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LUND
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DOCTORAL DISSERTATION

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Abstract

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This thesis aims to broaden the understanding of adipose tissue function, focusing on the importance of adipocyte cell-size and differences between visceral and subcutaneous adipocytes. This knowledge may in the future contribute to novel treatments that focus on improving function of specific fat depots.

Our first project (**Paper I**) examines the importance of EHD2, a caveolae-associated protein, and its influence on different fat depots by using knockout (*Ehd2*$^{-/-}$) mice. *Ehd2*$^{-/-}$ mice display impaired expansion of epididymal fat, most likely caused by weakened hyperplastic growth, while the inguinal adipocytes exhibit impaired lipolytic response. The results indicate that EHD2 is required for healthy fat expansion since both impaired lipolysis and reduced hyperplastic growth are associated with dysfunctional adipose tissue.

In **Paper II**, we examine the effect of the anti-diabetic drug, rosiglitazone, on hypertrophic adipocytes, and suggest a new mechanism of action for improving insulin-sensitivity in adipocytes. We propose that remodeling of the extracellular matrix (ECM), results in reduced stress-induced intracellular signaling, reduced filamentous (F)-actin and improved insulin-stimulated glucose uptake. The improvement in glucose uptake from rosiglitazone-treated mice are greater in epididymal adipocytes compared to inguinal, which is also reflected in larger changes in ECM expression, intracellular stress signaling and level of F-actin.

The last project (**Paper III**) examines the plasticity of adipose tissue, focusing on cell-size and actin dynamics during weight gain and weight loss. We examine differences between the inguinal and epididymal depots using temporal resolution and correlate the findings to changes in systemic insulin sensitivity. Our data suggest that there are fat depot-specific differences in cell-size plasticity during weight gain, but not during weight loss. Epididymal adipocytes expand in size early during weight gain (after 4 weeks) while inguinal adipocytes expand at a later timepoint (after 12 weeks). During weight loss, both fat depots display intact plasticity, regarding cell-size, weight, and level of F-actin, for short interventions (4 and 8 weeks) but not following prolonged overfeeding (12 weeks).The inguinal depot, in comparison to the epididymal depot, displays stronger correlation between insulin sensitivity and number of hypertrophic adipocytes, suggesting that hypertrophic inguinal adipocytes may contribute to the progression of insulin resistance in mice.

Key words Adipose tissue, adipocytes, fat depots, insulin-sensitivity, lipolysis, EHD2, rosiglitazone, Filamentous actin, adipose tissue plasticity

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Abbreviations

AC	Adenylyl cyclase
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
APC	Adipocyte progenitor cells
ARP2	Actin related protein 2
ARP3	Actin related protein 3
AS160	Akt substrate of 160 kDa
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
COLVI	Collagen VI
DcAMP	Dibutyryl cAMP
DIO	Diet-induced obesity
ECM	Extracellular matrix
EHD2	Eps15-homology domain containing protein 2
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FA	Fatty acids
F-actin	Filamentous actin
G-actin	Globular actin

GLUT4	Glucose transporter type 4
GSV	GLUT4 storage vesicles
HFD	High-fat diet
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
HSP90	Heat shock protein 90
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinases
mRNA	Messenger ribonucleic acid
NNT	Nicotinamide nucleotide transhydrogenase
NO	Nitric oxide
PDE	Phosphodiesterase
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKA	Protein kinase A
PLIN1	Perilipin-1
PPARγ	Peroxisome proliferator- activated receptor gamma
PTEN	Phosphatase and tensin homolog
RhoA	Ras homolog family member A
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
TG	Triglycerides
T2D	Type 2 diabetes
UCP-1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wild type

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Background

Overweight, obesity, insulin resistance and type 2 diabetes

Overweight (body mass index (BMI) ≥ 25) and obesity (BMI ≥ 30) are an increasing public health concern in most industrialized and developing countries. Obesity is a risk factor for severe diseases such as stroke, heart disease, some cancers and type 2 diabetes (T2D). The number of obese people has almost tripled since 1975 and nearly 60% of the adult population is projected to be overweight or obese by 2030 [1, 2]; public healthcare is facing an enormous challenge in treating obesity-related diseases. T2D is characterized by hyperglycemia that is caused by peripheral insulin resistance and an insufficient insulin production to maintain normoglycemia. The pathogenesis of T2D is initiated by peripheral insulin resistance and mild hyperinsulinemia, which progresses to impaired glucose tolerance characterized by hyperinsulinemia and elevated fasting blood glucose. Eventually, this leads to impaired β-cell function and insufficient insulin levels, hyperglycemia, and onset of T2D [3]. Insulin resistance is difficult to diagnose since affected individuals have normoglycemia and would require measurements of insulin levels to detect hyperinsulinemia, something that is rarely conducted clinically.

Adipose Tissue

The adipose tissue (AT) serves as the main energy reservoir, in which excess energy in the form of triglycerides (TG) is stored in adipocytes. TG yield more energy per gram compared to carbohydrates and due to their hydrophobic property form lipid droplets, that, unlike glycogen, are stored in the absence of water and are therefore more energy dense. During periods of low systemic energy, the AT provides energy to other organs by hydrolyzing TG into glycerol and free fatty acids (FA), which are released into the circulation and transported to target tissue for oxidation. During caloric surplus the AT stores energy by taking up lipids and glucose from the circulation that are incorporated into TG.

Adipose tissue dysfunction and insulin resistance

AT, skeletal muscles, and liver are the insulin-sensitive peripheral organs that are most affected by insulin resistance [4]. The function of the AT is important for maintaining systemic glucose tolerance. There are several proposed mechanisms for how the function of AT is linked to systemic glucose tolerance. First, it is important to highlight that postprandial glucose disposal is relatively minor in the AT, only accounting for 1-13% of all glucose disposal, in comparison to skeletal muscles (85%) [5-7]. However, preserved insulin-stimulated glucose uptake in adipocytes is required for systemic glucose tolerance, something that has been demonstrated in mice with selective reduction of glucose transporter type 4 (GLUT4) in adipocytes [8]. The same study showed that glucose uptake in skeletal muscle was significantly impaired *in vivo*, but not *ex vivo*, suggesting that dysfunctional adipocytes, influence function of other peripheral tissues, possibly through adipokines [8]. Selective overexpression of GLUT4 in adipocytes, in mice that lack GLUT4 in skeletal muscles, completely restores glucose tolerance, further emphasizing the importance of glucose transport in adipocytes for maintaining systemic insulin sensitivity [9]. Improving the insulin signaling transduction in adipocytes improves glucose tolerance; adipocyte-specific knockout of protein phosphatase and tensin homologue (PTEN) has been shown to improve insulin signaling in adipocytes and systemic insulin sensitivity in mice [10].

AT fibrosis is linked to AT inflammation and systemic insulin resistance. Suppressing pro-fibrotic gene-expression in mice improves glucose tolerance [11] which is also seen in specific collagen knockout mice [12]. AT inflammation and macrophage infiltration are suggested to contribute to systemic insulin resistance [13]. However, it is important to emphasize that a proinflammatory response is required for healthy AT expansion. Acute inflammation stimulates adipogenesis and mice with impaired proinflammatory response display reduced hyperplastic growth in response to high-fat diet (HFD)-feeding [14]. The proinflammatory response is likely necessary for proper extracellular matrix (ECM) remodeling and angiogenesis, which is required for adipogenesis. Impaired angiogenesis and consequent hypoxia are hallmarks of dysfunctional AT and adipocyte insulin resistance [15]. The sequential development of dysfunctional AT is not fully understood but fibrosis, inflammation, and impaired angiogenesis all contribute, reinforcing one another [16].

The AT expands by increasing volume of existing adipocytes, known as hypertrophy, and/or number of adipocytes, known as hyperplasia. Hypertrophic growth, in particular, has been associated with insulin resistance, while increased hyperplastic growth prevents insulin resistance [17, 18]. This has been demonstrated in the clinic, using thiazolidinediones, a class of drugs that act as agonists to peroxisome proliferator-activated receptor gamma (PPARγ), the master regulator of

adipogenesis [19]. Thiazolidinediones promote hyperplastic growth and improve systemic insulin sensitivity [20].

Hypertrophic adipocytes are less responsive to insulin compared to smaller adipocytes [21]. Furthermore, they display increased basal lipolysis leading to increased release of FA into the circulation [22]. This in combination with impaired expansion of the AT, leads to storage of lipids in non-adipose tissue, such as skeletal muscles and liver, referred to as ectopic fat. Accumulation of intramyocellular TG has been shown to negatively influence systemic insulin sensitivity [23].

Notably, not all peripheral tissues and signaling pathways are affected in the same way during the progression of insulin resistance. In the liver, gluconeogenesis, but not *de novo* lipogenesis, is affected by insulin resistance [24]. Adipocytes maintain their anti-lipolytic response to insulin while insulin-stimulated glucose uptake is impaired [25]. This is even observed in different fat depots where the anti-lipolytic effect of insulin in response to HFD-feeding differs between perigonadal and inguinal adipocytes [26].

Hormonal regulation of energy storage and release from adipocytes

Catecholamines and insulin tightly regulate the incorporation and release of TG in adipocytes.

Lipolysis

There is a constant cycling of TG/FA i.e., hydrolysis and re-esterification of TG in adipocytes [27]. This cycle is regulated by catecholamines and insulin, which promote liberation (lipolysis) and storage of energy, respectively (see **Figure 1**). Lipolysis is stimulated by catecholamine, such as norepinephrine, binding to beta adrenergic receptors, leading to activation of adenylyl cyclase (AC), and synthesis of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP binds and activates protein kinase A (PKA) which facilitates the phosphorylation of perilipin-1 (PLIN1), hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which all are required for maximal lipolytic response [28, 29]. Upon phosphorylation, HSL translocate from the cytosol to the lipid droplet and together with ATGL and monoacylglycerol lipase (MAGL) hydrolyze TG into FAs and glycerol [30]. Insulin promotes storage of TG by promoting glucose uptake and lipogenesis. It also inhibits lipolysis through Akt (protein kinase B)-mediated activation of phosphodiesterase (PDE) 3, which reduces the pool of cAMP and thus the activity of PKA [31]. During fasting, both glucose and insulin levels drop, and lipolysis is stimulated in the absence of the anti-lipolytic effect of insulin. FA are released into the circulation and serve as an energy source for other organs. Lipolysis is dysregulated in adipocytes from obese humans; non-stimulated (basal) lipolysis is increased while beta adrenergic-stimulated

lipolysis is reduced [32]. The re-esterification of FA's is impaired in T2D patients, leading to a 50% increase in release of FA for each TG that is hydrolyzed [33]. Furthermore, obese individuals also have reduced numbers of beta-adrenergic receptors. However, this alone cannot account for the impaired response to lipolytic agonists since lipolysis is also impaired when dibyturyl cAMP bypasses the adrenergic receptors and directly activates downstream targets [34].

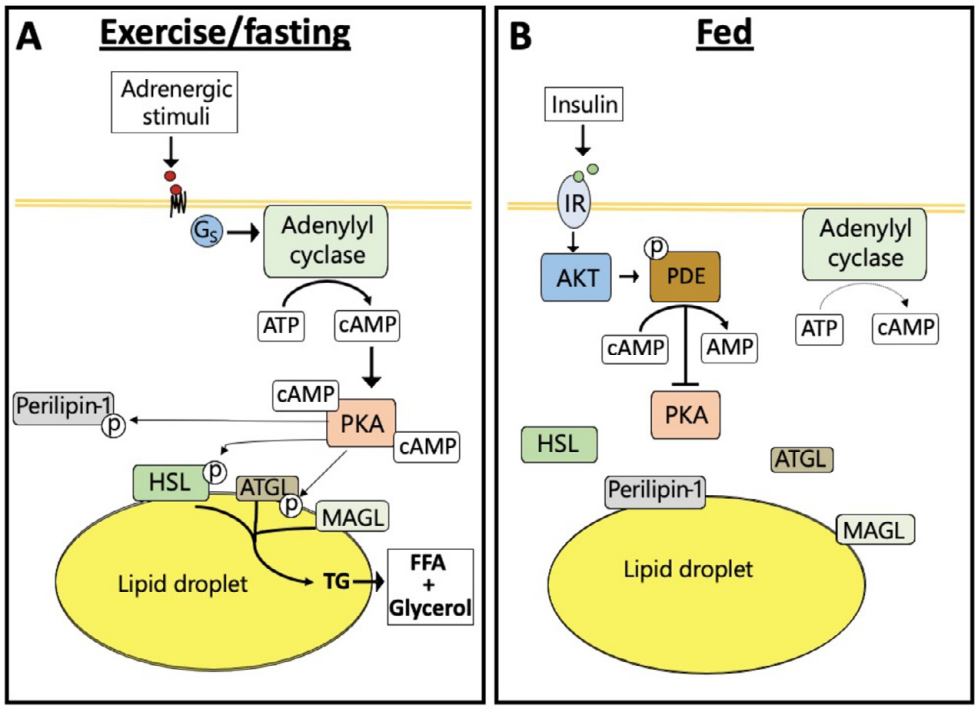

Figure 1. Hormonal regulation of lipolysis in adipocytes.
A) Liberation of energy from adipocytes during exercise and fasting. Catecholamines (adrenergic agonists) bind to the G protein-coupled adrenergic receptor; the G_s-protein activates adenylyl cyclase. Adenylyl cyclase converts ATP to cAMP which leads to an increase in intracellular cAMP. cAMP binds to and activates PKA, which facilitates phosphorylation and activation of perilipin-1, HSL and ATGL, which in turn catalyse the hydrolysis of TG into glycerol and FFA. **B)** The anti-lipolytic effect of insulin. In the fed state, circulating insulin levels increase and insulin binds to the insulin receptors on adipocytes. This initiates a signalling cascade which leads to phosphorylation and activation of phosphodiesterase (PDE). Activated PDE converts cAMP into AMP, thereby decreasing the intracellular level of cAMP which reduces the activity of PKA and its downstream targets, resulting in decreased lipolytic rate.

Glucose uptake

Insulin regulates glucose uptake in peripheral tissues, including adipocytes and skeletal muscles, by initiating a signal cascade that promotes the translocation and fusion of GLUT4 storage vesicles (GSV) to the plasma membrane (see **Figure 2**) [35]. Insulin stimulation increases glucose uptake in a dose dependent manner in adipocytes [36]. The signaling cascade is initiated when insulin binds to the insulin receptor (IR) at the cell surface, causing phosphorylation of the receptor and

recruitment, phosphorylation, and activation of insulin receptor substrate 1 (IRS-1). IRS-1 then binds and activates phosphatidylinositol 3-kinase (PI3K) which phosphorylates the membrane bound phosphatidylinositol-4, 5-bisphosphate (PIP2) into the second messenger phosphatidylinositol-3, 4, 5-triphosphate (PIP3). PIP3 recruits Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK1). Akt phosphorylates Akt substrate of 160 kDa (AS160) removing the inhibitory effect of AS160 on Rab proteins which promotes fusion of GSV with the plasma membrane [37]. In addition to the described signaling cascade required for GLUT4 translocation there are other factors that seem to be important for GLUT4 translocation and insulin induced glucose uptake in adipocytes. A dynamic turnover of the actin cytoskeleton (polymerization and depolymerization) has been suggested to be required for GLUT4 translocation and glucose uptake [38-41].

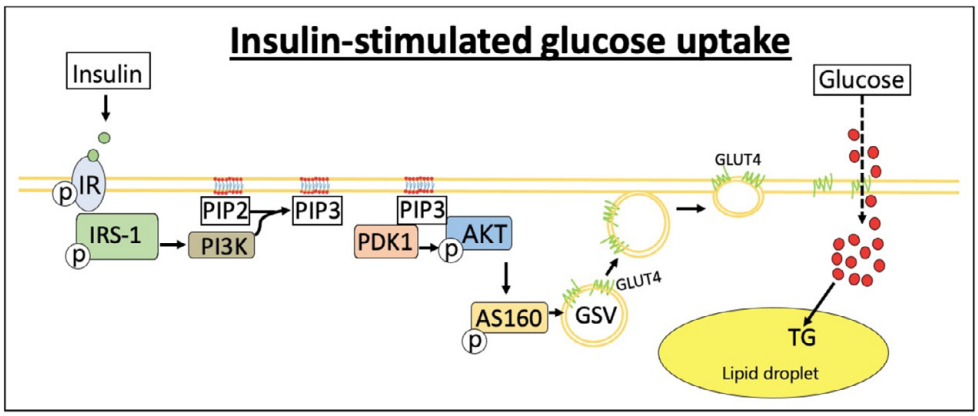

Figure 2. Insulin-stimulated glucose uptake in adipocytes.
Postprandial glucose uptake in adipocytes starts with an increase in blood insulin, secreted from the pancreas. Insulin binds to the insulin receptor (IR) and initiates a signalling cascade. IR recruits and phosphorylates IRS-1 which binds and activates PI3K. PI3K phosphorylates PIP2 into PIP3. PIP3 recruits Akt to the plasma membrane where it is phosphorylated and activated by PDK1. Akt phosphorylates AS160 which releases the inhibitory effect of AS160 on Rab proteins, located on GLUT4 storage vesicles (GSV). This leads to GSV translocation and fusion with the plasma membrane. At the plasma membrane, GLUT4 facilitates glucose uptake in adipocytes.

Adipose tissue composition, progenitors, and different types of fat

Adipocytes make up less than 50% of the total number of cells of the AT but due to their large size occupy 95% of the AT volume [42]. Apart from mature adipocytes, the AT constitutes endothelial cells, fibroblasts, adipocyte progenitor cells (APC) and immune cells. The cells are surrounded by ECM which undergoes remodeling during AT expansion and shrinkage. Both cell and ECM composition are altered in obesity and insulin resistance [43].

Adipocytes are renewed throughout life, with an estimated turnover of 10% annually [44]. However, the total number of adipocytes is set in childhood and does

not change in adulthood independent of weight gain or loss [44]. Adipocytes arise from APC, residing in the stroma vascular fraction of AT. The APC arise from either tissue resident mesenchymal stem cells or from bone marrow-derived myeloid cells recruited from the circulation. Bone marrow derived adipocytes are increased in obesity and more frequently found in the visceral AT, which displays a more pro-inflammatory phenotype [45].

There are different kinds of fat tissue that are characterized by morphology, origin, and function, commonly divided into three types: white adipose tissue (WAT), brown adipose tissue (BAT) and beige fat [46]. WAT is the predominant type of fat tissue and is characterized by adipocytes having a large unilocular lipid droplet, low number of mitochondria and a large cell size. WAT stores and supplies other organs with energy, serves as insulation and is an important endocrine organ secreting adipokines and cytokines [47]. BAT is characterized by adipocytes with small multilocular lipid droplets, a high number of mitochondria and smaller cell-size. Moreover, BAT expresses uncoupling protein 1 (UCP-1) which is a protein of the inner membrane of mitochondria that dissipates chemical energy and produces heat instead of ATP [48]. Therefore, BAT can use fat as a substrate to produce non-shivering thermogenesis instead of energy in the form of ATP [49]. Beige adipocytes are cells with a phenotype like white adipocytes, but upon adrenergic stimulation or cold exposure goes through "browning" and adapt a brown phenotype with high expression of UCP-1, thereby switching from energy storage to energy consumption [50, 51]. The work of this thesis focuses on WAT.

Visceral and subcutaneous WAT

WAT is further divided into visceral and subcutaneous fat which are defined by their anatomical location. Visceral fat is found within the abdominal cavity, surrounding organs, and is further subdivided into the following fat depots: omental, mesenteric, retroperitoneal, gonadal, and epicardial in humans [52]. Mice have the same fat depots and additionally perigonadal fat, which in males is called epididymal and in female periovarian. Perigonadal fat does not exist in humans and has therefore been termed peri-visceral fat. Despite this, it is the most studied fat depot in mice due to its accessibility and large size, allowing multiple cellular analyses [52]. The perigonadal fat drains systemically, in contrast to human visceral fat that drains into the portal vein, and thereby influences liver metabolism differently [53]. Mesenteric fat in mice drains into the portal vein and better mimics human visceral fat but is difficult to surgically remove. Subcutaneous fat is located under the dermis and is divided into deep or superficial subcutaneous fat depending on the location in relation to the fascia. In humans it is found throughout the body but in greater abundance in abdominal, gluteal, and femoral regions. Mice have two major subcutaneous fat depots, divided into anterior and posterior. The posterior depot, termed inguinal, is analogous (in terms of position) to the gluteofemoral fat depot

in humans [52]. The work presented in this thesis has characterized the cellular function of both epididymal and inguinal adipocytes in mice. Therefore, it is important to understand the inter-depot differences in mice, but also how this relates to human fat depots.

Visceral and subcutaneous fat in mice and humans

In humans, the regional distribution of fat, rather than the level of adiposity, is associated with metabolic complications. There is a strong negative correlation between percentage of central abdominal fat and insulin sensitivity in both overweight and normal weight humans [54]. This suggests that accumulation of abdominal fat, even for people within the range of normal weight, increases the risk of insulin resistance. Waist to hip circumference ratio, rather than BMI, is a better predictor for risk of cardiovascular disease [55]. Abdominal fat includes both visceral fat and subcutaneous abdominal fat, but only increase in visceral fat correlates with insulin resistance [56].

The relationship between accumulation of visceral fat and insulin resistance has been tested in mice by using fat depot transplantation and removal, reaching similar conclusions as in human studies. Removal of epididymal fat improves insulin sensitivity while removal of inguinal fat impairs insulin sensitivity [57, 58]. Transplantation of inguinal fat from a donor mouse into the abdominal cavity of a recipient mouse has also been shown to improve insulin sensitivity [57]. Overall, fat removal and transplantation studies in mice suggest that both the anatomical location and cell-intrinsic properties of the fat influence systemic insulin sensitivity, with inguinal fat proposed to be protective against insulin resistance. In contrast to studies in mice, removal of omental fat [59] or liposuction of abdominal subcutaneous fat [60] in obese humans does not improve insulin sensitivity. Therefore, conclusions from fat transplant studies in mice are likely not directly translatable to humans.

The relationship between adipocyte size and systemic metabolism has been studied extensively. In humans, increases in cell size of subcutaneous adipocytes, independent of fat percentage, correlates with risk of developing insulin resistance and T2D [18]. Visceral hypertrophy is also associated with metabolic diseases; metabolic unhealthy obese subjects have larger cell-size in omental fat compared to metabolic healthy obese subjects [61]. However, a few overfeeding studies in humans have challenged the view that small adipocytes are solely beneficial, at least during short-term diet interventions. Contrary to expectations, an increased number of small adipocytes, both before and after overfeeding, was associated with impaired metabolic outcome [62, 63]. Nevertheless, studies in transgenic modified mice displaying increased adipogenesis, show that hyperplastic growth is associated with improved systemic insulin sensitivity [17, 64, 65].

In humans, short-term overfeeding leads to hyperplastic growth of lower body subcutaneous fat [66]. In contrast, most *in vivo* studies in mice support that only the epididymal depot expands by hyperplasia in response to HFD-feeding [67-69], even though there are reports of hyperplastic growth also in the inguinal depot [70, 71]. Interestingly, APC isolated from the inguinal depot are more readily differentiated *in vitro* compared to epididymal APC, highlighting depot-specific discrepancies between *in vitro* and *in vivo* adipogenesis [72]. By transplanting and tracking adipogenesis in isolated APC from both fat depots, Jeffery *et al*. demonstrated that it is the microenvironment of the AT, not cell-intrinsic factors, that regulate adipogenesis *in vivo* in mice, and that the microenvironment of the epididymal AT stimulates adipogenesis [69].

Subcutaneous adipocytes are larger than visceral adipocytes in both obese and lean human subjects [73, 74] while the opposite is observed in mice. For subcutaneous fat, lower-body adipocytes are larger than upper-body abdominal adipocytes [74, 75]. In response to overfeeding in humans, lower-body adipocytes expand by hyperplasia and hypertrophy while upper-body only display hypertrophic growth [66]. Moreover, in humans, the number of omental adipocytes shows strong association with omental weight, suggesting that this visceral depot expands by hyperplasia [76]. In summary, visceral and subcutaneous fat display different characteristics with regards to cell-size and hypertrophic/hyperplastic growth in mice and humans (see **Figure 3**), of importance to consider when interpreting findings from studies in mice.

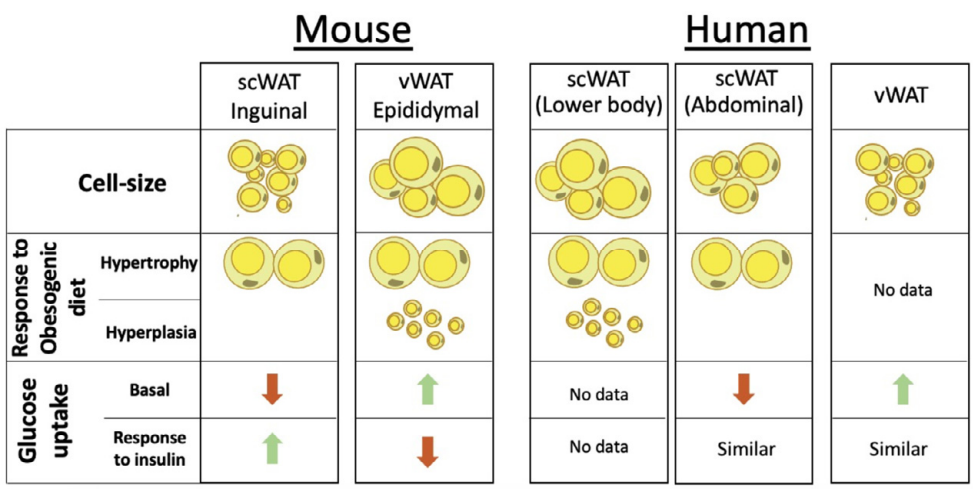

Figure 3. Characteristics of subcutaneous and visceral fat in mice and humans.
Adipocyte mean cell-size, hypertrophic/hyperplastic growth in response to overfeeding, cellular basal glucose uptake (non-stimulated) and response to insulin (fold insulin/basal) are displayed. Data only represent male mice. Human data are from lower body subcutaneous fat, upper body abdominal subcutaneous fat, and visceral fat.

There are some possible explanations of why epididymal fat displays more hyperplastic growth in comparison to inguinal fat. Adipogenesis is triggered in the proximity of dead adipocytes [77]. Adipocyte death following HFD-feeding reaches up to 80% in the epididymal depot while it remains marginal in the inguinal depot (< 3%) [78]. This could explain why only the epididymal depot, which has more cell death than the inguinal depot, displays hyperplastic growth in response to HFD-feeding [67]. Another possible explanation could be that very large adipocytes (>140 μm in cell diameter) secrete factors that induce adipogenesis, and such cell-sizes are only observed in the epididymal fat depot during HFD-feeding [79].

Cellular response in visceral and subcutaneous fat

Humans and mice display similarities in adipocyte glucose uptake, with higher non-stimulated uptake in the visceral depot (see **Figure 3**). In mice, basal glucose uptake (per cell) is higher in visceral (epididymal) compared to subcutaneous (inguinal) adipocytes, however, the responsiveness following insulin stimulation is higher in subcutaneous adipocytes [26]. Following HFD-feeding insulin responsiveness drops more in inguinal adipocytes [26], something also observed in the studies presented in this thesis. Humans display higher basal and insulin-stimulated glucose uptake in visceral compared to subcutaneous adipocytes, which is partially explained by an increase in GLUT4 expression in visceral adipocytes [80, 81].

HFD-feeding results in an increase in basal lipolysis, while beta adrenergic (epinephrine)-stimulated lipolysis is reduced in epididymal and inguinal adipocytes, with a more pronounced reduction in inguinal adipocytes [82]. Insulin's ability to suppress lipolysis is only impaired in epididymal, not inguinal, adipocytes following HFD-feeding [26].

Extracellular matrix

The ECM supports the adipocytes, provides structure and cell adhesion, prevents mechanical stress, and transduces intracellular signaling. The main components in the ECM of AT are collagens, fibronectin, and laminin [83]. Collagens are mainly produced by adipocytes but also by endothelial cells and preadipocytes [83]. For healthy expansion of the AT, the ECM requires remodeling and maintenance of high plasticity to provide space for hypertrophic/hyperplastic growth and proper angiogenesis [84]. Several different collagens of the ECM are upregulated in obese (db/db) mice [12]. Collagen VI (COLVI), in particular, has been demonstrated to be important during obesity-induced insulin resistance in mice. Knockout of COLVI promotes hypertrophic growth of adipocytes but surprisingly prevents AT inflammation and insulin resistance [12].

The ECM is important for hyperplastic growth and several collagens have been proposed to promote adipogenesis [85]. The origin of the ECM (epididymal or

inguinal) seems to heavily influence both adipogenesis and insulin sensitivity. Decellularized ECM isolated from epididymal AT of HFD-fed mice, has been shown to negatively influence insulin sensitivity and adipogenesis in cultured adipocytes [86]. Meanwhile, ECM from inguinal AT improves these parameters, suggesting that the ECM in epididymal AT contributes to insulin resistance. Remodeling of the ECM is regulated by matrix metalloproteases (MMPs), a family of enzymes involved in ECM degradation, while tissue inhibitor metalloproteinases (TIMPs) are inhibitors of MMPs [87]. The expression of MMPs and TIMPs in epididymal AT changes greatly during HFD-feeding in mice, reflecting a shift towards increased ECM degradation [88].

CD44 is a cell surface protein that interacts with several ECM proteins and has been linked to AT dysfunction and insulin resistance. The cytoplasmic domain of CD44 binds to a family protein that interacts with cortical actin, thereby linking the ECM to the actin cytoskeleton [89]. Like the COLVI knockout mice, the CD44 knockout mice exhibit hypertrophic adipocytes but retain intact insulin sensitivity [90].

Actin cytoskeleton

The actin cytoskeleton is a network of filaments important for cellular shape and movement, mitosis, intracellular transport and providing mechanical support [91]. The network is dynamic; filamentous (F)-actin is formed by polymerization of monomeric globular (G)-actin, and F-actin is depolymerized into G-actin. Cortical actin is a thin meshwork of F-actin that lies under the plasma membrane and has been shown to have an important role in regulating insulin-stimulated GLUT4 translocation and glucose uptake in adipocyte and myocyte models. We have previously shown that HFD-feeding leads to an increase of cortical F-actin, and that the level of F-actin correlates positively with adipocyte cell diameter [41]. Further, disrupting the cortical actin in adipocytes by using the actin filament disrupting agent Latrunculin A or B, leads to impaired insulin-stimulated glucose uptake and GLUT4 translocation [38, 41].

Several actin-regulating proteins have been demonstrated to influence GLUT4 translocation. Profilin-1 is an actin-binding protein, that regulates actin polymerization and is upregulated in epididymal adipocytes following HFD-feeding [92]. Mice with attenuated expression of profilin-1 are protected from HFD-induced glucose intolerance, suggesting that profilin-1 is important for insulin-sensitivity and adipocyte function [92]. Actin related protein 2 (ARP2), in complex with actin related protein 3 (ARP3), regulates actin polymerization and is required for adipogenesis as well as GLUT4 mediated glucose uptake in cultured 3T3-L1 cells [40]. Cofilin-1 is an actin depolymerizing protein and has been shown to be important for insulin-stimulated GLUT4 translocation in cultured myocytes. Silencing of cofilin-1 leads to increased F-actin and impaired GLUT4 translocation in response to insulin [39]. Collectively, the above-mentioned studies suggests that

a dynamic turnover of actin, via polymerization and depolymerization, is required for GLUT4 translocation [38-41].

Actin dynamics is also important during differentiation of adipocytes. This has been exemplified by silencing of ARP2/3 or cofilin-1, which both leads to impaired adipogenesis [40, 93].

Adipose tissue plasticity

Sakers *et al.* have thoroughly reviewed the importance of AT plasticity for adipocyte function [94]. In brief, they define AT plasticity as the capacity for the AT to dynamically change in response to weight gain or loss and suggest a distinction between metabolic plasticity and structural plasticity. Metabolic plasticity is the capacity to adapt and switch between energy storage and release, which is regulated by insulin and catecholamines. Structural plasticity is the capacity to expand and shrink the AT in response to over- and undernutrition by hyperplastic/hypertrophic growth, which involves ECM remodeling, angiogenesis, and adipocyte cell death. AT plasticity declines with age and in obesity, and loss of plasticity is associated with a dysfunctional AT. The structural plasticity is closely associated with the hallmarks of AT dysfunction: impaired angiogenesis, hypoxia, inflammation, hypertrophic growth, and fibrosis, all of which reinforce one another and contribute to an impaired AT function [16].

Caveolae and EHD2

In the plasma membrane of adipocytes there are small bulb-shaped invaginations, 25-150 mikrons in diameter [95], called caveolae, first described in the literature in 1955 [96]. Caveolae are lipid rafts, rich in cholesterol [97], and whose formation requires the proteins caveolin-1 [98] and cavin-1 [99]. In adipocytes, caveolae constitutes 50% of the plasma membrane [95] and have been shown to be important for several cellular processes: lipid transport [100], TG synthesis [101], lipolysis [102], glucose uptake [103], signaling transduction and enzyme activity [104], and protect against mechanical stress [105]. The importance of caveolae for TG storage in adipocytes has been demonstrated in mice models, where ablation of caveolin-1 and consequent caveolae depletion cause lipodystrophy and insulin resistance [106, 107]. The same phenotype is found in humans with mutations that causes caveolae depletion [108]. Eps15-homology domain containing protein 2 (EHD2) is an ATPase that binds to the neck of caveolae and is important for the stability of caveolae [109, 110]. Examinations of mice with global deletion of EHD2 have led to the proposal that EHD2 regulates FA uptake in adipocytes by stabilizing caveolae [111]. One study, using HeLa cells, that examined how the lipid composition of the plasma membrane influences caveolae stability, demonstrated that caveolae are destabilized and prone to scission when excess cholesterol is added to the plasma

membrane, but that this is counteracted by overexpression of EHD2 [112]. Together, evidence from *in vitro* experiments suggests that EHD2 is important for the stability of caveolae.

Methodology

DIO mouse model and primary mouse adipocytes

Most of the results in this thesis originate from isolated primary mouse adipocytes. In all publications, we have examined differences between adipocytes isolated from subcutaneous (inguinal) and visceral (epididymal) AT. Even though the epididymal fat has no corresponding anatomical depot in humans and has been suggested to be peri-visceral, it is still the most studied fat depot in mice and is commonly referred to as visceral [52]. The mesenteric fat would be a better fat depot for translational research but is more difficult to isolate and weighs less, which limits numbers of analysis.

The advantage of an *in vivo* model for studying fat metabolism and associated insulin resistance is the number of systemic and tissue-specific variables that can be assessed in a single animal. After all, the aim is to understand how insulin resistance emerges and progresses, and for that a complete overview of whole-body metabolism is desirable. In my third paper, we had this ambition and examined several parameters in each individual mouse and were able to correlate several parameters during different feeding interventions.

The C57BL/6J mouse model is frequently used as an animal model for studying diet-induced obesity (DIO) and associated metabolic diseases. C57BL/6J mice exhibit glucose intolerance when fed chow diet because of a mutation of the mitochondrial protein, nicotinamide nucleotide transhydrogenase (NNT), which causes impaired insulin secretion [113]. A comparative study with the substrain C57BL/6N, which has a functional NNT expression, revealed that both strains are similarly sensitive to HFD-induced glucose intolerance, but only C57BL/6J mice are glucose intolerant when fed chow diet [114]. Since humans express the NNT protein, the C57BL/6N mouse model may better reflect the disease progression of insulin resistance and T2D in humans. In the studies presented here, we have used the C57BL/6J strain, except in study I where *Ehd2*$^{-/-}$ mice and wild type (WT) *Ehd2*$^{+/+}$ mice were generated on a C57BL/6N background.

Interpretation and comparison of studies using HFD-feeding in mice is often made difficult by differences in study protocol e.g., mouse strain, diet composition (fat percentage/source), weeks on diet, fat depot examined and housing conditions [115, 116]. Of note, rodents also exhibit seasonal variation in cellular response e.g.,

lipolysis in adipocytes is increased in spring [117]. The body weight of mice has a strong correlation with systemic insulin sensitivity. Ideally, bodyweight of mice should be adjusted for as a covariate when interpreting results, to exclude body weight as a confounding variable [118].

It is important to emphasize the characteristics of visceral and subcutaneous fat and how they differ in humans and mice. As seen in **Figure 3**, several variables such as cell-size, hyperplastic growth and response to insulin display interspecies differences, and are important to bear in mind when interpreting results and translating them from mice to humans.

Coulter counter and adipocyte cell-size distribution

Several studies have investigated the relationship between adipocyte cell-size and function, and whole-body metabolism. Specifically, the ability to generate new adipocytes through adipogenesis (hyperplasia), rather than increasing cell-size of existing adipocytes (hypertrophy), is suggested to be beneficial for AT function and systemic metabolism. An accurate measurement of cell-size distribution from the AT is, therefore, a central analysis when studying adipocytes. Coulter counter is the most accurate method for obtaining a statistically robust result. This method requires the use of osmium tetroxide for fixation, a chemical that due to its toxicity and reactivity requires careful handling. After fixation, the cell-size distribution is obtained by counting fixed cells in a Coulter counter that accurately assesses the diameter of cells in a range of 20-240 μm. To gain a perspective of the volume that this range covers, one adipocyte, 240 μm in diameter, has the same volume as 1728 adipocytes, 20 μm in diameter. It also highlights the challenge of expressing cellular function e.g., lipolysis or glucose uptake, in the units of per cell or per cell suspension.

Statistical robustness of the distribution curve is obtained by counting 6000 particles in technical duplicates. Mathematical modelling has suggested that the epididymal fat depot expands by both hypertrophy and hyperplasia and that large and small cells can be separated and defined by the nadir in the bimodal distribution [119, 120]. This has been very useful for our studies when estimating the contribution of hypertrophic versus hyperplastic growth. One limitation with the Coulter counter method is that very small adipocytes (>20 mikrons) are not included in the analysis, so it is possible that we underestimate adipogenesis. Furthermore, there is a risk that cell debris contributes to the number of small cells detected [121], which could lead to an overestimation of hyperplastic growth. However, the Coulter counter method is superior to histology for determining cell-size distribution, since histology underestimates the true cell-size diameter, cannot detect very small cells, and is more tedious [74].

Alternative methods for assessing adipogenesis in vivo

The Coulter counter is an excellent method for detecting a wide range of cell sizes and estimating the total number of adipocytes in a fat depot. However, it only provides a snapshot of the AT and cannot be used to track adipogenesis *in vivo*. In mice, two methods have primarily been used for tracking adipogenesis: the Adipochaser mouse and the bromodeoxyuridine (BrdU) pulse chase method. The AdipoChaser mouse model uses a doxycycline inducible system reliant on the adipocyte specific expression of adiponectin and detects proliferating and differentiating APC [67]. The BrdU pulse chase method only detects actively proliferating APC, since BrdU is only incorporated in cells that are in an active cell cycle (S-phase). Therefore, the BrdU pulse-chase method fails to detect postmitotic, differentiating APC. This was nicely illustrated by a study in which the Adiponectin-Cre method and BrdU pulse chase method were compared, and the Adiponectin-Cre method detected nine times as many new adipocytes in response to HFD-feeding [69]. The different methods used for measuring hyperplasia may account for discrepancies among studies of hyperplastic growth in the epididymal and inguinal depots in response to HFD-feeding [67-71]. All things considered, the Coulter counter method serves as an adequate method for assessing adipocyte size-distribution and total number for the purposes of the studies presented in this thesis.

Cellular analysis

In all projects, we have used primary adipocytes and examined adipocyte function by assessing glucose uptake, lipolysis and signaling transduction.

Glucose uptake assay

The responsiveness to insulin, including GLUT4-mediated glucose uptake, is of major importance for adipocyte function and systemic metabolism [8, 17]. One way to examine this is to measure glucose uptake during insulin-stimulation in isolated adipocytes. For this, a cell suspension (containing 5-10% (v/v) cells) is prepared from floating adipocytes and incubated with or without insulin, followed by addition of radiolabeled (^{14}C) D-glucose, which is taken up and metabolized by the adipocytes. The adipocytes are separated from the incubation-media (containing radiolabeled glucose) by adding dinonylphtalate oil followed by centrifugation. The density of adipocytes makes them float on top of the dinonylphtalate oil and allows for the separation of the adipocytes from the media, using a razorblade. The amount of glucose taken up by the adipocytes is assessed by measuring the radioactive decay (beta-radiation) in the adipocyte-fraction, using a scintillation counter. Cytochalasin

B, which inhibits all glucose transport across the cell membrane, is used as a control for glucose binding to the cell-surface.

Lipolysis

Adipocytes supply other tissues with energy in the form of FA through lipolysis, which is regulated by both insulin and adrenergic agonists. Dysregulation of both basal and beta-adrenergic induced lipolysis are associated with risk of developing T2D and insulin resistance [122], and therefore of importance to examine. In a similar manner to the glucose uptake assay, the lipolysis assay is carried out using primary adipocytes in a cell-suspension (containing 10% (v/v) cells). Lipolysis is assessed by the release of glycerol into the incubation media during non-stimulation (basal), or stimulation with a beta-adrenergic agonist (isoprenaline) or dibutyryl cAMP (DcAMP). The glycerol content is analyzed with a commercial (enzymatic based) kit, measuring absorbance of a product, linear to glycerol content, with a spectrophotometer.

Western blot and signaling transduction

Western blot has been used to examine protein expression and signaling transduction (phospho-specific antibodies) in all papers presented herein. Equal amounts of protein from cell-lysates are loaded to a commercial precast SDS-PAGE, followed by transfer of proteins to a nitrocellulose membrane by dry transfer using Trans-Blot Turbo transfer system from Bio Rad. Blocking, preventing unspecific binding of antibody, is carried out in 5% milk, followed by overnight incubation (4°C) in primary antibody. The next day, membranes are washed (5x5 min) followed by 1h incubation in horseradish peroxidase (HRP)-coupled secondary antibody and then washed again (5x5 min) before proteins are detected by chemiluminescence after adding HRP-substrate. Proteins are normalized towards a house-keeping protein, most often heat shock protein 90 (HSP90). When examining signaling transduction, primary cells have been stimulated with insulin or isoprenaline before cell lysates were prepared, and phosphorylation levels were examined using phospho-specific antibodies.

RT-qPCR

To examine gene expression in AT we have used reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissues from different fat depots are immediately frozen in liquid nitrogen after excision. Extraction of messenger ribonucleic acid (mRNA) is carried out in QIAzol lysis reagent and mRNA is isolated using an RNeasy Mini Kit, concentration and quality of mRNA is determined by NanoDrop. We use a one-step RT-qPCR with mRNA as starting

material. Reverse transcription of RNA into DNA and amplification of DNA is performed in the same reaction-tube and in sequential events that are regulated by temperature. The reverse transcriptase is only active at low temperatures and the HotStarTaq DNA Polymerase is only active at high temperatures. The relative mRNA expression between groups was calculated using the ΔΔCT-method [123] and 18S ribosomal RNA has been used as a housekeeping gene. Statistical analysis was carried out on ΔCT values.

Presenting data as *cell volume* or *per cell*

We perform *ex vivo* measurement of lipolysis and glucose uptake using adipocytes in a cell suspension. Adipocytes form a layer of packed cells on top of media. Following adipocyte isolation, media is removed, and a volume of packed adipocytes is transferred to buffer to make a cell suspension (usually 5-10% v/v). The heterogeneity of adipocyte sizes, especially comparing chow to HFD-feeding, influences the number of adipocytes in a cell suspension. We report our results from glucose uptake and lipolysis as *per µl packed cells* instead of *per cell*. Reporting *per cell* would require examination of the cell-size distribution or DNA content before starting the cellular analysis or an estimation of these parameters beforehand and normalization to cell number afterwards. Since adipocytes survival is short *ex vivo* it is difficult to do beforehand. Presenting data as *per cell volume* rather than *per cell* has been suggested to be preferable when examine lipolytic activity [34]. In **paper I**, we examine lipolytic activity in inguinal adipocytes. The inguinal depot expands primarily by hypertrophic growth in response to HFD-feeding, therefore the change in weight of the AT is primarily due to an increase in cell-size. Multiplying results obtained from *per cell volume* with the depot weight could reflect the lipolytic activity *per cell*, assuming no hyperplastic growth, negligible contribution from non-adipocytes to the fat depot weight, and that our *ex vivo* packed cells reflect the density of adipocytes *in vivo*. Therefore, this would be an alternative way of presenting our data without having to examine cell-size in all samples. Using a fixed cell volume instead of fixed number of cells, allows for faster handling and shorter *ex vivo* exposure of isolated adipocytes. In my opinion, the increase in cell viability outweighs the negative consequences of not being able to express results *per cell*.

Present investigation

Overview and aims

An estimated 463 million people suffer from diabetes, 90% from T2D, a number which is projected to increase to 700 million by 2045 [124]. The cost for society, apart from individual suffering, is estimated to be 12% of global healthcare expenditure by 2030 [125]. This emphasizes the need for new strategies for treating T2D. The AT plays an important role in the progression of systemic insulin resistance. This thesis aims to broaden the understanding of AT function, focusing on cell-size and differences between visceral and subcutaneous adipocytes. This knowledge could in the future contribute to novel treatments that focus on improving the function of specific fat depots and thereby metabolic health.

Paper I aimed at examining the importance of caveolae-associate protein EHD2 during fat expansion, by using HFD-feeding in mice with global deletion of EHD2. We examined the cellular function in isolated adipocytes by measuring lipolysis, and hyperplastic/hypertrophic growth by measuring cell-size.

In **Paper II** we investigated the insulin-sensitizing effect of rosiglitazone on hypertrophic adipocytes. We designed a study protocol that minimized the adipogenic effect of rosiglitazone and were thereby able to examine its effect on mature hypertrophic adipocytes. We examined how cellular function (glucose uptake), ECM composition, signaling transduction and actin cytoskeleton were influenced by rosiglitazone treatment in both epididymal and inguinal AT.

Paper III aimed to explore AT plasticity in response to weight gain and loss, using HFD-feeding and diet reversal (switching from HFD to chow-feeding) in mice. The study design included a temporal resolution of 4, 8 and 12 weeks, to characterize adipocyte cell-size, actin cytoskeleton and depot weight and how this correlated with systemic parameters.

All papers have examined both visceral and subcutaneous adipocytes to explore inter-depot differences. Each study provided new evidence of these differences and highlighted the importance of studying both visceral and subcutaneous fat for understanding how AT dysfunction relates to systemic metabolism.

Paper I

The aim of **paper I** was to examine and characterize the importance of EHD2 for adipocyte function during fat expansion. Previously, our lab has shown that EHD2 is one of the most highly upregulated genes in epididymal AT in response to HFD-feeding [41]. Furthermore, we showed that EHD2 was important for lipolytic activity in 3T3-L1 cells and that EHD2 localizes to the lipid droplet in primary human adipocytes during beta-adrenergic induced lipolysis [126]. Therefore, we hypothesize that a short diet intervention could reveal novel importance of EHD2 during AT expansion. For that, we used a mouse model with global deletion of the EHD2 protein (*Ehd2*$^{-/-}$), kindly provided by Prof. Oliver Daumke [111] that were fed HFD for two weeks.

Main findings

We first observed that the epididymal fat depot in *Ehd2*$^{-/-}$ mice did not expand as much as WT in response to HFD-feeding, while inguinal expansion was similar comparing WT and *Ehd2*$^{-/-}$ mice. The cell-size distribution curves also revealed that *Ehd2*$^{-/-}$ mice had a reduced number of very small epididymal adipocytes, indicating that there could be impaired adipogenesis in response to HFD-feeding in this fat depot. This was further supported by our finding that the activity of PPARγ, master regulator of adipogenesis, was reduced in mature epididymal adipocytes of *Ehd2*$^{-/-}$ mice.

We also examined the lipolytic response in isolated primary adipocytes and found that beta-adrenergic stimulated lipolysis was impaired in inguinal, but not epididymal, adipocytes from *Ehd2*$^{-/-}$ mice following HFD-feeding. Beta-adrenergic receptors have been suggested to localize at caveolae [127]. Therefore, we hypothesized that there might be an impairment at the receptor level of the beta-adrenergic pathway in *Ehd2*$^{-/-}$ mice. However, when stimulated with a cell permeable cAMP analogue, dibutyryl-cAMP (DcAMP), which bypasses the adrenergic receptor, the *Ehd2*$^{-/-}$ adipocytes still had reduced lipolysis, similar to what was observed with a beta-adrenergic agonist. The results suggest that the impaired lipolytic response is downstream of PKA, which is directly activated by cAMP. In support of this, we found that phosphorylation of both PLIN1 and HSL was reduced in *Ehd2*$^{-/-}$ adipocytes in response to beta-adrenergic stimulation. We also excluded PDE3 and 4 as potential mediators of the impaired lipolysis by using inhibitors for PDE3 (OPC3911) and PDE4 (rolipram).

Discussion

The main findings from this paper, that *Ehd2*$^{-/-}$ mice have reduced adipose expansion and impaired lipolysis, were only observed after mice were fed HFD. This suggests that EHD2 is required for maintained adipocyte function during AT expansion. Gene expression data have revealed that caveolae-associated proteins, including EHD2, are upregulated in AT upon HFD-feeding [128, 129], which most likely reflects an increase in membrane area and requirement of increased number of caveolae per cell for maintained caveolae density and cellular function. Furthermore, *in vitro* data have shown that EHD2 prevents scission of caveolae from the plasma membrane [112].

It is possible that the findings we observed in the *Ehd2*$^{-/-}$ adipocytes are primarily a consequence of dysfunctional, detached, or reduced caveolae. This was supported by western blot where we found a reduction in caveolin-1 following HFD-feeding in both epididymal and inguinal adipocytes. In further support of this, Matthaeus et al. found that adipocytes from *Ehd2*$^{-/-}$ mice have an elevated number of caveolae detached from the plasma membrane [111].

EHD2 has also been linked to direct gene regulation in response to mechanical stress by being SUMOylated then translocating to the nucleus [130]. Possibly, EHD2 exerts transcriptional regulation during adipogenesis which could explain why it is one of the most highly upregulated genes in response to HFD-feeding [129]. If so, this would explain the reduced number of small adipocytes that we observed in the epididymal depot from *Ehd2*$^{-/-}$ mice in response to HFD-feeding.

Matthaeus et al., who first characterized the *Ehd2*$^{-/-}$ mouse, concluded that EHD2 regulates FA uptake in adipocytes, in a caveolae-dependent mechanism [111]. They propose that ablation of EHD2 increases caveolae-dependent lipid uptake. In contrast to us, they found an increase in perigonadal fat, and lipid droplet size, in *Ehd2*$^{-/-}$ mice. Their conclusions were based on visual inspection (no fat depot weight), a low number of mice (n=7) and use of histology to assess cell size. I think our data are more robust, given that they rely upon more observations (n=22) and a more accurate method for determining cell-size (Coulter counter). In addition, we used *Ehd2*$^{+/+}$ as control (WT) mice, while they used heterozygous (*Ehd2*$^{+/-}$) mice as control. However, data from the same group show that *in vitro* differentiated *Ehd2*$^{-/-}$ adipocytes had increased FA uptake, which supports their observations *in vivo*. The discrepancy between our results and theirs is likely related to the model used. Matthaeus et al. show that *Ehd2*$^{-/-}$ promotes FA transport in adipocytes cultured *in vitro*, however our data, from cell-size distribution, does not support this conclusion for *in vivo* adipocytes. It should also be noted that *in vitro* differentiated adipocytes more closely resemble brown adipocytes with small multilocular lipid droplets in comparison to *in vivo* differentiated white adipocytes that have a large unilocular lipid droplet.

We hypothesize that the impaired lipolysis in inguinal *Ehd2*$^{-/-}$ adipocytes was due to insufficient activation/phosphorylation of lipolytic proteins downstream of PKA, since the response to beta adrenergic agonist and DcAMP was similarly impaired in *Ehd2*$^{-/-}$ adipocytes. Caveolin-1 interacts with PKA and has been suggested to facilitate PKA-mediated phosphorylation of PLIN1 [102]. It is possible that *Ehd2*$^{-/-}$ adipocytes have reduced or dysfunctional caveolae which influence the adipocytes' capacity to activate targets downstream of PKA (see **Figure 4** for proposed mechanism).

The reason why lipolysis was only impaired in inguinal adipocytes from *Ehd2*$^{-/-}$ mice could be related to cell-size, which differed in the epididymal depot comparing *Ehd2*$^{-/-}$ to WT. If the results had been expressed in lipolytic rate per cell instead of packed cell volume, it may have revealed a difference in lipolysis between *Ehd2*$^{-/-}$ and WT in the epididymal depot (see method page 29). This is supported by the fact that we found a trend towards reduced stimulated lipolysis in the chow-fed state, where adipocytes from *Ehd2*$^{-/-}$ and WT were similar in cell-size. Since impaired stimulated lipolysis is a predictor for future weight gain and development of insulin resistance in humans [122], our results suggest that EHD2 could be an important factor for maintained lipolytic response and possibly for maintained metabolic health. The impaired response to diet-induced adipogenesis also suggests that EHD2 could have an important role in maintaining healthy AT expansion.

During the project we also found that the *Ehd2*$^{-/-}$ mice had reduced protein expression of endothelial nitric oxide synthase (eNOS) following HFD-feeding (non-published data) and that adenoviral overexpression of EHD2 in human adipocytes increases eNOS expression. eNOS expression is known to be induced during *in vitro* differentiation of 3T3-L1 [131]. Interestingly, transgenic mice that overexpress eNOS are protected against diet-induced insulin resistance [132]. eNOS converts L-arginine into nitric oxide (NO) [133], localize to caveolae and once bound to caveolin-1 is kept in an inactive state [134, 135]. NO has been shown to regulate both basal and adrenergic-stimulated lipolysis in human and rodent adipocytes [136-138]. Since we found altered protein expression of eNOS in *Ehd2*$^{-/-}$ adipocytes from HFD-fed mice, it is possible that this also influenced the lipolytic activity. However, to further dissect this would require extensive additional experiments, beyond the scope of this paper.

There was a trend towards increased level of PLIN1 in inguinal adipocytes from *Ehd2*$^{-/-}$ mice fed HFD. PLIN1 localizes to the lipid droplet and prevents lipolysis but is also required for maximal adrenergic-stimulated lipolysis [139]. This has been shown by overexpression of PLIN1, which results in decreased adrenergic-stimulated lipolysis [140]. Possibly, the increased PLIN1 levels in adipocytes from *Ehd2*$^{-/-}$ could have influenced the lipolytic rate in *Ehd2*$^{-/-}$ mice, however, it does not explain the lower phosphorylation and activation of both PLIN1 and HSL that we observed.

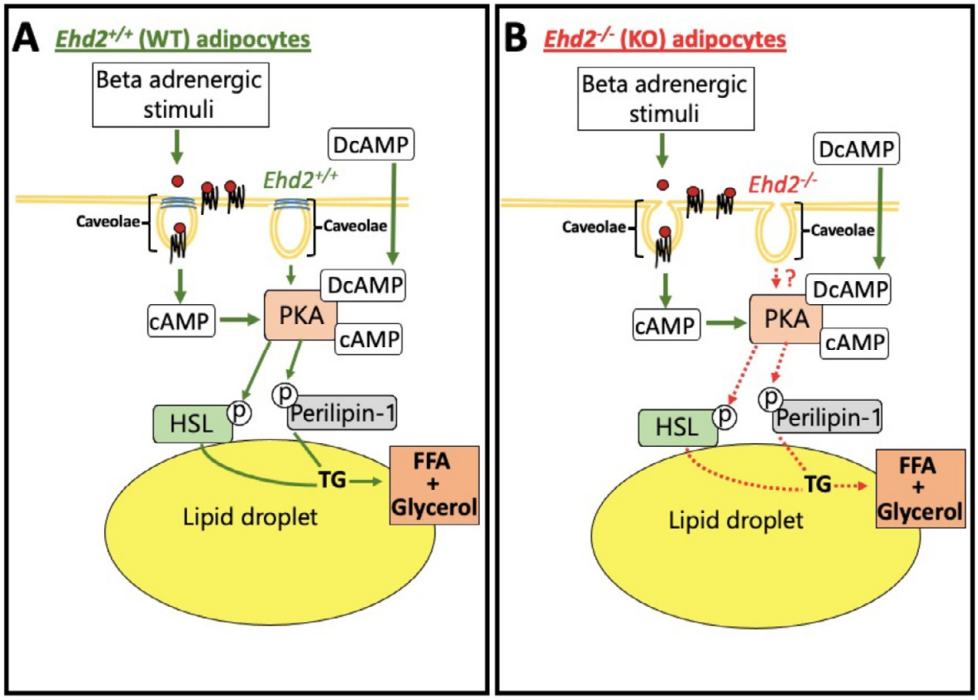

Figure 4. Proposed mechanism for impaired lipolysis in inguinal adipocytes from *Ehd2*$^{-/-}$ mice.
Stimulated lipolysis in **(A)** *Ehd2*$^{+/+}$ and **(B)** *Ehd2*$^{-/-}$ adipocytes. Lipolysis is impaired in *Ehd2*$^{-/-}$ inguinal adipocytes when using a beta-adrenergic agonist and when stimulating with dibutyryl cAMP (DcAMP), a cAMP analogue which bypasses the adrenergic receptor and directly activates PKA. Therefore, the impairment is likely downstream of cAMP in the beta-adrenergic signalling pathway. PKA-mediated phosphorylation of downstream targets (HSL and PLIN1) is reliant on caveolae. We propose that deletion of EHD2 influences caveolae stability/function and thereby PKA's ability to mediate phosphorylation of perilipin-1 and HSL.

Paper II

The findings from **paper I** revealed a reduced activity of PPARγ, the master regulator of adipogenesis, in *Ehd2*$^{-/-}$ adipocytes, both during basal condition and when stimulated with rosiglitazone. Rosiglitazone belongs to the family of thiazolidinediones and is an agonist to PPARγ. Rosiglitazone's insulin-sensitizing effect was first described in rats in 1994 and since 1999 it has been used in the clinic for treatment of T2D [141]. However, adverse side-effects, including cardiovascular complications, caused the European Medicines Agency to withdraw the drug from the market in 2010 [142]. Still, the beneficial effects mediated by rosiglitazone are of interest for further investigation in search of novel strategies for treatment of T2D.

Rosiglitazone stimulates adipogenesis, increasing the number of small adipocytes, one mechanism that improves systemic insulin sensitivity [143]. It has also been

shown to influence pyruvate metabolism in mitochondria, a potential cause of its pleotropic effect, and adverse side-effects [144]. Previous results from our group have shown that rosiglitazone drives the expression of cavin-2 [145]. In **paper II** we examined the beneficial effect that rosiglitazone exerts on hypertrophic adipocytes by using HFD-feeding in mice, combined with a short-term treatment of rosiglitazone. We propose a mechanism of action where rosiglitazone modulates the ECM composition, which in turn improves actin dynamics and thereby insulin-stimulated glucose uptake in hypertrophic adipocytes.

Main findings

To prevent undesired adipogenesis from rosiglitazone-treatment, and exclusively study the effect on mature adipocytes, we used a study protocol where mice were fed HFD for two weeks, to induce hyperplasia, followed by a short-term, daily treatment with rosiglitazone for one week. We examined the cell-size distribution using the Coulter counter method to assess adipogenesis. Treatment with rosiglitazone did not increase the number of small cells in HFD- or chow-fed mice in the epididymal or inguinal fat depot, confirming that rosiglitazone had no additive hyperplastic effect, using this study design. Therefore, subsequent cellular analysis where not affected by any hyperplastic effect. In isolated primary adipocytes from HFD-fed mice we found that rosiglitazone enhanced insulin-stimulated glucose uptake in all three fat depots examined: epididymal, inguinal and retroperitoneal. Interestingly, the improvement in insulin-stimulated glucose uptake in HFD-fed mice treated with rosiglitazone was more pronounced in visceral depots (epididymal: +66%, retroperitoneal: +76%) compared to the subcutaneous depot (inguinal: +51%).

GLUT4 expression and insulin signaling transduction were similar between control (HFD) and rosiglitazone-treated mice, which suggests that other mechanisms contribute to the improved insulin-stimulated glucose uptake. To further explore this, we examined the actin cytoskeleton in isolated adipocytes, which have previously been shown to be important for facilitating GLUT4 translocation and insulin-stimulated glucose uptake [40, 41]. As previously shown by our group [41], cortical F-actin increases upon HFD-feeding and correlates with cell diameter. However, the level of F-actin was reduced in hypertrophic epididymal adipocytes in mice treated with rosiglitazone. The gene expression of several ECM proteins was also reduced following rosiglitazone treatment. Simultaneously we found reduced activation of mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway in epididymal adipocytes, suggesting reduced mechanical stress. All together, we propose a novel mechanism of action for rosiglitazone to improve glucose uptake in hypertrophic adipocytes (see **Figure 5**).

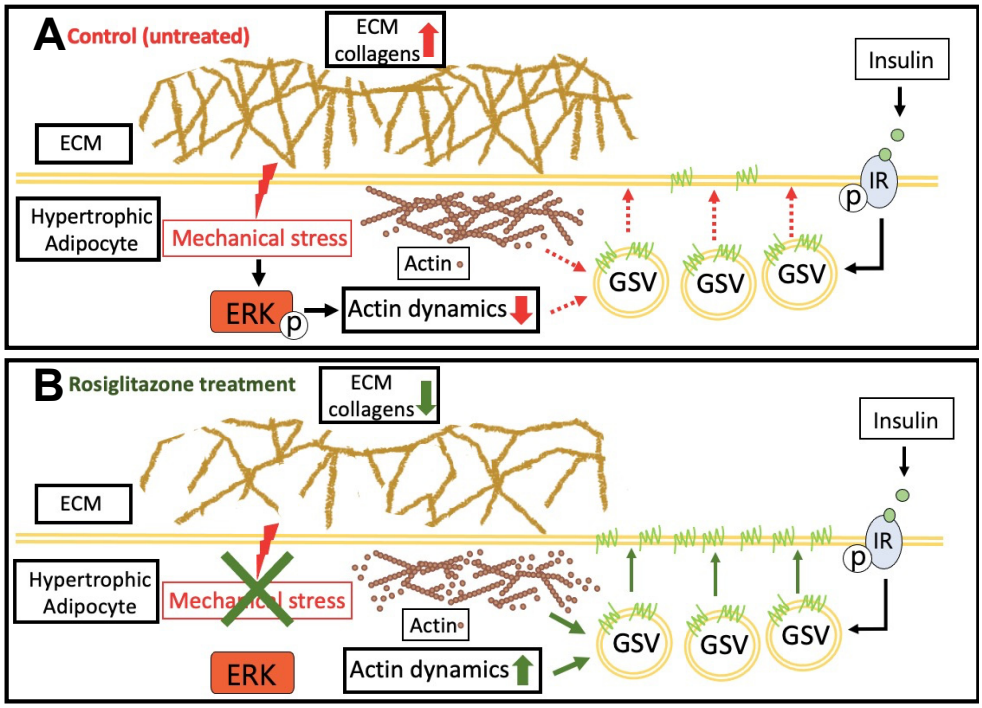

Figure 5. Proposed mechanism of rosiglitazone's insulin-sensitizing effect in hypertrophic adipocytes.
The upper panel **(A)** shows the ECM and a hypertrophic epididymal adipocyte after HFD-feeding from untreated mice and the lower panel **(B)** shows the ECM and a hypertrophic epididymal adipocyte after HFD-feeding from mice treated with rosiglitazone (7 days). **(A)** HFD-feeding causes an accumulation of ECM-proteins, including collagens, in epididymal AT. The large cell-size of hypertrophic adipocytes and augmented collagen deposit lead to increased intracellular stress-signalling, including phosphorylation and activation of ERK. Activated ERK reduces the dynamics of actin, promoting filamentous actin over monomeric actin. The final step of GLUT4-storage vesicle (GSV) exocytosis relies on actin dynamics and is impaired in hypertrophic adipocytes from HFD-fed mice. **(B)** Rosiglitazone treatment leads to reduced ECM collagens, lower phosphorylation of ERK, improved actin dynamics and increased insulin-stimulated glucose uptake.

Discussion

That hypertrophic adipocytes are associated with insulin resistance is well supported in the literature [18, 146-148]. Therefore, identifying mechanisms that improve the function of hypertrophic adipocytes offers an attractive approach to treating insulin resistance and T2D. In this paper we propose a novel mechanism of action for rosiglitazone: that remodeling of the ECM, induced by rosiglitazone, results in reduced stress-induced intracellular signaling, reduced F-actin and improved insulin-stimulated glucose uptake (see **Figure 5**).

A few studies have attempted to modulate the ECM composition to evaluate the role of ECM as a mediator of systemic insulin resistance. Khan et al. showed that modulation of the ECM, by knockout of COLVI, improved glucose tolerance in ob/ob mice despite increasing the number of hypertrophic adipocytes [12]. Similar

results, increased number of hypertrophic adipocytes and improved insulin sensitivity, were seen when knocking out CD44, a cell-surface receptor that binds to the proteins in the ECM and to the actin cytoskeleton [89, 90]. Both studies associated the improved insulin sensitivity with reduced inflammation in the AT. These studies highlight that the ECM could be a putative target for treatment of insulin resistance.

Collagens are primarily produced by adipocytes in the AT, and to lesser extent also by preadipocytes and endothelial cells [83]. Therefore, it is likely that the modification of ECM composition after rosiglitazone-treatment is mediated through adipocytes and not through other cell-types of the AT. Macrophages have been suggested to be a target for rosiglitazone treatment, as they express PPARγ, and macrophages-specific deletion of PPARγ leads to insulin resistance [149]. Therefore, macrophages could potentially contribute to the insulin-sensitizing effect of rosiglitazone. However, transgenic mice with a selective activation of PPARγ in mature adipocytes display improved insulin sensitivity to a similar extent as thiazolidinedione treatment, while selective activation of PPARγ in macrophages fails to do so [150].

A similar phenotype as observed in our study was reported in obesogenic mice that were treated with the antidiabetic drug metformin [151]. They found a reduction of collagens following metformin-treatment and improved systemic insulin sensitivity, possibly mediated through activation of AMP-activated protein kinase (AMPK). Still, further research is needed to explore if rosiglitazone, and other insulin-sensitizing drugs, are mediating their pharmacological effect through modification of the ECM.

Our study highlights fat depot-specific differences in response to rosiglitazone treatment. The improvement in insulin sensitivity was more pronounced in epididymal adipocytes even though the drop in insulin-stimulated glucose uptake, comparing chow with HFD-feeding, was greater in inguinal adipocytes. Therefore, in mice, rosiglitazone seems to primarily improve glucose uptake in the visceral fat depots, which stands in contrast to results obtained from human studies of T2D patients [152]. We also found that rosiglitazone had a greater impact on the level of F-actin and ECM remodeling in the epididymal depot compared to the inguinal. This strengthens the hypothesis that part of the improvement in insulin sensitivity from rosiglitazone arises from modulation of the ECM and improved actin dynamics. It would be interesting to examine subcutaneous and visceral human fat following rosiglitazone treatment to see if the greater improvement in glucose uptake in subcutaneous fat is mirrored in altered ECM composition of this fat depot [152].

The ERK signaling pathway has been shown to be activated in AT following HFD-feeding in mice. Moreover, pharmacological inhibition of ERK activity significantly improves glucose tolerance in DIO-mouse model [153]. Upon phosphorylation,

ERK activates Ras homolog family member A (RhoA) that in turn promotes formation of actin stress fibers [154]. Therefore, it is possible that ERK activation is mediating the increase in F-actin that we observe after HFD-feeding, and that this is counteracted when mice are treated with rosiglitazone. It would be interesting to see if coadministration of rosiglitazone and a MEK inhibitor has an additive effect on glucose uptake and systemic insulin sensitivity. If not, it is an indication that rosiglitazone mediates its insulin-sensitizing effect primarily through the ERK pathway.

One pathway, which we did not examine in this paper, involves PPARγ phosphorylation. In HFD-fed mice, PPARγ is phosphorylated at S273 which alters the gene expression profile and is thought to contribute to insulin resistance. Treatment with rosiglitazone prevents phosphorylation at this residue [155]. The phosphorylation of S273 is mediated through the MAPK/ERK pathway and can be blocked using a MAPK/ERK inhibitor [156]. Even though this offers a potential mechanism of action in our study, it is unlikely since an increase in PPARγ phosphorylation (S273) is only observed after prolonged HFD-feeding and not after 3 weeks, as in our study protocol [155]. Furthermore, mice with a modified PPARγ, that prevents phosphorylation of S273, still displayed improved insulin sensitivity following treatment with rosiglitazone [157]. This shows that rosiglitazone has other mechanisms of action that promote insulin sensitivity and are not only mediated through altering PPARγ phosphorylation.

Vascularization of the AT is increased in mice following prolonged rosiglitazone-treatment (2 weeks), a possible beneficial mechanism that we did not assess in this study [158].

Paper III

Our intention with **paper III** was to examine actin dynamics and cell size distribution in epididymal and inguinal fat during weight gain and weight loss. For that, we examined the AT expansion in response to overfeeding by employing a temporal resolution of 4, 8 and 12 weeks of HFD-feeding in mice. To evaluate AT plasticity, we had a group of mice for each timepoint that were switched from HFD to chow during the final two weeks of feeding (referred to as the reverse group). We have previously published a paper with a similar study design and found that F-actin, cell-size, and insulin sensitivity in adipocytes were completely reversible when switching from HFD to chow during a short diet intervention (4 weeks) [41].

Main findings

The temporal response to HFD-feeding revealed fat depot-specific differences regarding expansion and cell-size. While the epididymal fat reached its max in both depot weight and cell-size after 4 weeks of feeding, the inguinal depot expanded primarily between 8 and 12 weeks of feeding. The inguinal expansion coincided with increased liver TG and impaired systemic insulin sensitivity (QUICKI) between the 8- and 12-week timepoints. The epididymal depot reached its maximal weight at a body weight around 44g and thereafter declined.

The plasticity of the fat depots, as well as systemic parameters, were preserved in the 4- and 8-week groups, where depot weight, cell-size, body weight, liver TG and insulin sensitivity were all restored to chow level in the reverse group. However, for the 12-week timepoint, all these parameters were significantly altered comparing the reverse group with chow. The inguinal fat depot weight displayed stronger correlation with body weight, systemic insulin sensitivity, and liver TG compared with the epididymal and retroperitoneal depot weight.

In a similar nature to what was described in **paper II**, we examined the level of F-actin in the different feeding intervention from 4-, 8- and 12-week groups. Like most other variables examined, we found that F-actin increased with HFD-feeding but were restored to chow level in the 4- and 8-week, but not 12-week, reverse groups in both epididymal and inguinal adipocytes.

We also examined cellular response to insulin, by measuring glucose uptake in isolated adipocytes and found this to be impaired in the 12-week reverse group, at a comparable level to the HFD-fed group. Interestingly, GLUT4 levels were partially restored in the epididymal adipocytes in the 12-week reverse group, despite no improvement in insulin-stimulated glucose uptake.

Discussion

This study provided novel knowledge of the plasticity of cell-size in inguinal and epididymal AT during weight gain and weight loss. During weight gain the epididymal depot reaches maximal expansion, in terms of weight and cell-size, already after 4 weeks. Meanwhile, the inguinal depot expands massively in terms of cell-size and weight between 8 and 12 weeks of HFD-feeding. At this timepoint, the number of hypertrophic inguinal adipocytes (>70 μm) almost doubled, suggesting that the increase in mass is primarily caused by hypertrophic, rather than hyperplastic growth. However, the increase in mean adipocyte volume between 8 to 12 weeks in the inguinal AT was 10% less than the increase in depot weight (data not shown), suggesting that some hyperplastic growth might contribute to the inguinal expansion.

A study that has monitored adipogenesis in the inguinal depot, using the BrdU method, concluded that proliferation occurs after 12 weeks of HFD-feeding and that this coincides with a robust increase of inguinal weight in the following weeks [159]. If our study protocol were extended to 16 weeks of HFD-feeding, we would likely detect an increase of small adipocytes in the inguinal depot with the Coulter counter method. It would be interesting to examine whether a prolonged feeding intervention, with potential hyperplastic growth of the inguinal AT, would result in improved systemic insulin sensitivity. Using an alternative method to the Coulter counter would be required to further dissect the extent of hyperplastic growth in the inguinal depot.

In contrast to what we observed during weight gain, the plasticity of cell-size during weight loss was similar in both epididymal and inguinal AT. Cell-size, depot weight and number of hypertrophic adipocytes reverted to chow levels in both the 4- and 8-week groups but not in the 12-week reverse group.

Depot-specific weight loss and weight gain have been studied in non-obese mice by using fasting (72h) and refeeding [160]. The study revealed that epididymal fat has increased weight loss in response to fasting while the inguinal fat recovered in weight faster during refeeding [160]. In comparison to our results, this highlights that the level of obesity may influence the depot-specific differences in AT plasticity during weight gain and weight loss.

Collectively, our data suggest that there are fat depot-specific differences in cell-size plasticity during weight gain, but not during weight loss. However, the cell-size distribution may be misleading since it displays the diameter of adipocytes. As mentioned in the methodology section, the volume of a sphere (modeling the volume of adipocytes) is increased by 8 times when the diameter is doubled. In the 4-week group, the mean cell volume of inguinal adipocytes increased by 197%, comparing HFD with chow-fed mice, while the increase in epididymal adipocytes was only 172% (data not shown). In the same group, the epididymal AT increased by 0.98g (283% increase) while the inguinal AT only increased by 0.57g (219% increase), comparing HFD- to chow-fed mice. Thus, the inguinal AT displays high plasticity in terms of cell volume expansion, expressed as a percentage, but in actual volume and mass the epididymal depot expands more rapidly. This is likely related to the inguinal AT initially having smaller adipocytes compared with epididymal AT.

The impaired recovery of systemic metabolism in the 12-week reverse group was also reflected at a cellular level, where adipocytes had impaired insulin-stimulated glucose uptake. The fact that the GLUT4 expression in epididymal adipocytes was partly restored in the reverse group at this timepoint, without an improvement in insulin-stimulated glucose uptake, suggests that there are mechanisms other than GLUT4 expression, that influence glucose uptake. One such mechanism could be related to the level of cortical F-actin, which we found to be similar in epididymal

adipocytes in the 12-week HFD and reverse group. Possibly, in hypertrophic adipocytes, the limiting factor for GLUT4 translocation is actin dynamic rather than expression of GLUT4, which would fit our observations. An interesting continuation of this study would be to examine whether overexpression of GLUT4 in already hypertrophic adipocytes improves insulin-stimulated glucose uptake.

We found that epididymal and inguinal fat depots displayed similar plasticity in the reverse groups with regards to levels of F-actin, which fully reverted to chow level at the 4- and 8-week timepoints but only partly was restored at the 12-week timepoint. This suggests that the plasticity of the depots is similar during weight loss and that level of F-actin correlates well with cell-size, something we observed in a previous study [41].

Since bodyweight strongly correlates with several metabolic parameters, including insulin resistance, it is possible that the strong association between inguinal depot weight and bodyweight is a confounder in our dataset. The correlation between inguinal depot weight and QUICKI does not prove that the inguinal depot expansion causes impaired insulin sensitivity. However, we showed that the number of hypertrophic inguinal adipocytes correlated more strongly with QUICKI than the number of hypertrophic epididymal adipocytes. This association would be interesting to further investigate.

Subcutaneous lower-body fat has been suggested to function as a “metabolic sink” for storage of excess lipids. When this “sink” has reached its maximal expansion, due to limits to hypertrophic/hyperplastic growth, lipids start to accumulate as visceral and ectopic fat, leading to deteriorating insulin sensitivity [161]. However, our study shows that systemic insulin sensitivity progressively worsens as the inguinal depot expands in mass and cell-size, challenging the view of inguinal fat as a “metabolic sink” in male mice. It has previously been demonstrated that limited expansion of the epididymal fat directs the progression of insulin resistance, while the inguinal fat displays no limitation in expansion and continues expanding with increasing body weight [162].

Fat transplantation studies in mice have shown that inguinal fat is protective against metabolic diseases [57, 163]. It is important to emphasize that inguinal adipocytes are much smaller than epididymal adipocytes, and thereby likely possess a greater capacity to expand. It would be interesting to conduct a fat transplantation with hypertrophic inguinal adipocytes, similar to the sizes we observed after 12 weeks of HFD-feeding to see whether the beneficial effect persisted.

Alterations in the ECM composition and macrophage infiltration, measured as mRNA levels of *Cd44*, *Col6a3* and *Cd68,* were observed in the epididymal but not inguinal AT following 12 weeks of HFD-feeding and did not revert in the reverse group. These findings suggest that the plasticity in ECM remodeling and cell composition is impaired in the epididymal AT following prolonged feeding. However, this may not persist if the time of reversing were to be extended.

Interestingly, the mRNA expression of *Col6a3* was almost 8-fold higher in the inguinal AT compared to the epididymal AT in chow-fed mice and did not increase in response to HFD-feeding. This is an indication that COLVIα3 has a less important role for development of dysfunctional adipocytes in the inguinal AT. When the COLVI knockout mouse model was evaluated only epididymal and mesenteric fat was characterized [12]. It would be interesting to evaluate the inguinal AT using this model.

Summary and future perspectives

This thesis has provided further knowledge of adipocyte function, specifically regarding regulation of lipolysis (**paper I**), insulin-stimulated glucose uptake (**paper II**) and adipocyte plasticity (**paper III**). It highlights depot-differences of inguinal and epididymal adipocytes, and the importance of examining both visceral and subcutaneous fat in understanding the complexity of AT biology. There are several observations found during this work that would be interesting to investigate further.

Paper I describes the importance of EHD2 for sustained adipogenesis in the epididymal AT, although the mechanism remains unclear. It is uncertain whether this observation was restricted to the epididymal depot because of the low hyperplastic growth in inguinal AT in response to HFD-feeding, or whether EHD2 have different functions in the two fat depots. One possible explanation for the depot-differences we observed could be that the importance of EHD2 may be cell-size dependent. Inguinal adipocytes are smaller and EHD2 may be more important for sustaining function in hypertrophic adipocytes. EHD2 stabilize caveolae at the plasma membrane and when adipocytes expand in size the invaginations of caveolae serve as a membrane reservoir, protecting cells from mechanical stress [105, 164]. Therefore, EHD2 may be more important in the larger epididymal adipocytes. EHD2 have been reported to exert transcriptional regulation. As such, further research needs to be conducted to elucidate whether our observations are mediated directly through EHD2 or indirectly via the influence of caveolae stability and function.

In **paper II**, we propose a novel insulin-sensitizing mechanism in adipocytes, mediated via rosiglitazone. Rosiglitazone alters the ECM composition, which influences intracellular signaling transduction, and thereby promotes a more dynamic actin turnover, which leads to improved glucose uptake. This mechanism of action offers several potential drug targets for improving glucose uptake in hypertrophic adipocytes, something that could be tested in future studies. Modifying the ECM, favoring a less fibrotic AT, is of interest to future evaluations. New strategies, employing nanoparticle techniques have been tested for modulating the ECM in cancer tissue, with promising results [165]. Furthermore, collagenase coated micelles have been used to modify the ECM in hepatic fibrosis [166]. However, the broad spectrum of tissues affected by ECM modulating drugs makes selectively targeting the AT very challenging. Similarly, actin is expressed in all

cells and actin modifying drugs are toxic and have off-targets effect that make them unlikely to be of use clinically. It would be interesting to study actin modulating drugs in more detail in adipocytes *ex vivo*, but so far, most actin modulating drugs seem to impair insulin-stimulated glucose uptake [40, 41]. To validate the link between the ECM and level of cortical F-actin in hypertrophic adipocytes it would be of value to study the level of cortical F-actin in HFD-fed COLVI knockout mice. Since these mice exhibit less collagen deposition in the ECM, increased hypertrophic growth of the AT, but sustained insulin sensitivity, they may have a reduced level of cortical F-actin in the adipocytes. If so, this would strengthen our proposed mechanism of action for rosiglitazone. It would be valuable to examine whether the level of cortical F-actin in adipocytes correlates with BMI/insulin sensitivity in humans.

In the final project, **paper III**, we examined AT plasticity, focusing on cell-size, level of F-actin and association with systemic insulin sensitivity. We found fat-depot differences in cell-size plasticity, and expandability, during weight gain, but not during weight loss. The DIO-mouse model serves as a valuable tool for examining the relationship between AT and systemic metabolism. Our study provided further knowledge of the temporal cellular adaptations during weight gain and weight loss that can be used for deciding length of HFD-intervention in future studies. Our findings challenge the view of the subcutaneous fat depot acting as a metabolic "sink" in mice, which would be interesting to examine in future studies. One way to study how epididymal and inguinal fat, respectively, contribute to systemic insulin resistance during HFD-feeding would be to perform fat transplantation studies in mice. By using inguinal fat from donor mice that are very obese (45-50g), when the cell-size distribution of inguinal fat resembles that of epididymal fat, could reveal whether cell size, rather than depot-specific cell-intrinsic factors, is the most important variable influencing insulin resistance.

Populärvetenskaplig sammanfattning

Bakgrund

Antalet människor som lider av övervikt och fetma har öka kraftigt senaste årtiondena och väntas inom snar framtid omfatta mer än hälften av världens vuxna befolkning. Fetmarelaterade sjukdomar, inklusive typ 2 diabetes (T2D) har också ökat explosionsartat. T2D innebär att kroppen har förhöjt blodsocker p.g.a. för låg produktion av hormonet insulin, samt att kroppens muskler, lever och fettceller inte längre reagerar på insulin, s.k. insulinresistens. När normalt fungerande muskel och fettceller utsätts för insulin så ska de öka transporten av blodsocker (glukos) in i cellerna och på så sätt sänka blodsockret, men detta fungerar inte optimal hos T2D patienter.

Det finns ett samband mellan ökad fettmassa och risken att drabbas av T2D. Vidare är det även viktigt var på kroppen man lagrar in fett. Ökad mängd underhudsfett, så kallad subkutant fett, framförallt från midjan och nedåt, anses vara mer fördelaktig i jämförelse med bukfetma, så kallat visceralt fett. Det har även visats att storleken på fettcellerna är avgörande för risken att drabbas av T2D. När fettvävnaden ökar i storlek gör den det genom att nybilda fettceller (öka antalet fettceller) eller öka storleken på redan existerande fettceller. Om man har en ansamling av väldigt stora fettceller och inte kan skapa nya små fettceller, så ökar också risken för T2D.

I denna avhandling så har jag kartlagt förändringarna som sker i fettväven och fettcellerna under fettvävsexpansion. För detta används en djurmodell, bestående av möss som får en fettrik diet, vilket leder till viktuppgång och fettvävsexpansion. Extra fokus har lagts på förändring i cellstorlek samt att kartlägga skillnader mellan visceralt och subkutant fett, för att bättre förstå hur olika typer av fett regleras under fettvävsexpansion, och vad som kan gå fel.

Resultat

I det första arbetet så undersökte vi relevansen av ett protein som kallas för EHD2. Det finns mycket EHD2 i fettceller, och dess funktion är att binda till små inbuktningar vid cellens yta, så kallade caveolae, och därigenom stabilisera caveolae vid cellytan. För att undersöka betydelsen av EHD2 i fettceller så använde

vi oss av s.k. knockoutmöss, vilket innebar att mössen helt saknar proteinet EHD2. Vi observerade att dessa möss hade mindre visceralt fett medan mängden subkutant fett var intakt. Vidare så hade det viscerala fettet mindre mängd av väldigt små celler vilket tyder på att EHD2 är viktigt för nybildandet av fettceller. Vi fann även att EHD2 var viktigt för lipolys i subkutana fettceller. Lipolys är när fettcellerna utsöndrar fett som via blodet sedan transporteras till andra vävnader för att där användas som energi. Båda dessa fynd, försämrad lipolys och försämrad nybildande av fettceller är förknippade med dysfunktionell fettväv och kan medverka till progressionen av T2D, vilket tyder på att EHD2 är viktigt för hälsosam fettvävsexpansion.

I den andra studien så studerade vi mekanismen bakom ett anti-diabetesläkemedel, rosiglitazon, som tidigare använts för att bota T2D. Det är sedan tidigare känt att rosiglitazon verkar genom att öka nybildandet av fettceller, men hur läkemedlet påverkar stora fettceller är okänt, vilket var målet att utreda med denna studie. Vi använde oss återigen av möss som fick fettrik kost, för att sedan behandlas med rosiglitazon. Möss som hade fått behandling hade lägre mängd av proteiner i utrymmet som ligger mellan cellerna, även kallat extracellulär matrix. Minskad mängd proteiner i extracellulär matrix gör att fettcellerna utsätts för mindre stress, vilket vi också kunde se genom att stress-aktiveringen var lägre inne i fettcellerna efter behandling med rosiglitazon. Vi såg även en förändring av strukturen av cellskelettet, något som kan förändras av stress-aktivering och som medverkar i flera processer inne i cellerna. Cellskelettet medverkar bland annat till att föra ut molekyler till cellytan som hjälper till att transportera glukos (socker) in i fettcellen, detta sker som respons när cellerna stimuleras med insulin. Vi såg att glukos-transporten in i fettcellerna ökade efter behandling med rosiglitazon. Sammanfattningsvis så föreslår vi följande mekanism hur rosiglitazon förbättrar insulin responsen i stora fettceller, som i kronologisk ordning innefattar följande steg: minskad mängd extracellulär matrix proteiner, vilket leder till minskad stress-aktiveringen i fettcellerna, som i sin tur förändrar cellskelettet, vilket i sin tur förbättrar cellens förmåga att, i respons till insulin, föra ut molekyler till cellytan som ökar transporten av glukos in i cellerna. Effekterna av rosiglitazon var större i viscerala fettceller jämfört med subkutana fettceller, vilket tyder på att den ovanbeskrivna mekanismen har större relevans för viscerala fettväven.

I sista projektet så studerade vi hur cellstorleken och cellskelettet i fettceller förändras under viktuppgång och viktnedgång, samt hur det skiljer sig mellan subkutant och visceralt fett. Vi använde oss återigen av möss som fick fettrik diet i 4, 8 eller 12 veckor, för att studera viktuppgång. Vi hade även en grupp möss som först fick fettrik diet men sedan bytte tillbaka till vanlig diet, för att studera viktnedgång. Under det initiala skedet av viktuppgång så var det främst det viscerala fettet som expanderade och fick stora fettceller, medan subkutan fettcellerna framför allt blev stora i ett senare skede av viktuppgången. Under viktnedgång så betedde sig subkutant och visceralt fett liknande och minskade kraftigt i cellstorlek.

Cellskelettet förändras i samband med viktuppgång och viktnedgång, i både subkutant och visceralt fett, och speglade förändringar i cellstorlek. Vi mätte även insulinresistens i mössen och hittade att framförallt antalet stora subkutana fettceller, i större utsträckning än viscerala fettceller, var förenat med försämrad insulinkänslighet.

Slutsats

Studierna i denna avhandling visar att subkutant och visceralt fett skiljer sig på flera viktiga punkter under fettvävsexpansion i möss och att det därför är viktigt att studera båda fettvävnaderna för att få en klargörande helhetsbild. Specifikt belyses följande skillnader mellan subkutant och visceralt fett. EHD2 är viktigt för fettcellfunktion under fettvävsexpansion, för nybildning av fettceller i viscerala fettet och för lipolys i det subkutana fettet. Rosiglitazon har störst fördelaktig effekt i viscerala fettväven, där även extracellulär matrix ändras mest. Insulinresistensen i möss är förenat med stora fettceller i främst subkutana fettväven och i mindre utsträckning i visceral fettväven. Sammantaget så framstår det som att subkutant och visceralt fett verkligen påverkas olika under fettvävsexpansion. Att identifiera olikheter gör att framtida nya läkemedel kan inriktas mot en selektiv typ av fetma baserat på hur fettdistributionen på kroppen ser ut.

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