). It has been known since the 1970s that large adipocytes correlate with pernicious metabolic profile in cross-sectional studies[78-80]. Adipocyte size has also been shown to predict future development of T2D independent of fat mass[81, 82]. After weight loss, fat cell size decreases but the fat cell number remains unaltered[77].

As adipocyte size is closely related to fat mass it has been difficult to determine the impact of fat cell size independent of body fat mass. A model based on the curvilinear relationship between fat cell size and fat mass has previously been suggested to isolate the effect of fat cell size independent of fat mass (Figure 4)[77]. If a curve is fitted into this graph, individuals can be described as having larger or smaller adipocytes than expected at any given fat mass. Thus, those above the fitted line have larger but fewer fat cells than expected (hypertrophy) whereas those below have more but smaller fat cells than expected (hyperplasia)[77]. The degree of hyperplasia or hypertrophy can be quantified using a *morphology value* which reflects the distance from the curvilinear fit.

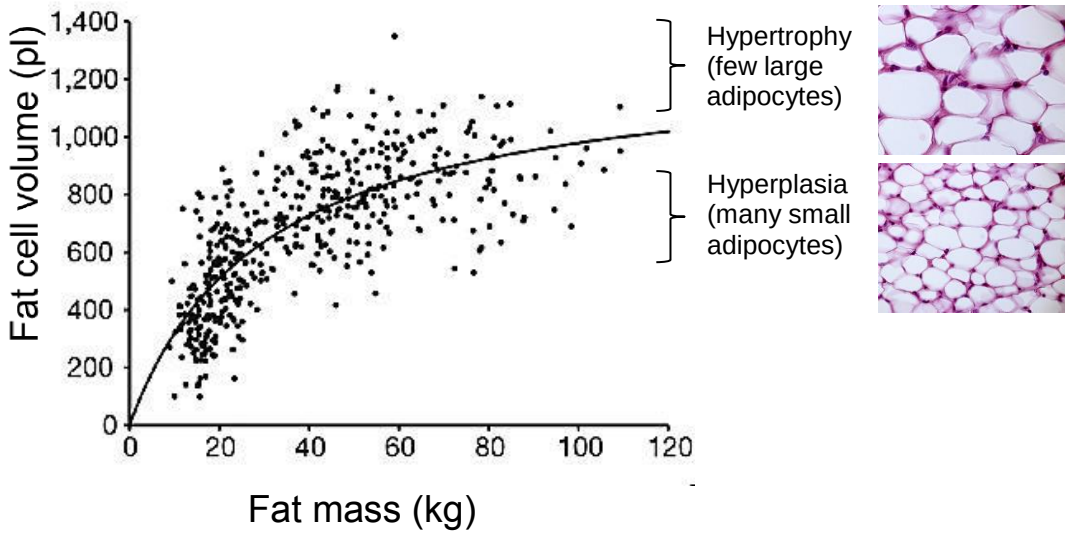


Figure 4. Adipose tissue morphology illustrated by the curvilinear relationship between fat mass and fat cell volume. At any given fat mass adipose tissue may consist of few but large adipocytes or many small adipocytes. Subjects above the fitted line can be classified as having an adipose tissue morphology characterized by hypertrophy, whereas those below as having hyperplasia. The morphology can be quantified by a morphology value, i.e. the vertical distance from the fitted line. Graph published with permission from the publisher[77], pictures published with permission from Shutterstock.com.

What mechanisms may explain the association between fat cell size and metabolic disturbances? Two principal different mechanisms are mainly suggested, the first involving local changes in adipose tissue phenotype and the other related to ectopic fat accumulation. With increasing fat cell size, adipokine expression turns into a more proinflammatory pattern with increased release of adipokines such as monocyte chemoattractant protein-1 (MCP-1) which recruits macrophages and induces a local low-grade inflammation promoting insulin

resistance[83]. Hypertrophic adipocytes develop insulin resistance and are less sensitive to the inhibiting effect of insulin on the lipolytic processes resulting in increased basal lipolysis[84] and thus increased release of FFA. Another important role of subcutaneous adipose tissue is to store excess lipids to prevent ectopic lipid accumulation in liver, skeletal muscle and other organs where they may cause metabolic disturbances[85]. Inability of subcutaneous WAT to act as an "energy sink" promotes ectopic fat accumulation including visceral obesity with its associated negative metabolic consequences[86].

The process in which preadipocytes are differentiated into adipocytes is referred to as adipogenesis. The main regulator of adipocyte differentiation is the peroxisome proliferator-activated receptor-γ (PPAR-γ)[87], although other factors including insulin-like growth factor 1 (IGF-1), insulin and glucocorticoids also induce differentiation[72]. The mechanisms determining development of hyperplasia or hypertrophy are not fully understood. It is likely to involve an inability to recruit and/or differentiate progenitor cells into mature adipocytes, i.e. impaired adipogenesis. The transcription factor early B-cell factor 1 (EBF1) has been shown to correlate with adipose morphology, with decreased expression and activity in hypertrophy[88], and transforming growth factor-beta 3 has recently been shown to regulate subcutaneous adipocyte number[89].

In summary, impaired expandability of WAT by recruiting new adipocytes leads to WAT characterized by few but large adipocytes accompanied by a metabolically unfavorable clinical phenotype.

1.2.3 Adipocyte function

1.2.3.1 Lipogenesis and lipolysis

Storage and release of lipids are major functions of adipocytes. Lipids are stored in the adipocytes in the form of triacylglycerides (TAG), and the controlled release of lipids through enzymatic breakdown of TAG, *lipolysis*, is an important mechanism in lipid metabolism.

In times of energy excess, non-esterified FFA from the diet or hepatic de novo lipogenesis are taken up by the adipocyte through fatty acid transporter protein, and glucose enter the cell through the glucose transporter (GLUT) 4 upon insulin stimulation. FFA can also be synthesized from glucose through de novo lipogenesis within the adipocyte, although this process is probably of minor importance in adults. Glucose is metabolized into glycerol-3-phosphate which is esterified with FFA eventually forming TAG that are stored in the large intracellular lipid droplets pushing the intracellular organelles to the periphery[90] (Figure 2).

In lipolysis, TAG are metabolized in three major steps by three distinct lipases, each releasing one fatty acid from the glycerol body (Figure 5). Glycerol is then released from the adipocyte since the cell lacks significant amounts of glycerol kinase and is thus unable to metabolize the glycerol molecule[91]. The rate-limiting step in human lipolysis is the release of the second fatty acid by hormone sensitive lipase (HSL) which is inhibited by insulin and stimulated primarily by catecholamines but also by natriuretic peptides, tumor necrosis factor alpha

(TNF-α) and other factors[91, 92]. Catecholamines primarily stimulate lipolysis through β-adrenergic receptors, but may also inhibit lipolysis through α-adrenergic receptors to a lower extent. Differences in lipolytic response to catecholamines between body fat regions are partly explained by variations in receptor density[74].

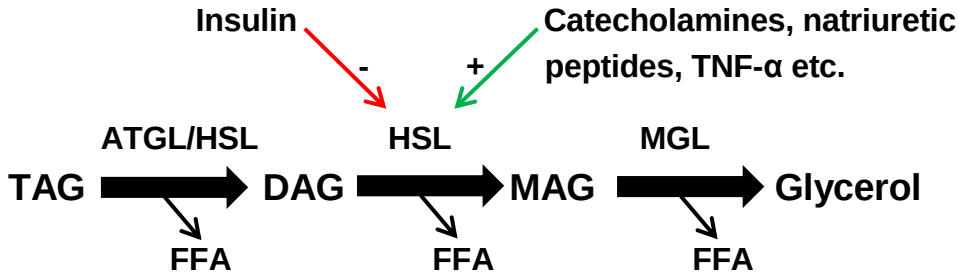


Figure 5. Schematic illustration of the hormonal regulation of human lipolysis. TAG are metabolized in three steps, each releasing one FFA. Insulin inhibits HSL while catecholamines and other substances stimulate lipolysis through HSL. ATGL, adipose triacylglyceride lipase; DAG, diacylglycerol; FFA, free fatty acid; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; TAG, triacylglycerol; TNF, tumor necrosis factor.

In patients with obesity, the basal lipolysis is increased. Concerning the catecholamine stimulated lipolysis in obesity, regional differences exists where visceral stimulated is increased while subcutaneous catecholamine stimulated lipolysis is decreased[92]. In the insulin resistant state, the activity of HSL is altered resulting in increased basal lipolysis and thus increased release of FFA further contributing to insulin resistance. In contrast, stimulated lipolysis is decreased which implies a decreased adaptability in the lipid metabolism[91]. These changes are believed to be a major contribution to the development of insulin resistance as further described below. The physiological importance of a functional lipolysis is illustrated in humans with mutations in the gene encoding HSL, which display insulin resistance, dyslipidemia and early development of T2D[93]. With increasing age, basal lipolysis is increased and stimulated lipolysis inhibited[92]. Lipolysis has been linked to long term development of T2D and weight development. Inflexible lipolysis, i.e. low stimulated and high basal lipolysis, predicts weight gain and insulin resistance[94].

1.2.3.2 Adipokines

Adipose tissue is now recognized as an important endocrine organ. During the last decades more than 600 polypeptides have been described to be released from adipose tissue[57]. These factors are collectively termed adipokines. There is no consensus on whether this term should be reserved for factors only released from adipocytes or if those released from any cell type in adipose tissue could also be included[95]. Adipokines act within a broad range of different functional areas, including lipid turnover, inflammation, angiogenesis and regulation of blood pressure. However, the function of many adipokines still remains to be characterized.

TNF-α was the first cytokine to be described as a WAT-secreted factor and associated with insulin resistance, secreted from adipocytes but primarily adipose tissue macrophages[96]. Adipose expression and levels of TNF-α have been shown to be increased in obesity and

correlate with insulin resistance, and decrease after weight loss[97]. TNF-α acts in a paracrine fashion in adipose tissue, interfering with several parts of intracellular insulin signaling, including insulin receptor substrate 1 (IRS-1)[98], downregulating factors involved in the effects of insulin and inhibiting transcription of factors associated with insulin sensitivity [99].

Leptin[100], which is released from WAT and acts on the hypothalamus to regulate appetite and thus energy balance in an endocrine manner, was a seminal discover. Leptin levels are highly correlated to degree of obesity. Its implication as a therapeutic substance in obesity has been limited by the leptin resistance that quickly develops with increasing leptin levels[101]. Leptin levels in plasma correlate well with adipocyte size[102]. After weight loss, adipocyte leptin release is decreased below the control state[103].

Adiponectin is another well characterized adipokine. It displays an inverse relation to BMI and acts in an anti-diabetic manner by decreasing hepatic glucose production and increasing skeletal muscle fatty acid oxidation[104]. Low levels of adiponectin have been shown to predict future T2D development[105]. Although its effects are potent in murine models, the clinical relevance in man is disputed, and there are no therapeutic options targeting adiponectin.

Interleukin 6 (IL-6) is released from adipose tissue and induces insulin resistance. Systemic levels of IL-6 can predict future T2D development[106]. IL-6 is also released from skeletal muscle and may mediate cross-talk between WAT and skeletal muscle[95]. The metabolic effects of IL-6 is probably dependent on whether the elevation of IL-6 is acute or chronic, as reviewed[107]. Acute IL-6 rise as in exercising muscle seem to reduce inflammation and promote insulin sensitivity whereas a chronic rise is associated with inflammation and reduced insulin sensitivity. Acute hyperinsulinemia induces elevated plasma IL-6 levels[108].

MCP-1 is a proinflammatory chemokine encoded by the *CCL2* gene. MCP-1 is expressed in adipose tissue and attracts macrophages into adipose tissue. Its expression in adipose tissue is higher in obese state and higher in visceral than subcutaneous WAT. Circulating MCP-1 is elevated in patients with T2D[109].

1.2.4 Adipose inflammation

As mentioned above, obesity is characterized by a chronic low grade inflammation in WAT induced by para- and autocrine pro-inflammatory molecules such as MCP-1, IL-6 and TNF-α. These factors are released from the fat cells themselves as well as immune cells in the stromal vascular fraction, including macrophages recruited into WAT. Inflammatory molecules interfere with insulin signaling and induce insulin resistance as described above. The levels of MCP-1 correlate significantly with the percentage of infiltrating macrophages[83]. Traditionally, it has been considered that obesity induces a switch from alternatively activated anti-inflammatory "M2" macrophages to classically activated proinflammatory "M1" macrophages. The M1 phenotype is characterized by expression of proinflammatory markers such as TNF-α and IL-6, and is especially abundant in the "crown like structures" surrounding dead adipocytes (Figure 3). On the opposite, the M2 phenotype is characterized by anti-inflammatory markers[110]. However, this view seems to be simplistic and it has

recently been shown that metabolic dysfunction may drive the development of a specific metabolically activated macrophage phenotype[111]. In vitro experiments have shown that co-culture of M1 macrophages and adipocytes make macrophages turn in to a more M2 like phenotype[112].

WAT inflammation is diminished after weight loss, with decreased number of macrophages[113] and decreased adipocyte expression of inflammatory proteins such as TNF-α[97, 114].

1.2.5 Adipose fibrosis

Development of fibrosis is another characteristic of metabolically unhealthy expanded WAT. Rapidly expanding adipose tissue leads to hypoxia which induces excessive synthesis of extracellular matrix components including collagens. Fibrosis is linked to inflammation and infiltration of macrophages[115]. In fibrotic areas, alternatively activated M2 macrophages are abundant in the adipose tissue of obese subjects and correlate with insulin resistance[112].

1.3 INSULIN

1.3.1 Insulin synthesis and secretion

Insulin is a 5.8 kDa protein composed of 51 amino acids synthesized in the pancreatic β-cells. It is initially synthesized as preproinsulin, and then processed to proinsulin in the endoplasmic reticulum before cleaved into insulin and C-peptide in the Golgi apparatus and eventually stored in granules awaiting proper stimulus for release. Insulin synthesis and release into the circulation is primarily regulated by glucose. Glucose enters the beta cells through GLUT2, which responds even to small changes in the blood glucose concentration, thus tightly regulating insulin secretion during physiological conditions. Glucose is metabolized through glycolysis after entering the cell, and ATP formed which in turn acts on ATP-sensitive potassium channels which increases intracellular levels of potassium and depolarizes the cell. This causes voltage-sensitive calcium channels to increase calcium influx which finally induces exocytosis of granules containing insulin, releasing insulin into the circulation. Insulin secretion is also regulated by FFA, GLP-1 and other factors. GLP-1 increases insulin secretion.[116]

1.3.2 Insulin signaling and effects

Insulin exerts its effects through a tetrameric membrane spanning tyrosine kinase receptor. Upon insulin binding, the receptor is autophosphorylated which activates the tyrosine kinase activity of the receptor. This initiates the intracellular signaling pathway, including phosphorylation of IRS-1, activating phosphatidylinositol-3 kinase and eventually the protein kinase Akt, mediating the intracellular effects of insulin. Intracellular insulin signaling is complex and involves many different pathways resulting in many different events.

In the liver, insulin inhibits glycogenolysis and gluconeogenesis and thereby the subsequent efflux of glucose from hepatocytes through the GLUT2 into the circulation. In skeletal

muscle, insulin (and physical activity) induces the translocation of GLUT4 to the cell membrane which facilitates glucose uptake into myocytes. Although skeletal muscle is the major target organ for insulin stimulated glucose uptake, GLUT4 is also responsible for the insulin stimulated glucose uptake into adipocytes[117]. By inhibiting HSL, insulin promotes the storage of lipids in the adipocytes.

1.3.3 Insulin resistance

The progress from normal glucose metabolism to T2D involves a progressive impairment of insulin sensitivity in different organs paralleled by increased endogenous insulin release from the pancreatic beta cells that initially compensate for the insulin resistance. But with long-standing insulin resistance the beta cells gradually fail in this compensation and relative insulin deficiency develops which results in impaired fasting glucose, impaired glucose tolerance and eventually T2D[118].

Central organs involved in the development of and affected by the consequences of insulin resistance include the pancreas, liver, skeletal muscle and adipose tissue[119], as illustrated in Figure 6. Chronic elevation of circulating FFA causes ectopic lipid accumulation in peripheral organs such as liver, skeletal muscle and pancreas. This induces negative metabolic consequences described as lipotoxicity. The consequences of hepatic insulin resistance include decreased glycogenesis and decreased inhibition of glycolysis and gluconeogenesis resulting in increased glucose production and output, and increased release of VLDL. In skeletal muscle, insulin resistance causes decreased uptake of glucose. In adipose tissue, the effects of insulin resistance include decreased inhibition of basal lipolysis leading to excess release of FFA.

FFA impair insulin sensitivity in a dose-dependent manner in humans[120, 121]. One of the first proposed mechanisms to explain insulin resistance was the Randle hypothesis[122], stating that increased FFA influx and fatty acid oxidation cause increased levels of acetyl coenzyme A, inhibit pyruvate dehydrogenase and thus reduce glucose oxidation and increase levels of citrate. This causes inhibition of the rate limiting step in glycolysis (phosphofructokinase), eventually causing increased intracellular glucose levels and decreased glucose uptake. Later studies have shown that this mechanism may not be entirely correct, and rather stress the importance of diacylglycerols (DAG) arising from increased influx of FFA into the cell. DAG activate protein kinase C, which in turn inactivate IRS-1 through phosphorylation and thereby inhibit the insulin signaling pathway causing decreased glucose uptake and breakdown[123].

As described in previous sections, inflammation is another feature promoting insulin resistance. TNF-α interferes with insulin signaling through interaction with IRS-1[98] and stimulates lipolysis through HSL[99]. MCP-1 induces insulin resistance in adipocytes with reduced glucose uptake, and also promotes insulin resistance in non-adipose tissue[83].

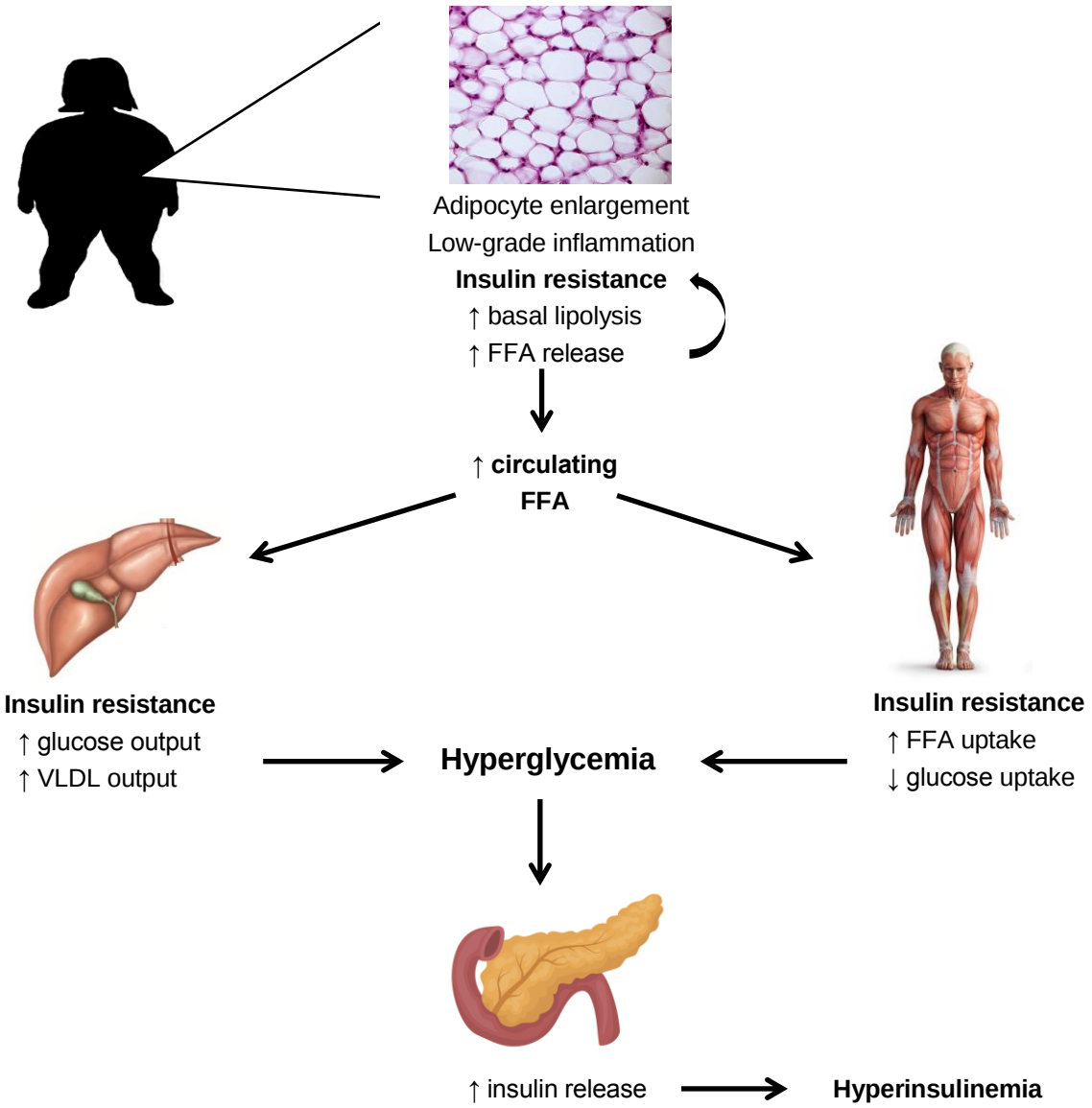


Figure 6. Causes and consequences of insulin resistance in obesity. In the obese state, the release of free fatty acids (FFA) from adipose tissue increases. By paracrine actions, FFA and proinflammatory adipokines from enlarged adipocytes induce insulin resistance in adipose tissue which increases lipolysis enhancing the flux of FFA tissue into the circulation. By interfering with intracellular insulin signaling pathways, FFA induce insulin resistance in liver, muscle, pancreas and other organs. In the liver, insulin resistance induces increased glucose output and release of very-low-density lipoproteins. In skeletal muscle, insulin resistance leads to increased FFA utilization and decreased glucose uptake. These changes induce hyperglycemia, which triggers increased insulin release from the pancreas resulting in hyperinsulinemia. FFA, free fatty acids; VLDL, very-low-density lipoproteins. Pictures published with permission from Shutterstock.com.

The hyperinsulinemia resulting from insulin resistance is also *per se* considered to drive the development of insulin resistance. Chronic hyperinsulinemia is believed to contribute to insulin resistance by inducing inflammation in adipose tissue and thereby increasing lipolysis; by increasing lactate formation in muscle and thereby increasing hepatic gluconeogenesis; and by stimulating hepatic lipogenesis[117].

Taken together, all the changes discussed above promote insulin resistance if energy excess is sustained.

The clinical consequences of insulin resistance and T2D include the micro- and macrovascular complications of diabetes. T2D is associated with an approximate two-fold increased risk for cardiovascular disease[124]. Even hyperinsulinemia has been linked to increased risk of cardiovascular disease[125], and a slight increased risk for cardiovascular disease has been observed even at modest elevated blood glucose levels[124].

1.3.4 Measuring insulin sensitivity

1.3.4.1 Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp method to measure insulin sensitivity was presented in 1979[126]. During a continuous insulin infusion, a simultaneous glucose infusion maintains euglycemia. High levels of insulin are required to fully suppress the hepatic glucose production. Since the hepatic glucose production is assumed to be switched off, the procedure reflects whole body insulin sensitivity. The amount of glucose infused corresponds to the glucose disposal rate (GDR) or metabolizable glucose (M), reflecting whole body insulin sensitivity. A GDR of 4.9 mg/kg/min has been suggested as a cut-off for insulin resistance in the 0.12 U insulin/m^2/min setup[127]. Hyperinsulinemic euglycemic clamp is further discussed in chapter 3.5.

1.3.4.2 Homeostatsis Model Assessment of Insulin Resistance (HOMA-IR)

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)[128] is a mathematical formula to estimate insulin sensitivity, based on fasting plasma levels of glucose and insulin. It is calculated as follows:

HOMA-IR = fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/l) / 22.5

HOMA-IR correlates well with hyperinsulinemic euglycemic clamp[128]. Reflecting normal fasting values for insulin and glucose, HOMA-IR is around 1 at physiological conditions. Proposed limit for insulin resistance has been a value above the 75th percentile in a population without diabetes[8], usually HOMA-IR $\geq$ 2.5[64, 129].

1.3.4.3 Other methods to determine insulin sensitivity

Oral Glucose Tolerance Test (OGTT) assesses the blood glucose response to an oral bolus of glucose and provides a relatively simple method able to detect early disturbances in glucose metabolism[130].

Glucose tolerance tests can also be performed through intravenous administration of glucose, a method less influenced by gastrointestinal glucose uptake. In the *Frequently Sampled Intravenous Glucose Tolerance Test* (FSIVGTT), glucose is infused and frequent samples of insulin and glucose collected. Using the *minimal model assessment*, a sensitivity index reflecting insulin sensitivity can be calculated[131].

Insulin Tolerance Test (ITT) is another measure of insulin sensitivity validated against clamp[132]. After an intravenous insulin infusion, the measured plasma glucose disappearance rate offers an assessment of insulin sensitivity.

In addition to HOMA-IR, there is another fasting index to determine insulin sensitivity. In the *Quantitative Insulin Sensitivity Check Index (QUICKI)* fasting values of insulin and glucose are log-transformed, and the index can be calculated using the formula 1 / [log (fasting plasma insulin) + log (fasting plasma glucose)]. QUICKI has been validated against hyperinsulinemic clamp and FSIVGTT[133].

2 HYPOTHESES AND AIMS

2.1 GENERAL AIM

The general aim of this thesis is to further characterize the relationship between WAT characteristics and metabolic parameters, cross-sectionally before and after weight loss, and to investigate if WAT phenotype can predict changes in metabolic parameters after weight loss.

2.2 SPECIFIC HYPOTHESES AND AIMS

2.2.1 Study I

Rationale: Proper selection criteria for bariatric surgery are of great importance from individual and societal view in order to make proper risk-benefit analyses. Current selection criteria based on BMI has a limited ability to predict metabolic outcome after RYGB, and better predictors of metabolic outcome are needed.

Hypothesis: Large adipocytes and adipose hypertrophy predicts improved insulin sensitivity after weight loss.

Aim: To investigate if adipose tissue morphology or fat cell size can predict improved insulin sensitivity after weight loss.

2.2.2 Study II

Rationale: WAT and clinical phenotype improve after RYGB. However, less is known about the degree of improvement in relation to weight stable subjects.

Hypothesis: Subjects that have undergone RYGB have a more favorable metabolic and WAT phenotype than weight stable control subjects.

Aim: To compare the clinical and WAT phenotype in RYGB patients after weight loss with weight stable control subjects.

2.2.3 Study III

Rationale: Characterization of protein release from WAT may facilitate further understanding of the mechanisms behind metabolic alterations in obesity and identify markers of metabolic disturbance. CC chemokine ligand 18 (CCL18) expression in WAT has been reported to be upregulated in obesity and diabetes, and related to inflammation and fibrotic disease in other tissue types.

Hypothesis: CCL18 is associated with metabolic disturbance, secreted from subcutaneous adipose tissue and exert effects on WAT cells promoting extracellular matrix formation and a proinflammatory response.

Aim: To investigate if CCL18 is secreted from subcutaneous adipose tissue. To determine if adipose secreted or circulating CCL18 correlate with metabolic parameters. To determine the cellular origin of CCL18 in WAT and investigate the potential effects of CCL18 on adipocytes and macrophages in vitro.

2.2.4 Study IV

Rationale: Better predictors of metabolic outcome and weight loss after RYGB are needed to improve candidate selection for RYGB. Body fat distribution is associated with metabolic dysfunction.

Hypothesis: DXA measured body fat mass and distribution can predict weight loss and improved insulin sensitivity, hypertension or dyslipidemia after RYGB. DXA measures are superior to basic anthropometric measures as predictors of these outcomes.

Aim: To investigate if preoperative body fat mass and/or distribution determined by DXA can predict improved metabolic parameters and/or weight loss after RYGB, and to compare predictive value of DXA with basic anthropometric measures.

3 METHODOLOGICAL ASPECTS

3.1 STUDY DESIGN

Study I was a prospective interventional cohort study in which two cohorts were examined before and after weight loss induced by diet or RYGB respectively. Baseline adipose tissue morphology and adipocyte size were studied as predictors of changes in insulin sensitivity after weight loss. A third cohort was used to enable calculations of adipose morphology.

Study II was a prospective interventional cohort study with matched controls. Patients were examined before, two and five years after weight loss. At the five year follow-up, patients were matched on BMI, sex and age with control subjects and clinical and adipose parameters compared.

Study III was a cross-sectional cohort study. CCL18 expression and release were correlated to clinical parameters, and effects of recombinant CCL18 on adipocytes and macrophages studied in vitro.

Study IV was a prospective interventional cohort study where patients were examined before and two years after RYGB. Baseline DXA measures and anthropometric measures were studied as predictors of improved metabolic parameters after weight loss.

3.2 CLINICAL COHORTS

Several cohorts are included in this thesis and one of the cohorts is used in three studies herein, as presented in Table 3 and the text below. More detailed clinical characteristics of each cohort are described in each manuscript respectively.

Table 3. Study specific presentation of cohorts included in this thesis. Cohorts are further described in the text following the table.

	Longitudinal cohorts			**Cross-sectional cohorts**			
	NUGENOB	**DEOSH**	**NEFA**	**Morphology cohort**	**DPP4**	**RIKEN**	**EMIF**
Study I	x	x		x			
Study II		x					
Study III					x	x	x
Study IV		x	x				

NUGENOB (Nutrient-Gene Interactions in Human Obesity) was a European randomized study on the metabolic effect of either of two hypocaloric diets in overweight or obese but otherwise healthy subjects[134]. In study I, 21 men and 79 women from the NUGENOB study were included.

DEOSH (Danderyd Ersta Oment Södertälje Huddinge) is a multi-center randomized controlled trial primarily investigating the metabolic effects of omentectomy in addition to RYGB[60, 135] (clinicaltrials.gov NCT01785134). 82 women with obesity were examined at baseline, 62 women two years after RYGB and 49 women five years after follow-up. Exclusion criteria were T2D with insulin or glitazone treatment, oral or parenteral steroid treatment, complicated psychiatric disease and warfarin use. This study also includes a matched control group.

NEFA (Non-Esterified Fatty Acids / New Fat study) is a longitudinal study of the metabolic and adipose tissue effects after RYGB (clinicaltrials.gov NCT01727245). Exclusion criteria were the same as in *DEOSH* described above. 133 women that were examined on baseline and 104 followed up after two years are included in study IV.

The *DPP4* (Dipeptidyl Peptidase 4) cohort was originally used in a study that identified DPP4 as a novel adipokine[136]. It consisted of 10 non-obese patients and 19 obese women.

The *RIKEN* cohort included 30 obese and 26 non-obese patients, original involved in a study of adipose tissue microRNA as regulators of CCL2 production[137]. Transcriptomic and other analyses where performed at the RIKEN research institution in Japan.

EMIF (European Medical Information Framework) is a large pan-European research collaboration in which this cohort has been a part. The cohort used herein is based on a group of 220 women in which the upper and lower extremes (n = 40 + 40) of insulin sensitivity as measured by HOMA-IR are compared. Further clinical details on this cohort have been published[138].

3.3 CLINICAL EXAMINATIONS

Subjects arrived at the research center in the morning after an overnight fast. They had been instructed to avoid physical activity from the day before the examinations and smoking or other nicotine use in the morning. Patients in the bariatric surgery cohorts were examined within three weeks before surgery. A digital scale (TANITATBF-305) was used to measure weight to the nearest 0.5 kg, and height was measured to the nearest 0.5 cm. Waist and hip circumferences were measured. Venous blood samples were drawn for further analyses. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald´s formula with values expressed in mmol/L (cholesterol - high-density lipoprotein - 0.45 x triglycerides)[139]. An automatic device was used to measure blood pressure in the right arm (Omron M10-IT, Omron Health Care, Hoofddorp, the Netherlands). ATP III-score used in Study III were based on the ATP III definition of the metabolic syndrome (Table 1), with one point given for each criteria fulfilled or medication for any risk factors.

3.4 MEASURING BODY FAT MASS AND DISTRIBUTION

In this thesis, body fat mass was measured by DXA or bioelectrical impedance, and calculated by the use of a formula as described below.

3.4.1 Dual-energy X-ray absorptiometry (DXA)

DXA was used to determine body fat mass and distribution (GE Lunar iDXA, GE Healthcare, Madison, WI, USA). The enCORE software (version 14.10.022, GE Healthcare, Madison, WI, USA) enabled determination of fat mass and percentage within different fat depots, i.e. android and gynoid fat. The android region is defined by the software as the region between the pelvis cut line, a line 20 % of the distance between the pelvis and neck cut line and laterally by the arm cut line (Figure 7). "Estimated subcutaneous adipose tissue" (ESAT) is assessed within the android region. The CoreScan feature (GE Medical Systems, Chalfont St. Giles, UK) was used to estimate visceral fat amount. "Estimated visceral adipose tissue" (EVAT) mass is assessed within the android region (total android fat mass - ESAT)[140], thus only constituting the visceral fat within the android region as illustrated in Figure 7. Throughout the study, daily automatic calibration checks of the DXA machine were performed. A spine phantom provided by the manufacturer was used for regular calibrations three times a week.

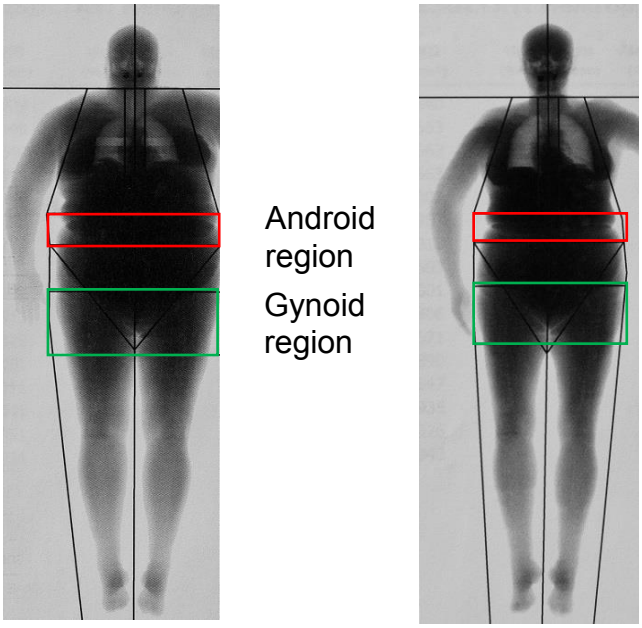


Figure 7. Dual-energy X-ray absorptiometry examination before (left) and after (right) Roux-en-Y gastric bypass. The android (red box) and gynoid region (green box) is indicated as defined by the software. Published with permission from the patient.

DXA measure of body fat allows fast examinations with low radiation exposure in comparison to CT scan, one of the reference methods. In a comparative study, DXA measures of visceral fat correlated well with values from CT scan (r^2 value 0.959 for women)[140], and this assessment of visceral fat mass is approved by the US Food and Drug Administration for clinical use. In comparison to MRI in the study of patients with obesity, DXA do not suffer from the limitation that a substantial proportion of patients do not fit into the MRI scan[141].

3.4.2 Bioelectrical impedance

In study I, body fat percentage was determined by bioelectrical impedance (Body Stat, Quad Scan 4000, Isle of Man, British Isles). The precision of bioelectrical impedance analyses is considered sufficient to estimate body composition at group level, although a tendency towards a few percent underestimation of fat mass in the obese and overestimation to a similar degree in the lean is reported in comparison to DXA. This trend towards underestimation of fat mass in comparison to DXA was also seen in a cohort included in the same multicenter study as cohort 1 in study I, although there was a strong correlation between bioelectrical impedance and DXA values (r=0.78)[142] .

3.4.3 Formula

In study IV, body fat percentage was also calculated in addition to being measured by DXA. The following formula, based on BMI, age and gender was used[76]:

age x 0.13 + 1.5 x BMI -11.5 (for women)

3.5 DETERMINATION OF INSULIN SENSITIVITY

Insulin sensitivity was determined by HOMA-IR and hyperinsulinemic euglycemic clamp in this thesis.

Insulin levels were determined by enzyme-linked immunosorbent assay (ELISA) as described below, and *HOMA-IR* calculated using the formula (fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/l) / 22.5).

During the *hyperinsulinemic euglycemic clamp,* a constant insulin infusion is administered paralleled by a variable glucose infusion to maintain euglycemia. In our setup, a 1.6 units/m^2 body surface area bolus dose of insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) was administered intravenously, followed by a continuous intravenous insulin infusion at a rate of 0.12 units/m^2 body surface area/minute during two hours. The insulin was suspended in 82 ml sodium chloride (9 mg/ml), 2 ml albumin (200 g/l, Albunorm Octapharma, Stockholm, Sweden) and 16 ml potassium chloride (2 mmol/ml) to avoid hypokalemia. The infusion rate was 25 ml/h. This was accompanied by a 200 mg/ml variable glucose infusion to maintain euglycemia (4.5 - 5.5 mmol/L). Arterialized blood samples were collected from the dorsal side of the hand, kept in a heating box (63^o C), and analyzed in duplicates every fifth minute (Hemocue, Ängelholm, Sweden) to determine blood glucose values throughout the procedure. Glucose disposal rate during steady state was quantified by the calculated M-value (mg/kg*min), based on the glucose infusion rate during steady state the last hour of the clamp.

Hyperinsulinemic euglycemic clamp has been considered gold standard to measure insulin sensitivity, although disadvantages of the method include that it is quite time consuming and expensive. Besides, the term “gold standard” may be misleading since different measures of insulin sensitivity reflect different aspects of insulin sensitivity. Since skeletal muscle is the

primary target for insulin stimulated glucose uptake, clamp measurement of insulin sensitivity are highly dependent on factors associated with glucose uptake by skeletal muscle including muscle mass and physical activity. In contrast to the hyperinsulinemic euglycemic clamp, HOMA-IR relies on the relationship between hepatic glucose output and insulin secretion in the fasting state and primarily reflects hepatic insulin sensitivity. HOMA-IR has been reported to correlate well with hyperinsulinemic clamp[143], although another study found a lower correlation and suggests that oral glucose tolerance test may better reflect insulin stimulated glucose uptake[144]. The M-value can be indexed on insulin levels during the clamp or lean body mass, and thereby reflecting slightly different aspects.

In our clamp setup, the insulin infusion rate used was 0.12 U/m^2/min. This relatively high rate was chosen to achieve a complete suppression of endogenous glucose production even in morbid obesity, and to reach steady state more quickly as discussed[127]. Since endogenous hepatic glucose production is assumed to be shut off due to the high concentration of insulin, administered exogenous glucose is expected to reflect whole body glucose uptake (primarily by skeletal muscle), but to be sure would require studies involving radioactively labelled glucose.

3.6 ADIPOSE TISSUE EXAMINATIONS

3.6.1 Subcutaneous adipose tissue biopsies

Biopsies were performed approximately 15- 30 cm laterally from the umbilicus. Under sterile conditions, 20 ml of prilocaine hydrochloride (5 mg/ml) without epinephrine was injected subcutaneously. After local anesthesia was achieved, a minor incision was made and subcutaneous adipose tissue vacuum aspired using a needle and a 10 ml syringe. Tissue samples were rinsed with saline over a plastic filter (Sefar Nitex 06-210/33, Bigman AB, Sweden) and contaminating coagulated blood removed manually using a spatula. Samples were immediately transported to lab, rinsed in saline and analyzed immediately or frozen in liquid nitrogen for storage in -70 degrees C for later protein analyses. The biopsy procedure with needle aspiration under local anesthesia has previously been shown not to affect adipocyte metabolism[145].

3.6.2 Fat cell isolation and determination of adipocyte size

Subcutaneous adipose tissue samples were kept in sodium chloride during transport to the lipid laboratory for further analyses. Samples were washed again as described above, and thereafter further washed several times with a washing buffer (Krebs Ringer Phosphate buffer with 1 % and 0.1% bovine serum albumin respectively). To separate adipocytes from the stromal vascular fraction, samples were incubated in a buffer with 0.5 mg/ml collagenase and 4 % bovine serum albumin in a 37°C water bath. Thereafter, cells were washed again and filtered through a nylon filter three times.

Determination of fat cell size was made in accordance to Rodbell[146]. After collagenase treatment, a droplet of the cell suspension was examined in direct light microscope where the

diameter of 100 cells was measured using a ruler in the ocular of the microscope. The mean adipocyte volume was estimated from the diameter by a formula taking into account that the cells are spheric[147]. The method used for determination of adipocyte size has been used by our laboratory for decades and correlates well to measure of adipocyte size in sliced sections[148].

In study I, a morphology value (i.e. quantification of the deviation of adipocyte size from the expected size at any given fat mass) was calculated for each subject. For cohort I, morphology values were available from a previous study[149]. To enable calculations of morphology values in cohort 2 where fat mass was measured by DXA, a third cohort with subjects having a broad range of fat mass was added and a line fitted to describe the curvilinear relationship between fat mass and adipocyte size. The least squares non-linear fitting was used to describe the curve with the following formula:

$V = (31.6 \times m)/(1 + 0.0161 \times m)$

Where V = mean fat cell volume (picoliters), and m = fat mass (kilograms). Morphology value was calculated by subtracting the expected fat cell mass from the measured.

3.6.3 Measurement of lipolysis

Lipolysis is assessed through the measurement of glycerol release. Since glycerol is not metabolized in adipocytes due to the lack of glycerol kinase, glycerol release is directly related to the lipolytic activity in contrast to the other end product of lipolysis, FFA, which may become re-esterified. In study II, glycerol release from intact pieces of subcutaneous adipose tissue was analyzed. Pieces of adipose tissue (100 mg/ml medium) were put into Krebs-Ringer phosphate buffer (pH 7.4) supplemented with glucose (1 mg/ml), bovine serum albumin (20 mg/ml) and ascorbic acid (0.1 mg/ml), and incubated at 37°C in 2 hours. Aliquots of the medium were subsequently removed for determination of glycerol release. Glycerol release was determined by bioluminescence as described[150]. In this assay, glycerol kinase consumes added ATP when breaking down glycerol, and the residual ATP is quantified through a Luciferase catalyzed reaction of ATP and luciferin in which light is emitted.

In study II, glycerol release was assessed from tissue pieces rather than isolated fat cells, which enables assessment under more physiological conditions, with the stromal vascular fraction and the structure of the tissue better preserved. However, assessment of stimulated lipolysis would require isolation of adipocytes. Glycerol release is usually normalized on triglyceride mass or number of fat cells. In study II, fat cell number is calculated based on lipid weight of the incubated WAT divided by mean fat cell weight. Glycerol release was also expressed in relation to the amount of ESAT, providing a potential estimate of the systemic effects.

3.6.4 Gene expression analyses

Polymerase chain reaction (PCR) was used in study III to determine expression of the specific genes of interest. The qPCR technique enables a quantitative determination of gene expression. RNA was isolated, cDNA synthesized and quantitative PCR performed. The ΔΔCt method was used to analyze gene expression, and *LRP10* and *18S* rRNA and were used to normalize the expression of analyzed genes in adipocytes and THP-1 cells respectively.

Microarray data were used for analyses of gene expression in intact adipose tissue in cohort 2 in study III. Microarray data were obtained by the usage of Human gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA, US), and processes were performed using standardized protocols. Microarray offers the possibility to perform a genome wide characterization of gene expression and to perform gene ontology analyses. In comparison to other approaches such as Cap Analyses of Gene Expression (CAGE), microarray is cheaper and requires a smaller sample size.

3.6.5 Determination of protein release

ELISA was used to determine levels of serum insulin and levels of proteins released from adipose tissue, including CCL18. ELISA enables a quantitative determination of protein levels. In comparison to Western blot, ELISA is faster to perform and offers a highly quantitative approach. Depending on factors including antibody clonality, analyses may vary in terms of sensitivity and specificity. All analyses were performed according to the instructions from the manufacturer. The CCL18 assay used (R&D Systems Inc., Minneapolis, USA) is based on the quantitative sandwich enzyme immunoassay approach, with monoclonal antibodies covering the microplate and polyclonal enzyme-linked antibodies used for detection. Linear increase of protein with incubation time indicates secretion from WAT.

3.6.6 Cell cultures

In study III, cell cultures of *in vitro differentiated preadipocytes* were used. In contrast to freshly isolated adipocytes, in vitro differentiated preadipocytes offers the advantage of being able to attach to wells and being cultured for longer time. Additionally, freshly isolated adipocytes are fragile although they may better reflect in vivo conditions. *THP-1 cells*, also used in study III, are derived from an immortalized monocyte cell line that can be differentiated into macrophages in the M0, M1 or M2 spectrum[110], offering an in vitro model of macrophages although there may be differences in comparison to adipose tissue derived macrophages.

3.7 ROUX-EN-Y GASTRIC BYPASS

In the *DEOSH* cohort, open RYGB was performed as described[60]. After an upper midline incision, a small gastric pouch (20–30 ml) was created and sealed by linear staples. The gastro-entero anastomosis was created by linear or circular staples, and the entero-entero anastomosis by linear staples. The alimentary and biliopancreatic limbs were typically 120

and 75 cm long, respectively. Half of the patients in the *DEOSH* study were randomized to omentectomy, where the entire greater omentum was surgically resected during the same session. In the *NEFA* study, patients primarily underwent laparoscopic surgery.

3.8 STATISTICAL ANALYSES

In all studies, absolute values were presented as mean ± standard deviations. Depending on data distribution and whether data were dependent or not, different tests were used to compare data as presented in Table 4.

Table 4. Statistical tests used to compare two data sets

Data type	**Parametric**	**Non-parametric**
Unpaired/independent	(Student´s) t-test	Mann-Whitney U-test (Wilcoxon rank sum test)
Paired/dependent	Paired (Student´s) t-test	Wilcoxon signed rank test

Study I. Comparisons between groups were made by unpaired t-test or Mann-Whitney U-test depending on data distribution, and by paired t-test for intraindividual comparisons of values before and after weight loss. Spearman correlation tests were used to determine the correlation between continuous predictors and outcomes.

Study II. Longitudinal comparisons between intraindividual values and values between post-obese patients and matched controls were made using paired Students t-test or Wilcoxon signed rank test. Comparisons between post-obese subjects and controls were also made by unpaired t-test. Analysis of covariance (ANCOVA) was used for comparison of the relation between serum adiponectin and HOMA-IR between the post-obese subjects and controls. Linear regression was used to investigate potential predictive properties of values at two years versus changes between two and five years, and to determine potential associations between changes in different parameters between two and five years.

Study III. Comparisons between groups were made with tests in accordance to Table 4. Non-parametric data were log10 transformed to achieve normal distribution. Linear regression was used to determine potential correlations between different parameters. Multiple regression analyses were used to adjust for BMI.

Study IV. Shapiro-Wilks test was used for assessment of normal distribution, and two-sided statistical tests were chosen in accordance to Table 4. Linear regression was used to correlate predictors (baseline parameters) with outcome (changes in metabolic parameters). Multiple regression analyses were used to adjust for potential confounders (BMI and age) and to compare the predictive value of DXA measures with basic anthropometric values.

Both study I and IV revolves around prediction. In study I, the predictors are reported both as a binary and continuous variable, whereas study IV investigates continuous predictors. Both

studies have continuous outcomes. Alternatively, the outcome could have been dichotomized into a binary outcome, enabling the construction of a receiver operating characteristic (ROC) curve which offers a measure of predictive accuracy, i.e. the area under the curve. Disadvantages of dichotomizing the outcome may however include the risk of an arbitrary cut-off and that information about degrees within the parameter. Dichotomizing both the predictors and outcomes would enable assessment of predictive accuracy in terms of sensitivity, specificity and positive and negative predictive value. It may be an alternative approach but would expand the challenges discussed above to the predictor as well. To assess prediction, Spearman correlation test was used in study I and linear regression in study IV. Spearman correlation test makes fewer assumptions than linear regression. A linear regression model enables (more or less precise and valid) reproductions of the prediction. The rho and r^2 values offer an indication of the precision of the predictor.

4 RESULTS

Findings are presented in detail in each respective manuscript. Study specific summary of the results are presented in the following sections. Figure 10 summarizes the major findings of the studies.

4.1 STUDY I

In cohort I, 100 patients (79 women and 21 men) were included. 61 patients were included in cohort II and examined before and two years after RYGB. At baseline, patients were classified as having an adipose morphology characterized by hypertrophy or hyperplasia. In both cohort I and II, patients with hypertrophy displayed higher HOMA-IR (P = 0.003 in cohort 1 and 0.0006 in cohort 2), insulin (P < 0.0001 and 0.004 respectively) and WHR (P = 0.013 and 0.0066) despite similar BMI (P = 0.46 and 0.97). In cohort 2, glucose was higher in the group with hypertrophy (P = 0.008). After weight loss no statistically significant difference in HOMA-IR remained between the groups in the two cohorts.

Average weight loss in cohort 1 was 7 ± 3 %, and 33 ± 9 % in cohort 2. BMI, waist circumference, WHR, fat mass, insulin, glucose and HOMA-IR decreased in all subgroups in the two cohorts (P <0.0001 for all values).

Baseline morphology could predict changes in (Δ) insulin levels and HOMA-IR in both cohorts, regardless of whether morphology was studied as a dichotomous variable (P = 0.0073 and 0.047 for Δ insulin in cohort 1 and 2; and P = 0.013 and 0.0012 for Δ HOMA-IR) or continuous variable (P = 0.015, Rho = 0.25 and P = 0.01, Rho = 0.35 for Δ insulin; and P = 0.027, Rho = 0.23 and P = 0.0002, Rho = 0.50 for Δ HOMA-IR) as illustrated for cohort 2 in Figure 8. Adipose morphology could not predict weight loss. Neither baseline BMI, total fat mass, WHR nor waist circumference correlated with improved HOMA-IR or insulin. Fat cell size alone predicted improved HOMA-IR only in cohort 2 (P = 0.005, Rho = 0.38).

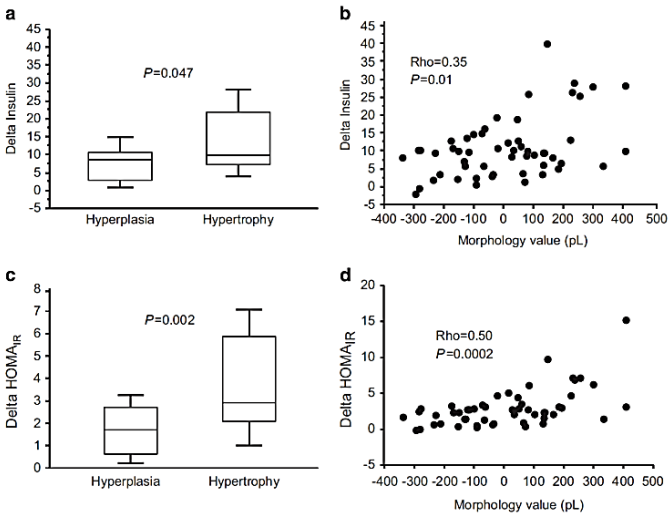


Figure 8. The relationship between adipose morphology and changes in (delta) insulin (a, b) and HOMA-IR (c, d) in cohort 2. P and Rho values from Mann-Whitney U-test and Spearman correlation test respectively. HOMA-IR, homeostasis model assessment of insulin resistance. Figure 2 from study I.

4.2 STUDY II

Forty nine patients were examined before, two and five years after RYGB. 30 patients were individually matched for sex, BMI and age with a weight stable control subject at the five year follow-up.

Average BMI was reduced from 43.0 ± 4.8 kg/m^2 at baseline to 28.8 ± 4.2 kg/m^2 two years postoperatively ($P < 0.0001$), followed by a slight weight regain to 31.3 kg/m^2 ($P < 0.0001$) at the five year follow-up. As expected, all clinical parameters improved after two years, including average HOMA-IR that decreased from 3.9 to 1.0 (all $P < 0.0001$). Fat cell volume decreased ($P < 0.0001$) but fat cell number did not change significantly. There was a marked decrease in total and regional fat mass and a slight increase between two and five years (all $P < 0.0001$). S-adiponectin increased ($P = 0.001$) and remained stable at the five year follow-up. Lipolysis measured as glycerol release decreased two years after surgery and experienced an increase up to levels above baseline five years after surgery (both $P < 0.0001$ when normalized per number of cells). Self-reported physical activity increased after RYGB and remained stable at the five year follow-up. Between two and five years after RYGB, most clinical parameters deteriorated slightly following the weight regain. Fat cell number but not volume increased between 2 and five years ($P = 0.019$ and 0.19).

In relation to the individually age- and BMI-matched control group, the women examined five years after RYGB had lower HOMA-IR ($P = 0.002$), lower triglycerides ($P = 0.02$) and cholesterol levels ($P = 0.003$) and higher circulating adiponectin ($P = 0.0009$) and HDL-levels ($P = 0.02$) as seen in Table 5. EVAT was significantly lower than in controls ($P = 0.00006$), despite similar WHR and waist circumference.

Analyses of WAT protein release showed no significant differences between two and five years after RYGB for MCP-1 and adiponectin but higher levels for TNF-α ($P = 0.003$) and IL-6 when expressed per ESAT ($P = 0.02$). In comparison to controls, subjects that had undergone RYGB had lower release of TNF-α ($P = 0.005$ and 0.004) and higher adiponectin release when expressed per number of cells ($P < 0.0001$).

Serum adiponectin levels and HOMA-IR correlated negatively, both five years after RYGB and in the control group. However, in the control group the correlation was steeper and HOMA-IR was higher with low adiponectin levels.

Analyses of distribution of fat cell volume showed normal distributions before RYGB, two and five years after as well as in the control group.

Table 5. Findings 5 years after gastric bypass in 30 obese women compared with 30 control women pair-matched for age and BMI

Measure	5 years after RYGB	Control	P-value
Age, years	49 ± 9	48 ± 9	-
BMI, kg/m^2	32 ± 7	32 ± 7	-
Waist-to-hip ratio	0.93 ± 0.07	0.94 ± 0.06	0.76 (0.86)
Waist circumference, cm	104 ± 17	106 ±17	0.25 (0.65)
Hip circumference, cm	111 ± 14	112 ±13	0.23 (0.68)
Body fat, percentage	44 ± 9	45 ± 10	0.19 (0.66)
Android fat, kg	35 ± 18	37 ± 18	0.25 (0.58)
Gynoid fat, kg	7 ± 3	6 ± 2	0.23 (0.62)
EVAT, kg	1.0 ± 0.7	1.5 ± 0.9	0.00006 (0.02)
ESAT, kg	2.6 ± 1.4	2.4 ± 1.0	0.21 (0.62)
S-insulin, mU/l	5.9 ± 2.7	10.6 ± 7.3	0.001 (0.003)
P-glucose, mmol/l	5.1 ± 0.6	5.1 ± 0.4	0.48 (0.49)
HOMA-IR, units	1.4 ± 0.7	2.5 ± 1.7	0.002 (0.003)
P-triglycerides, mmol/l	0.9 ± 0.5	1.2 ± 0.6	0.02 (0.04)
P-HDL cholesterol, mmol/l	1.63 ± 0.51	1.34 ± 0.36	0.02 (0.001)
P-total cholesterol, mmol/l	4.2 ± 0.7	4.8 ± 1.0	0.003 (0.003)
P-glycerol, µmol/l	116 ± 42	108 ± 69	0.59 (0.57)
P-adiponectin, µg/ml	12.9 ± 5.5	9.1 ± 3.1	0.0009 (0.001)
Fat cell volume, pl	492 ± 210	711 ± 243	< 0.0001 (0.0008)
Fat cell number x 10^7	653 ± 518	366 ±122	0.02 (0.06)
Glycerol release, µmol/10^7cells	4.5 ± 1.9	4.7 ± 2.72	0.45 (0.68)
Glycerol release, mmol/total ESAT	2.4 ±2.0	1.9± 0.8	0.08 (0.20)
Physical activity, score	2.3 ±0.5	2.1 ±0.7	0.74 (0.21)

Values are mean ± SD and compared by paired and unpaired (within parenthesis) Students t-test. In some cases, n < 30 due to missing values. BMI, body mass index; ESAT, estimated subcutaneous adipose tissue; EVAT, estimated visceral adipose tissue; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; RYGB, roux-en-Y gastric bypass. Table 2 from study II.

4.3 STUDY III

Levels of CCL18 in WAT incubate increased statistically significant over time (Figure 9).

Serum CCL18 levels where compared between the 40 most insulin sensitive versus 40 most insulin resistant women in a cohort of 220 women with obesity (average HOMA-IR 1.1 and 5.6 in each group respectively). Serum CCL18 levels were significantly higher in the insulin

resistant group (P = 0.0005, Figure 9), in contrast to the serum levels of TNF-α and IL-6 that barely differed significantly.

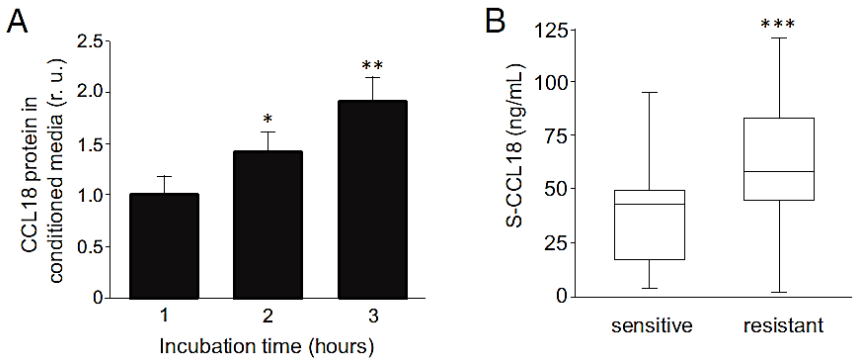


Figure 9. Adipose secreted and circulating CCL18 levels. (A) CCL18 levels from subcutaneous adipose tissue incubate collected at baseline and after 1 and 2 hours. n=7 * = P < 0.05 and ** = P = < 0.001. r.u. = relative units. (B) Serum CCL18 levels in insulin sensitive versus insulin resistant obese subjects. *** = P < 0.001. Figure 1 from study III.

Secretion of CCL18 from subcutaneous WAT was correlated to metabolic parameters in two cohorts. In cohort 1 (n = 29), linear regression showed a significant relationship between CCL18 secretion and BMI (P = 0.0009, r = 0.60), M-value (P < 0.0001, r = -0.75), ATP III risk score (P <0.0001, r = 0.81) and triglycerides in plasma (P < 0.0001, r = 0.70). These correlations remained significant after adjustment for BMI in multiple regression analyses. Independent of BMI, WAT CCL18 secretion correlated with TNF-α and IL-6 secretion (P = 0.0093 and 0.016). The findings in cohort 1 were validated in cohort 2 (n = 56) where similar correlations were observed besides that insulin sensitivity was determined by HOMA-IR instead of hyperinsulinemic euglycemic clamp. Circulating CCL18 correlated with circulating TNF-α (P = 0.018, Rho = 0.28) but not IL-6.

To determine the primary cellular source of CCL18 in subcutaneous WAT, CCL18 mRNA levels were determined in purified fractions of mature adipocytes, macrophages and leukocytes. Results showed that CCL18 was enriched in macrophages (P < 0.001 in comparison to leukocytes and adipocytes). In cohort 2, transcription of CCL18 correlated with subcutaneous WAT secretion (P < 0.0001, r = 0.68). Gene ontology analyses showed that CCL18 expression correlated with expression of genes in the immune system spectrum. When correlating CCL18 expression with expression of genes reported to be enriched in M1 an M2 macrophages respectively, stronger correlations were seen with those in the M2 spectrum.

Determination of mRNA levels in M0, M1 and M2 macrophages derived from the human THP-1 cell line revealed that *CCL18* expression was induced in M2 cells. Recombinant CCL18 induced expression of the proinflammatory genes *CCL2* and *CCL3* in M0 an M2 cells. There were no effects of recombinant CCL18 on neither adipocyte nor preadipocyte lipolysis (assessed as glycerol release), expression of inflammatory genes (*TNF-α* and *IL-6*) or genes related to extracellular matrix formation (*COL1A1*, *CTSB* and *COL6A1*). Among the

genes encoding the previously reported receptors for CCL18, *GPER1* expression was higher than *PITPNM3* in all subtypes, and *CCR8* was not detectable. A similar pattern was observed in vitro-differentiated adipocytes, where *GPER1* expression was higher than in preadipocytes.

4.4 STUDY IV

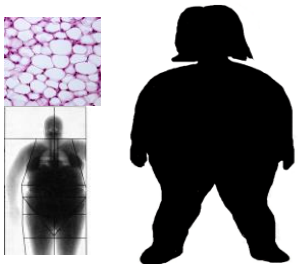
At baseline, 215 women scheduled for RYGB were included. Two years after surgery, 166 patients were followed-up (77.2 %) and included in further analyses. Omentectomy had been performed in 33 patients in addition to RYGB, and these patients were excluded from analyses of changes in fat mass and distribution.

As expected, all clinical and anthropometric parameters improved significantly after weight loss (all $P < 0.0001$). Android-gynoid (AG) and EVAT-total fat mass ratios decreased (both $P < 0.0001$)

Improved HOMA-IR was predicted by baseline AG-ratio ($P = 0.0028$, $r^2 = 0.056$) and WHR ($P = 0.0014$; $r^2 = 0.063$), independent of BMI and age ($P = 0.0044$ and 0.0023 respectively in multiple regression). In multiple regression analyses with BMI and age, AG-ratio and WHR remained significant versus changes in HOMA-IR ($P = 0.0044$ and 0.0023 respectively).

Weight loss (%) was predicted by DXA measured baseline body fat percentage ($P < 0.0001$, $r^2 = 0.090$) and fat percentage in the android region ($P = 0.0055$, $r^2 = 0.046$), and among the basic anthropometric measures by BMI ($P = 0.0022$, $r^2 = 0.056$) and body fat percentage calculated by formula ($P = 0.0083$, $r^2 = 0.042$). DXA measured fat percentage remained a significant predictor in multiple regression with age and BMI ($P = 0.0033$), as did BMI in multiple regression with age ($P = 0.0086$). Excess BMI lost (%) could not be predicted by any DXA measures, but by BMI ($P < 0.0001$, $r^2 = 0.10$), waist circumference ($P = 0.0087$, $r^2 = 0.042$) and fat percentage calculated by formula ($P < 0.0001$, $r^2 = 0.11$). BMI remained a significant predictor of percentage excess BMI lost after adjusting for age in multiple regression ($P < 0.0001$).

Finally, comparisons between the predictive properties of DXA measures versus basic anthropometric measures were made in multiple regressions analyses. In multiple regression with AG-ratio and WHR versus changes in HOMA-IR, none of these predictors remained significant. In a multiple regression analysis of DXA measured fat percentage, fat percentage calculated by formula and BMI versus weight loss (%), only DXA measured fat percentage remained significant ($P = 0.0057$).



Study I
Study IV

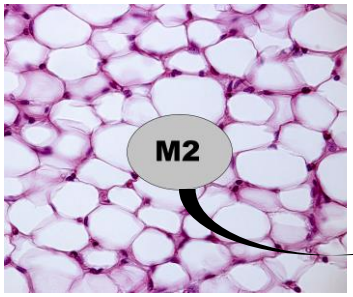
Prediction of outcome after weight loss

Before weight loss

Study II

After weight loss		**Weight stable control**
↑Insulin sensitivity	⟺	Lower insulin sensitivity
Better lipid profile		Worse lipid profile
Better adipokine secretion		Worse adipokine secretion
Smaller adipocytes		Larger adipocytes
Less visceral fat		More visceral fat

Study III



CCL18 ⟺ Metabolic risk factors

Figure 10. Overview summarizing the main findings in this thesis. *Study I* and *IV* revolves around prediction, illustrated by the Clairvoyant ("Fjärrskådaren"). *Study I* identified baseline adipose tissue morphology, i.e. hypertrophy or hyperplasia, as a predictor of improved insulin sensitivity after weight loss through gastric bypass or diet. In *study IV*, baseline body fat distribution predicted improved insulin sensitivity after gastric bypass, and body fat percentage predicted weight loss. In *study II*, gastric bypass patients were compared to a matched control group after weight loss. Results showed that clinical and adipose tissue phenotype was better after gastric bypass than in the matched control group. *Study III* identified CC Chemokine Ligand 18 (CCL18) to be secreted from M2 macrophages in adipose tissue. Adipose secreted and circulating CCL18 correlated with metabolic risk factors in women.

5 DISCUSSION

5.1 STUDY SPECIFIC ASPECTS

Study I showed that adipose tissue morphology can predict improved insulin sensitivity after moderate weight loss following a diet program, and after pronounced weight loss following RYGB. Proper selection of patients for bariatric surgery is of great importance since not all patients with obesity can undergo surgery. The fact that current selection criteria still rely on BMI despite that BMI has a limited ability to predict metabolic outcome[13, 28], illustrates the lack of proper predictors for metabolic outcome. Adipose morphology adds predictive information concerning expected improvement in insulin sensitivity after RYGB, although the predictive value is limited in a clinical context. Combining adipose morphology with other factors may potentially improve prediction of improved insulin sensitivity after weight loss. The importance of weight loss independent mechanisms behind metabolic improvements was highlighted by the finding that WAT morphology predicted improved insulin sensitivity but not weight loss.

Study II compared the WAT and metabolic phenotype of patients that had lost weight through RYGB with an individually BMI-, age- and sex-matched control group. Interestingly, this comparison revealed that after RYGB, patients had reached a "supra-normal" state with better insulin sensitivity, lipid profile, higher adiponectin levels and less visceral fat than the controls. Adipose secreted TNF-α was lower than in controls and adiponectin higher. Our findings suggest that adipose factors including adiponectin levels, adipose hyperplasia and body fat distribution may be involved in the metabolic improvement beyond the control state after RYGB. Adipose inflammation may be another factor involved in this improvement. While adipose release of MCP-1 and IL-6 was unaltered in comparison to controls, TNF-α levels where lower. As previously discussed, TNF-α induces insulin resistance in adipose tissue, and this decrease after weight loss is likely to be protective. The pattern of IL-6 and MCP-1 release may on the other hand potentially reflect a less dynamic and less reversible response in expanded fat mass or be less associated with adipocyte size. Furthermore, the study confirms the previously demonstrated metabolic improvement after weight loss following RYGB, including increased circulating adiponectin, reduced basal lipolysis and relative hyperplasia. Between two and five years, weight regain was observed and insulin sensitivity decreased slightly and fat cell number but not size increased. The increased fat cell number between two and five years after weight loss indicate that adipogenesis has been triggered after weight regain, a phenomenon described previously[151]. However, we did not find any pool of small fat cells (i.e. bimodal distribution of adipocytes), as have been reported by some groups previously[152]. This may be interpreted in the way that newly formed adipocytes increase their size relatively fast and that this pool is not identified by size after long-term weight regain. These discrepancies may also be attributed to different approaches to determine adipocyte size. Although patients were examined after relatively long term, the finding of weight regain may indicate that the hyperplastic WAT after weight loss represents a "hungry" adipose tissue prone to regain its mass. After weight loss, lipolysis[153] and WAT

leptin release[103] has been shown to decrease below the control state which may promote weight regain.

Study III demonstrated that CCL18 is secreted from subcutaneous WAT, primarily from macrophages in the M2 spectrum, and affects macrophage expression of inflammatory factors. However, no effects could be demonstrated on adipocyte expression of classical inflammatory or extracellular matrix genes in vitro. WAT release of CCL18 and circulating levels correlated with insulin sensitivity and metabolic disturbances better than the established proinflammatory cytokines TNF-α and IL-6. Subcutaneous WAT *CCL18* expression has previously been reported to be upregulated in obesity[154, 155] and diabetes[156], the latter being in line with our findings on the correlation between CCL18 expression and secretion from WAT and between WAT release of CCL18 and insulin sensitivity. Our findings indicate that CCL18 may be a novel circulating marker of insulin resistance and WAT inflammation, better than the established markers IL-6 and TNF-α. The primary source of CCL18 is M2 macrophages, but WAT release of CCL18 was highly correlated to classic proinflammatory cytokines as well. This questions the traditional division of macrophages into M1 and M2 phenotype, and rather supports more lately notions of intermediate macrophage states[157], including metabolically activated macrophages in obesity[111]. CCL18 is known to be involved in development of pulmonary fibrosis[158], and we hypothesized that potential metabolic effects in adipose tissue may be mediated by development of fibrosis, i.e. excess extracellular matrix formation from preadipocytes. However, no such effects could be demonstrated in vitro although different responses may still potentially be induced in vivo. The signaling pathway for CCL18 is not fully elucidated, and our results concerning expression of the previously described receptors did not provide any clear pattern supporting any of these as the likeliest receptor although *GPER1* was most highly expressed.

Study IV identified body fat percentage as a predictor of weight loss after RYGB and high android/gynoid fat accumulation as a predictor of improved insulin sensitivity after weight loss. When comparing the predictive value of DXA measures with basic anthropometric assessments, body fat percentage measured by DXA performed slightly better than estimated by formula or BMI as predictor of weight loss. For prediction of improved insulin sensitivity, no significant difference between DXA measured AG-ratio and WHR was observed, indicating similar predictive properties of these measures. Predictive values were independent of BMI and age. These findings suggest a limited value of DXA examination in addition to anthropometric measures to predict metabolic outcome after RYGB. EVAT/total fat mass ratio did not predict improved insulin sensitivity after adjustment for multiple testing, although this might have been expected given the negative metabolic impact attributed to visceral adiposity. This may reflect that, in contrast to AG-ratio, EVAT/total fat mass ratio does not take the protective gynoid fat mass[68] into account to the same extent. Finally, favorable redistribution of body fat after RYGB was observed. These results are in line with previous findings[141, 159, 160], and may potentially involve a higher lipid turn-over rate in visceral adipose tissue[161].

5.2 GENERAL ASPECTS

The findings in this thesis highlight the BMI-independent correlation between intrinsic factors in adipose tissue and metabolic disturbance before and after weight loss, and put these findings in a clinical context.

As demonstrated in study II, loss of fat mass after weight loss is achieved by decreased adipocyte size but not number. Adipocyte size is highly correlated to insulin resistance and predicts improved insulin sensitivity after weight loss as seen in study I. We have previously shown that reduction in subcutaneous adipocyte size after weight loss correlates with improved insulin sensitivity[162]. It may be discussed whether there is a causal relationship between adipocyte size and insulin sensitivity, or if they are parallel phenomena with a common underlying cause. However, several observations suggest a causal link. According to the adipose tissue expandability hypothesis[163], metabolic disturbances in obesity appears as a consequence when adipose tissue reaches a state where it can no longer harbor excess fat. Lipids are then instead stored intraabdominal and in muscle, liver, pancreas and other ectopic foci where lipotoxicity induces metabolic disturbances. Decreased ability to recruit new adipocytes when expanding fat mass will thus result in hypertrophic adipose tissue associated with disturbed metabolism. Our observation in study I, that subjects with hypertrophy have higher waist circumference and WHR despite similar BMI, may be considered to be in line with this hypothesis; approaching the subcutaneous expansion limit early will promote visceral and other ectopic fat accumulation. Further notions suggesting a causal link include the observation that treatment with thiazolidinediones induce WAT hyperplasia and improves insulin sensitivity[164]. In mice, inducing hyperplasia chemically has been reported to improve insulin sensitivity[165]. An alternative or additional explanation may be that intrinsic adipocyte factors are of importance for the development of insulin resistance. Large adipocytes are reported to have higher lipolysis rate[84, 92], and more proinflammatory adipokines such as MCP-1 and IL-6 are released from larger adipocytes[166]. These factors contribute to insulin resistance.

What may explain the identified predictive properties of adipose morphology and body fat distribution? One potential explanation of the findings could be that most WAT changes in the group studied herein are reversible after weight loss. If deteriorated WAT phenotype is a major contributor to metabolic disturbance and normalization (or supra-normalization) of WAT phenotype occurs after weight loss, then normalization of metabolic phenotype would be expected after weight loss. Inversely, if WAT phenotype in the obese state is “benign” and metabolic disturbance still occurs this may indicate other causes outside of WAT, potentially less reversible after weight loss. As discussed in the background, preserved beta cell function seems central to enable reversal of disturbed glucose metabolism after weight loss. The high degree of normalization of insulin sensitivity after weight loss in our cohorts may potentially be attributed to preserved beta cell function (only a minority had T2D, and patients with insulin treatment were excluded), although this was not studied specifically herein. With a high degree of reversibility after weight loss, worse metabolic status before weight loss will be associated with larger improvement after weight loss. Thus, factors closely related to

metabolic disturbances will likely be predictors of improved metabolic outcome after weight loss in this group which may also explain our findings. However, the results herein shows that several factors considered to associate with metabolic disturbance (including BMI) do not display these properties, whereas intrinsic adipose factors including fat cell size in fact do, and thus seem more associated with insulin sensitivity and the potential to reverse insulin resistance after weight loss.

When comparing our results in study I with other studies we found some conflicting results concerning adipocyte size and metabolic improvement. Another study[46] showed a decreased resolution of T2D with increasing fat cell size, whereas we observed a higher degree of improved insulin sensitivity with increasing fat cell size. If a cohort of patients with diabetes is selected; adipocyte hypertrophy may reflect a more advanced disease stage and a higher probability of irreversible beta cell failure, and thus decreased chance of T2D remission. In our cohorts, including primarily patients with insulin resistance but not T2D, the reversibility of insulin resistance is still high. Displaying the phenotype associated with insulin resistance in this cohort will thus be associated with the highest potential for improved insulin sensitivity. Analogously, this "switch" from positive to negative predictors when studying subjects with insulin resistance but without T2D in comparison to patients with T2D may also be applicable for the findings in study IV, since a negative relation between visceral obesity and diabetes remission has been reported[167]. Differences between patients with and without T2D have been reported previously in the association between adipocyte size and insulin sensitivity[102]. To summarize, the seemingly conflicting findings described above may not be conflicting but only reflecting different relations between predictors and improved insulin sensitivity or diabetes status depending on whether the patients display impaired insulin sensitivity alone or have progressed into T2D. Our studies highlight this phenomenon and add information on predictors for improved insulin sensitivity after weight loss in patients without T2D.

Many previous studies on improvements in glucose metabolism after bariatric surgery have focused on T2D remission. In the ongoing debate concerning indications for bariatric surgery[30, 168], focus is shifting from weight loss to the positive metabolic effects of surgery. Since the degree of T2D remission is reduced in more advanced T2D stages[43, 44] it may be argued that interventions should be initiated earlier to reverse metabolic disturbances with sustained efficacy. Patients like the majority included in this study; with obesity and insulin resistance but without established T2D, may thus be considered particularly suitable for bariatric surgery in terms of reversibility of metabolic disturbances and prevention of future comorbidity. Previous studies on the elevated T2D incidence in patients with hypertrophic adipose tissue[81, 82] highlight the elevated risk of metabolic disease in this specific group, which may strengthen the indication for preventive interventions.

In a clinical context, the value of the predictors identified in *study I* and *IV* is limited although they may give an indication on average expected weight loss or improvement of insulin sensitivity despite large inter-individual differences. The reason for the weak correlations

between the predictors and outcome probably reflect the fact that the mechanisms behind improved insulin sensitivity and weight loss after RYGB (and diet interventions) are highly complex, involving many factors with a large inter-individual variation including eating behavior, physical activity, genetic predisposition, hormonal factors and nicotine use etcetera. To identify one factor summarizing all these aspects would probably be difficult and weak associations are likely to be expected for many single predictors. Using a combination of independent predictors could potentially improve the predictive precision. Preoperative BMI is included in many predictive models of T2D remission after bariatric surgery. We did not find BMI to predict improved insulin sensitivity, but WHR did. Could replacing BMI with WHR in these models sharpen the predictive accuracy?

5.3 STRENGTHS AND LIMITATIONS

One major general strength of the cohorts in this thesis include the extensive translational characterization of the human study participants; ranging from clinical parameters through anthropometric measures to studies of adipose tissue structure and function, and adipocyte gene expression and protein secretion. Another strength is the longitudinal approach with a long follow-up time after weight loss, in the weight stable phase after two years and after some weight regain.

The detailed characterization is expensive as well as time and labor consuming which limits the number of patients included in the cohorts, although still relatively large when taking the extensive characterization into account. The number of patients does however limit the ability to perform subgroup analyses and extensively correct for possible confounding with sustained precision and power. Potential confounders that have not been taken into account may include physical activity, diet and nicotine use. Physical activity affects insulin sensitivity and has been reported to induce a more pronounced loss of visceral mass than hypocaloric diets[19]. Altered food preferences have been reported after RYGB[169], which may potentially influence metabolic status independent of weight loss. As reviewed[170], nicotine has been linked to visceral fat accumulation, increased adipocyte differentiation, increased lipolysis and weight loss. Thus, these factors may potentially influence some of the exposures and outcomes in the studies herein and thereby act as confounders. Although self-reported physical activity did not differ significantly between groups in study II, self-reported physical activity may suffer from limited validity.

Many organs besides WAT, including skeletal muscle, liver and pancreas as described, contribute to the development of metabolic disturbances in obesity and the subsequent reversal after weight loss. The relative contribution of adipose factors to whole-body effects is hard to quantify and some effects observed may admittedly not primarily be associated with WAT factors. Furthermore, the primary model for weight loss in our studies is RYGB. RYGB induces other alterations than weight loss that may affects metabolism[39], including hormonal changes. For example, the early diabetes remission before substantial weight loss has to a large extent been attributed to incretin effects[40-42]. Thus, other mechanisms independent of weight loss or WAT phenotype may contribute the positive effects on

metabolism observed, including the supranormality after RYGB in study II. These factors are not studied in this thesis.

Some patients were lost to follow-up in the longitudinal studies, which may potentially have influenced the results by introducing selection bias. For example, 22.8 % were lost to follow-up in study IV. Patients that are lost to follow-up may differ in their outcome from those followed up. It might be that those with worse outcome are less prone to reappear at the follow-up examinations. However, the only baseline characteristics differing between those followed-up and not in study IV were slightly higher weight. Another source of selection bias may be at the point of inclusion, were some patients may be considered less suitable to participate for some reason not reflected by the exclusion criteria.

The generalizability of our findings deserves discussion. The studies herein are all based on abdominal subcutaneous WAT and no other subcutaneous or visceral depots. Depot-specific differences in adipocyte size[171] and lipolysis[75] are known to exist, and the findings are probably not directly generalizable from one depot to another. In the case of *CCL18*, the expression in patients with insulin resistance is reported to be doubled in visceral WAT[172]. Furthermore, the study participants in our studies are primarily women, reflecting a consistent difficulty in recruiting enough men in this kind of studies since the majority of patients that undergo bariatric surgery are women[31]. The generalizability of the findings to men is not known. Body fat distribution differs between men and women, and change into a more android pattern after menopause in women accompanied by increased risk of metabolic disturbance[69, 173]. Menopause constitutes another factor that has not been taken into account which may have influenced the results. Another factor that has not been considered and that may limit the generalizability is ethnicity. The majority of patients included are of Swedish origin and ethnic specific differences in for example body fat distribution are known to exist[174]. Moreover, the great majority of the included patients do not have T2D and none are on insulin therapy. Correlations found herein may look different in patients with T2D as discussed above.

Strengths in study I and III include that the findings are validated in several cohorts. Our findings concerning supra-normality metabolic and WAT findings after RYGB have been validated in an additional study including a matched control group. In this study, patients were matched two years after RYGB and insulin sensitivity was also measured by clamp[175]. This study confirmed higher HOMA-IR and more hyperplastic adipose tissue in the post–obese state, although clamp values did not differ significantly between groups. A limitation of the findings in study IV is the lack of a validation cohort.

When interpreting the results in the studies herein, it should be taken into account that the results are sometimes secondary analyses of data with a different pre-specified primary outcome (for example metabolic effects of omentectomy and effects on adipose tissue of different diets in study I).

5.4 ETHICAL ASPECTS

All four studies in this thesis include human participants, and the ethical considerations are of greatest importance. All studies were performed in agreement with the principles of the Declaration of Helsinki and approved by the regional ethics committee in Stockholm. Informed written consent was received from all participants before examinations, and patients were free to withdraw their participation without further explanation throughout the examinations.

The examinations include subcutaneous adipose tissue biopsies. Although performed under local anesthesia, minor discomfort or pain may be experienced. A hematoma is expected after the biopsy, and sometimes a minor scar remains. It may be argued that this would interfere with the "do no harm" principle, but could be considered acceptable weighing in the benefits of the study including increased knowledge of the mechanisms behind the metabolic syndrome. All examinations are voluntary to the participants and no serious complications have been experienced during the more than 2000 biopsies performed at our research center to date. Besides, the principle of autonomy is always respected since patients are free to discontinue their participation whenever they wish.

Exposing healthy volunteers to radiation may be ethically complicated. However, the DXA examinations in our projects only confer a minor dose of radiation, equal to the background radiation of one day in the Stockholm area which is considered harmless within healthcare. A special permit from the Authority for Radiation Protection was achieved before the DXA examinations took place.

Analyzing blood samples and performing examinations concerning health status infers a risk or opportunity that previous unknown disease or risk factors for future disease are discovered, such as diabetes or hypertension. Patients are informed in advance that participation includes extensive health controls. All results are analyzed by medical doctors, and when necessary patients are referred to suitable health care instance to ensure adequate follow-up.

Handling of personal data and tissue samples demand ethical considerations not to interfere with the integrity of the patients. In our study, data from the examinations are coded and the key kept locked in. Only decoded material was then analyzed. Blood and tissue samples are also decoded in the handling and cannot be associated with a specific participant. Patients have given their written consent to the data handling.

Taken together, the ethical aspects discussed above are considered to be handled in a reasonable way in our studies and the risk-benefit balance acceptable.

6 CONCLUSIONS

Study I. Adipose tissue morphology predicts improved insulin sensitivity following moderate or pronounced weight loss, although the clinical implication may be limited. In patients without T2DM, adipose tissue hypertrophy correlates with larger improvement in insulin sensitivity after weight loss.

Study II. Weight loss through RYGB improves several metabolic and adipose tissue characteristics beyond the control state in women, despite weight regain. These include improved insulin sensitivity, better lipid profile, higher circulating and adipose secreted adiponectin levels, more and smaller fat cells (i.e. hyperplasia) and less visceral adipose tissue.

Study III. CCL18 is released from adipose tissue in a time-dependent manner. The primary source of CCL18 in adipose tissue is macrophages in the M2 spectrum. Adipose secreted and serum levels of CCL18 correlate positively with metabolic parameters in women. In vitro, recombinant CCL18 does not affect adipocyte expression of inflammatory proteins or extracellular matrix components, but increases expression of inflammatory proteins in macrophages. CCL18 may constitute a novel marker of metabolic disturbance and WAT inflammation in obesity.

Study IV. Independent of BMI and age, DXA measured AG-ratio and WHR predict improved insulin sensitivity after RYGB and BMI and body fat percentage measured by DXA or estimated by formula predicts weight loss after RYGB. However, DXA measurement of body fat mass and distribution has a limited clinical value for prediction of metabolic outcome or weight loss after RYGB in women since the precision is low and DXA do not perform much better than basic anthropometric measures. Body fat distribution is altered in a metabolically favorable manner after weight loss.

7 FUTURE PERSPECTIVES

Several questions concerning prediction of metabolic outcome after RYGB remains. What are the best predictors of outcome? Can the predictors identified herein improve existing predictive models or be useful in models including other factors as well? Can long-term (5 or 10 years or more) metabolic outcome be predicted by adipose parameters at baseline or two years after surgery?

There is limited knowledge about BMI-independent predictors of long-term mortality after RYGB. Can long-term incident cardiovascular disease or mortality after RYGB be predicted by WAT characteristics?

Further validation of the findings herein would be interesting in other populations including men, in particular in study IV where a validation cohort is lacking.

BMI is still widely used in the follow-up of patients after bariatric surgery, despite its limitations as a tool for cardiovascular risk stratification. What characterize patients with a BMI within the non-obese range but with a high body fat percentage after RYGB?

In study II, it was shown that patients displayed a more hyperplastic adipose tissue after RYGB. What are the long-term consequences of these changes? Is it really protective or does it leave patients with a "hungry" adipose tissue prone to weight regain? The longitudinal follow-up of the cohorts including RYGB patients continues, enabling future long-term follow-up. Does T2D incidence differ between the weight loss group and the matched controls?

Patients usually regain weight after initial weight loss after RYGB. What characterizes patients with a high degree of weight regain? Can they be identified early or do their adipose tissue display certain characteristics? Does weight regain and spontaneous weight gain follow the same pattern in adipose tissue?

Can CCL18 predict metabolic outcome after weight loss? Validation of the correlation between CCL18 and insulin resistance in other cohorts would be of interest.

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