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IMMUNE CELL-DERIVED ADIPOKINES: ROLE IN WHITE ADIPOSE TISSUE FUNCTION AND LINK TO METABOLIC HEALTH

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Cover illustration: Graphical illustration depicting adipocytes and other cell types residing in the white adipose tissue. Created by Narmadha Subramanian.

Immune cell-derived adipokines: role in white adipose tissue function and link to metabolic health

Thesis for Doctoral Degree (Ph.D.)

By

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Popular science summary

The white adipose tissue (WAT) is specialized at safely storing excess energy in the form of lipids, thereby protecting other organs like liver, heart and muscle from lipid toxicity, which would impair their function and eventually lead to metabolic diseases. Thus, a functioning WAT is crucial for maintained metabolic health. The lipid storing cells of the WAT are the fat cells, which make up the vast volume of the tissue, but the WAT is also home to many types of immune cells. Fat cells and immune cells communicate with each other through secreted factors (adipokines), and this interaction is crucial to uphold the function of the WAT. In obesity, the enlarged fat cells reach their capacity to further expand properly. The increased stress of nutrient overload causes dysfunction and “leakage” of lipids into the blood stream, and both fat cells and immune cells start to secrete many more pro-inflammatory adipokines, resulting in a state of chronic low-grade inflammation, which further disrupts WAT function and metabolic health. These dysfunctional features are a major link between obesity and metabolic diseases.

In this thesis, we studied both physiological and pathological aspects of adipokines secreted by immune cells in the WAT. We focused on three different factors secreted under different circumstances and wanted to understand their impact on WAT function and dysfunction.

In **study I**, we investigated the pro-inflammatory factor IL-1 β , which is known to be chronically elevated in the obese WAT, where it can cause metabolic dysfunction. However, we discovered that acute, short-term elevations of IL-1 β can have a more beneficial effect; transient, but not chronic levels stimulated the formation of new fat cells. This is crucial to preserve metabolic health during obesity development, because it distributes lipid storage over a larger number of fat cells, thereby preventing individual cells from becoming excessively large and thus avoiding many of the dysfunctions associated to an obese WAT. IL-1 β has previously been shown to be transiently elevated in the WAT after food intake. Based on our results, we therefore believe that this meal-induced acute IL-1 β may function to stimulate formation of new fat cells, thus allowing the WAT to adapt to excessive energy intake.

Although obesity is a major risk factor for cardiometabolic diseases, a subset of non-obese individuals also suffers from these disorders. In **study II**, we wanted to investigate whether WAT inflammation plays a role in the manifestation of these conditions even in the absence of obesity. We identified the immune cell-secreted factor CCL18 as elevated in these individuals and found that it recruits other types of immune cells to the WAT, where it activates them to secrete pro-inflammatory factors. These factors, in turn, act on fat cells to release more lipids out into the circulation,

thereby contributing to elevated blood lipids. Thus, this study identified a novel communication occurring between different immune cells and fat cells in the WAT that could contribute to cardiometabolic diseases even in the absence of obesity.

Despite a larger fat mass, women are more protected than men from developing metabolic diseases. In **study III**, we wanted to investigate if this phenomenon could be due to sex differences in WAT inflammation. We found that the anti-inflammatory factor IL-10 was elevated in the WAT of obese women with type 2 diabetes compared to non-obese women with or without type 2 diabetes. Interestingly, no such elevation was seen for men. Since IL-10 is known to dampen inflammation, we therefore propose that this sex-specific production may be one reason that women are generally metabolically healthier than men.

In summary, this thesis has provided new insights on how immune cell-derived adipokines may influence, or be influenced by, WAT expansion and function. As such, they have contributed to our understanding of the role of WAT-resident immune cells in the development or prevention of metabolic diseases under different contexts.

Abstract

The white adipose tissue (WAT) is home to a vast array of immune cells that control local homeostasis and metabolism by engaging in intricate crosstalk with adipocytes and their precursors through secreted factors (adipokines). In the obese WAT, these immune cells adopt a pro-inflammatory profile, resulting in a state of chronic low-grade inflammation that can perturb local and systemic metabolic function. This thesis aimed to study physiological and pathological aspects of the interplay between immune cell-derived adipokines and processes pertaining to the expansion and metabolic function of the WAT.

A postprandial induction of the pro-inflammatory cytokine interleukin (IL)-1 β was previously demonstrated in macrophages of the WAT, but not other tissues, hinting to a possible involvement in local energy handling. Therefore, in **study I**, we employed *in vivo* and *in vitro* models to investigate a physiological, metabolic role of IL-1 β in the WAT. To our surprise, IL-1 signaling was of minor importance in mature adipocytes. Instead, we identified adipocyte precursors as the major target of IL-1 β in the WAT, where it stimulated the formation of new fat cells, a process linked to preserved metabolic health during obesity development. Strikingly distinct effects caused by acute and chronic treatments led us to propose that postprandial surges in IL-1 β has a physiological role in promoting healthy WAT expansion but that this effect may be lost in the chronically inflamed obese WAT.

Study II set out to identify WAT-secreted factors involved in cardiometabolic diseases of non-obese individuals. An adipokine screen identified the chemokine CCL18 as significantly elevated in cardiometabolic disease groups compared to healthy controls (all non-obese). Further explorations led us to conclude that CCL18 recruits CD4⁺ T cells and activates them to secrete interferon γ and transforming growth factor β 1, which in turn stimulate lipolysis of adipocytes, thereby contributing to cardiometabolic disease through release of fatty acids into circulation.

Women generally display a healthier metabolic phenotype than men. Whether differences in WAT inflammatory status may account for this is unknown. The anti-inflammatory cytokine IL-10 was previously shown to be elevated in WAT of obese women. **Study III** explored possible sex differences in WAT production of this cytokine. IL-10 secretion and IL-10-producing macrophages were elevated in WAT of obese women, but not men, with type 2 diabetes. Estrogens had no direct effect on IL-10 expression *in vitro*. This reveals a sex-specific regulation of IL-10 production in obesity, which may provide women with relative protection from obesity-associated inflammation.

Overall, this thesis has provided new insights on the complex role of immune cell-derived adipokines in metabolism and expansion of the WAT under different contexts, and how this may be linked to metabolic health.

List of scientific papers

I. IL-1 β promotes adipogenesis by directly targeting adipocyte precursors

Hofwimmer K¹, de Paula Souza J¹, Subramanian N, Rachid L, Méreau H, Zhao C, Vujičić M, Wernstedt Asterholm I, Böni-Schnetzler M, Meier DT², Donath MY², Laurencikienė J². Manuscript.

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II. Adipose tissue specific CCL18 associates with cardiometabolic diseases in non-obese individuals implicating CD4⁺ T cells

Subramanian N, Hofwimmer K, Tavira B, Massier L, Andersson DP, Arner P, Laurencikienė J.

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III. Sex-specific regulation of IL-10 production in human adipose tissue in obesity.

Subramanian N, Tavira B, Hofwimmer K, Gutschmann B, Massier L, Abildgaard J, Juul A, Rydén M, Arner P, Laurencikienė J.

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List of abbreviations

ADIPOQ	Adiponectin
ATGL	Adipose triglyceride lipase
ATM	Adipose tissue macrophage
BMI	Body mass index
BMP	Bone morphogenetic protein
C/EBP	CCAAT/enhancer-binding protein
cAMP	cyclic adenosine monophosphate
CCL	CC motif chemokine ligand
CCR	C-C chemokine receptor
cDNA	Complementary DNA
CGI-58	Comparative gene identification 58
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
CRE	cAMP response element
CREB	cAMP response element-binding protein
CVD	Cardiovascular disease
E1	Estrone
E2	Estradiol
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
epiWAT	Epididymal WAT
ERK	Extracellular signal-regulated kinase
FABP4	Fatty acid-binding protein 4
FACS	Fluorescence-activated cell sorting
FASN	Fatty acid synthase
FFA	Free fatty acid
FoxO1	Forkhead box protein 1
GSK3 β	Glycogen synthase kinase 3 β
gWAT	Perigonadal WAT

hASC	Human adipose-derived stem cell
HFD	High-fat diet
HSL	Hormone-sensitive lipase
IBMX	3-Isobutyl-1-methylxanthine
IFN- γ	Interferon γ
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
I κ B	Inhibitor of κ B
IKK	I κ B kinase
IL	Interleukin
IL-10R	IL-10 receptor
IL-1R1	IL-1 receptor type 1
IL-1Ra	IL-1 receptor antagonist
IR	Insulin receptor
IRAK	Interleukin 1 receptor-associated kinase
IRS	Insulin receptor substrate
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen-activated kinase
MCP-1	Monocyte chemoattractant protein-1
MSD	Meso Scale Discovery [®]
MyD88	Myeloid differentiation primary response 88
mTOR	Mammalian target of rapamycin
NF κ B	Nuclear factor κ -light-chain-enhancer of activated B cells
PI3K	Phosphoinositide 3 kinase
PKA	Protein kinase A
PLIN-1	Perilipin-1
PPAR γ	Peroxisome proliferator-activated receptor γ
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
scWAT	Subcutaneous WAT
STAT	Signal transducer and activator of transcription

SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TAG	Triacylglycerol
TGF- β	Transforming growth factor β
TNF- α	Tumor necrosis factor α
TZD	Thiazolidinedione
vWAT	Visceral WAT
WAT	White adipose tissue
WT	Wild-type

1 INTRODUCTION

1.1 Obesity

From an evolutionary perspective, our understanding of the white adipose tissue (WAT) as an energy reservoir is quite straightforward; during times of temporary food abundance, the WAT safely stores excess calories and later, during famine, releases them to provide energy for other organs according to their needs. The environment in large parts of the world today is drastically different, with a constant abundance of available energy. Consequently, obesity, characterized by excessive adiposity, has reached epidemic proportions and is now one of the leading health concerns of our time. Body mass index (BMI) calculated from height and body weight (m^2/kg) is used to clinically define obesity ($\text{BMI} \geq 30$) and overweight ($\text{BMI} \geq 25$)¹. In 2016, an estimated 26% of the adult world population was overweight, and an additional 13% obese², and numbers are higher in developed countries³. Obesity is a major risk factor for hypertension, dyslipidemia, and insulin resistance and ultimately for the development of metabolic diseases, such as type 2 diabetes (T2D), cardiovascular diseases (CVDs), non-alcoholic fatty liver disease, and many cancer forms³. Understanding the role of excessive adiposity in the pathophysiology of these conditions is therefore of crucial importance.

1.2 Resident cells of the adipose tissue

Since the adipocytes are the lipid storing cells and consequently make up the vast majority ($> 90\%$) of the WAT volume⁴, they have naturally gained the most attention among the WAT-resident cells. However, adipocytes have been reported to only constitute around 20-40% of the cells in the WAT⁴⁻⁶, and many additional cell types reside in the stromal vascular fraction (SVF) of the tissue. In addition to fibroblasts and cells belonging to the vasculature (e.g. endothelial cells, pericytes and smooth muscle cells), this fraction mainly consists of mesenchymal stem cells and immune cells⁷.

1.2.1 Adipocytes and their precursors

Since adipocytes are considered post-mitotic cells, the differentiation of resident progenitors (adipogenesis) is crucial both to maintain and increase fat cell number⁸. Adipocyte turnover, in which dying cells are replaced with new ones, occurs at an annual rate of around 8% in humans⁹, and during caloric excess, an increased adipocyte number enhances lipid storage capacity. Adipocyte progenitors constitute a highly heterogeneous population, and much remains to be elucidated about specific subtypes and their distinct functions. The WAT contains progenitors with different levels of commitment to the adipocyte lineage^{5,10}, and the ones with a high adipogenic propensity are generally found in the perivascular region of the tissue¹⁰⁻¹². There are also subtypes with a more regulatory, anti-adipogenic role^{10,13}. Adipocytes, on the

other hand, were long viewed as a rather homogenous group of cells. However, recent spatial transcriptomics suggest otherwise, as three distinct subtypes could be identified, only one of which responded to insulin¹⁴. Murine single-nuclei data also reported three adipocyte subtypes with distinct metabolic profiles, which were altered in obesity¹⁵.

1.2.2 Immune cells

The immune cell compartment of the WAT consists of a vast array of immune cells that engage in intricate crosstalk with each other as well as with other resident cells, including adipocytes and progenitors. Among these, different subtypes of adipose tissue macrophages (ATMs) and T cells are predominant, but smaller proportions of monocytes, natural killer cells, dendritic cells, B cells, mast cells, neutrophils, innate lymphoid cells, and eosinophils are also present^{5,16,17}. The development of single-cell technologies has revealed the heterogeneity of these cells and enabled identification of many new subtypes^{18,19}. In the healthy state, these cells are involved in tissue repair and remodeling as well as metabolic and inflammatory regulation^{20,21}, and thus play a crucial physiological role for overall tissue homeostasis. However, both the abundance and phenotype of immune cells are drastically different in the obese WAT, and obesity-associated metabolic disorders are largely attributed to the state of chronic low-grade inflammation propagated by these cells (discussed in later sections).

ATMs are the most abundant and consequently the most studied immune cells of the WAT. They are important for WAT remodeling, clearance of dead fat cells, and regulation of adipocyte lipolysis²¹, and they also engage in many lipid-handling activities, both in lean and obese WAT (discussed in later sections). ATMs have traditionally been divided into classically (M1) and alternatively (M2) activated macrophages; the latter are the predominant ATMs in lean WAT²² and produce anti-inflammatory cytokines, whereas M1 ATMs are markedly increased in obesity²³ and display a highly pro-inflammatory profile, such as secretion of reactive oxygen species, nitric oxide, tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), and interleukin 1 β (IL-1 β)²⁴. However, these classifications are oversimplified and have been challenged at least in human WAT, where M2-like ATMs exhibit many M1-like features^{25,26}, in line with the identification of a mixed M1/M2 subtype²⁷. Further, although the pro-inflammatory ATMs seen in the obese WAT indeed have many M1-like characteristics, there are several distinctions between classically activated M1 macrophages and these obesity-associated ATMs^{28,29}, which are heterogeneous, and some major subgroups have instead been termed metabolically activated²⁸ and, more recently, lipid-associated macrophages (LAMs)¹⁹.

Among WAT-resident T cells, T helper (Th) cells (CD4⁺) and cytotoxic T lymphocytes (CD8⁺) are predominant⁵. The latter are enriched in the obese WAT³⁰ and highly contributing to its pro-inflammatory state, partly through recruitment of M1-like ATMs to the WAT^{31,32}. CD4⁺ T cells can be further divided into several subtypes, including

Th1, Th2, Th17 and regulatory T cells (Tregs). Th1 and Th17 are enriched in the obese WAT and release pro-inflammatory factors like interferon γ (IFN- γ) and interleukin 17 (IL-17), respectively, both of which are involved in obesity-associated WAT inflammation³³. For instance, IFN- γ stimulates ATM polarization into an M1-like phenotype³⁴ and IL-17 can induce expression of chemokines important for immune cell infiltration, including monocyte chemoattractant protein-1 (MCP-1)³⁵. Tregs and Th2 cells, on the other hand, display a more anti-inflammatory profile and seem to be important for inflammatory and metabolic homeostasis³³. The latter secrete IL-4, IL-5 and IL-13, which can polarize ATMs into an M2-like phenotype³³. Tregs secrete IL-10, thus attenuating inflammation³⁶. They are much more prominent in visceral than subcutaneous depots and most research has thus been focused on the former, where their depletion rapidly (four days) leads to increased WAT inflammation and elevated plasma insulin levels in lean mice³⁷, highlighting a crucial role in tissue homeostasis.

1.3 Main functions of the adipose tissue

For a long time, the WAT was considered a rather passive organ, with the main purpose of storing lipids, which, in addition to providing physical cushioning and thermal insulation, acts as an energy deposit for times of need. More recently, its additional role as an endocrine organ has become increasingly appreciated; since the discovery of the leptin gene in 1994³⁸, a plethora of WAT-derived factors known as adipokines have been described. These include hormones, cytokines, chemokines, and other factors that can act in auto- para- or endocrine manners to regulate a wide range of physiological and pathological processes both locally and throughout the body, including the brain.

1.3.1 Lipid storage and release

Adipocytes are responsible for the central actions of the WAT, namely storing and releasing lipids. Consequently, fat cells are highly dynamic and capable of remarkable expansion, and diameters can range from < 20 to 300 μm in humans³⁹. Lipid storage is mediated through the process of lipogenesis, in which adipocytes convert free fatty acids (FFAs) to triacylglycerols (TAGs) through enzymatic esterification of three FFAs to a glycerol backbone, the latter of which is produced intracellularly through the glycolytic pathway⁴⁰. The TAGs are stored in the large lipid droplet that takes up almost the entire intracellular space. The FFAs can be taken up directly from circulation⁴¹, but most are obtained from lipoprotein lipase-mediated cleavage of circulating lipoproteins in the capillary lumen⁴⁰. Alternatively, FFAs can be intracellularly synthesized from acetyl coenzyme A (acetyl-CoA) produced from glycolysis- and TCA-cycle-derived citrate, in which case it is called *de novo* lipogenesis. Acetyl-CoA is enzymatically converted to a FFA in a stepwise reaction including fatty acid synthase (FASN), which is the rate-limiting enzyme of *de novo* lipogenesis⁴².

Conversely, in the process of lipolysis, TAGs are hydrolyzed into three FFAs and one glycerol molecule, all of which are released into the circulation. This process involves three main enzymatic steps⁴³; first, one TAG molecule is converted into diacylglycerol and one FFA by adipose triglyceride lipase (ATGL), which requires the co-activator comparative gene identification 58 (CGI-58) for optimal activity. In the second step, hormone-sensitive lipase (HSL) hydrolyzes the diacylglycerol into monoacylglycerol and one FFA. Lastly, monoacylglycerol lipase converts the monoacylglycerol into one FFA and one glycerol molecule. HSL can also hydrolyze both TAG and monoacylglycerol, but it shows the highest activity against diacylglycerol⁴⁴. Lipolysis can be stimulated by a variety of factors, including natriuretic peptides, growth hormone, glucocorticoids, cytokines, and thyroid hormone, but the primary stimulators are catecholamines. Norepinephrine and epinephrine are increased through sympathetic nerve input and in circulation⁴⁵, respectively, during e.g. exercise⁴⁶ and fasting⁴⁷ and bind to adrenoceptors on adipocytes. This leads to activation of adenylate cyclase and elevated cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA)⁴⁵. PKA phosphorylates perilipin-1 (PLIN-1), a lipid droplet-associated protein that binds to CGI-58 in the basal state⁴⁸. PLIN-1 phosphorylation releases CGI-58, thus allowing it to interact with ATGL⁴⁸. In addition, PKA also phosphorylates HSL⁴⁹, thereby enabling its binding to the lipid droplet. Spontaneous (often termed basal) lipolysis in the fed, resting state also occurs and can be promoted by e.g. pro-inflammatory cytokines⁵⁰, possibly through activation of extracellular signal-regulated kinase (Erk) 1/2, which can phosphorylate HSL⁵¹. Basal, but not catecholamine-stimulated, lipolysis is associated to dyslipidemia⁵².

Both lipogenesis and lipolysis are also regulated by insulin, which acts directly on adipocytes through the insulin receptor (IR). Canonical insulin signaling is mediated through the IR substrate (IRS)/phosphoinositide 3 kinase (PI3K)/Akt pathway. PI3K activated by insulin increases lipoprotein lipase activity⁵³, thus increasing FFA uptake. Additionally, insulin promotes *de novo* lipogenesis through Akt-mediated translocation of glucose transporter 4 (GLUT4) to the plasma membrane and thus enhanced uptake of glucose to be utilized for production of both acetyl-CoA and glycerol. Mice with adipocyte-specific knockout of GLUT4 develop insulin resistance⁵⁴. Conversely, the same signaling arm inhibits the lipolytic pathway, possibly partly explaining why basal lipolysis is increased in insulin resistant individuals. Akt activates phosphodiesterase 3B through phosphorylation, which leads to decreased cAMP levels and ultimately inhibition of ATGL and HSL activity. Insulin also downregulates ATGL expression through inhibition of its transcriptional activator forkhead box protein 1 (FoxO1)⁵⁵. Thus, insulin is an important regulator of the main adipocyte functions, and preserving insulin sensitivity of adipocytes is therefore crucial for the metabolic function of the WAT, and ultimately the whole body.

1.3.2 Adipokines

In addition to functions related to lipid mobilization, the WAT also produces and secretes a vast array of factors. These are central to the crosstalk between resident cells and thereby regulate essentially all local processes taking place in the tissue, but they are also important endocrine factors influencing metabolic and inflammatory processes in cells throughout the body. Adipocytes and immune cells are the major sources of adipokines, which include hormones and other peptides such as leptin³⁸, adiponectin⁵⁶, resistin⁵⁷, and apelin⁵⁸, as well as classical cytokines and chemokines like TNF- α , monocyte chemoattractant protein-1 (MCP-1), IFN- γ , IL-6, IL-1 β , IL-10, and transforming growth factor β 1 (TGF- β 1)⁵⁹. Leptin and adiponectin have important metabolic functions and are the most extensively researched adipocyte-derived hormones.

Leptin was first identified when it was discovered that the extreme overeating and obesity displayed by *ob/ob* mice are due to genetic deficiency of this hormone³⁸, which acts in the brain to promote satiety and energy expenditure⁶⁰. Circulating leptin levels are increased in proportion to total fat mass⁶¹ but are also highly influenced by feeding state, with marked elevations and declines in the fed and fasted states, respectively⁶²⁻⁶⁴. However, the elevated levels in obesity are clearly not sufficient to return to a normal body weight. This has been attributed to central leptin desensitization, at least partly due to a saturated blood-brain barrier transport⁶⁵. Instead, in obesity, the increased levels aggravate the pro-inflammatory actions of leptin on peripheral immune cells, on which leptin act directly through the leptin receptor, both in WAT and other tissues, and thereby appears to be contributing to the obesity-associated chronic inflammation with adverse metabolic outcomes⁶⁶. Conversely, as a direct consequence of plummeted leptin levels, such as during starvation, central production of reproductive and thyroid hormones is decreased, which preserves energy by preventing pregnancy and reducing metabolic rate, respectively. Thus, leptin has likely evolved as a marker of *sufficient*, rather than *excessive*, net energy intake⁶⁷.

In contrast to leptin, adiponectin expression and circulating levels are decreased in obesity and strongly linked to preserved metabolic health⁶⁸. *ob/ob* mice overexpressing adiponectin display markedly improved metabolic health despite a more extreme obesity⁶⁹. A single adiponectin injection in mice has glucose- and lipid-lowering effects^{70,71}, and long-term treatment reduces triglyceride content in liver and muscle⁷². These effects have been attributed to direct regulation of the hormone on glucose- and lipid-handling processes in all major metabolic tissues. For instance, adiponectin induces insulin secretion and expression in pancreatic β -cells^{73,74}, promotes GLUT4 membrane translocation and glucose uptake as well as fatty acid oxidation in skeletal muscle cells^{70,75,76}, and enhances insulin-induced suppression of glucose production by hepatocytes at least partly through downregulation of gluconeogenic enzymes^{71,77}. In adipocytes, adiponectin improves insulin-induced

glucose uptake by promoting translocation and expression of GLUT4⁷⁸. Further, adiponectin appears to promote healthy WAT expansion and storage of lipids⁷⁸, thereby preserving the function of the adipocytes and reducing lipid accumulation in liver⁶⁹.

1.4 Role of adiposity in metabolic diseases

1.4.1 Adipose tissue dysfunction links obesity to metabolic diseases

The obese WAT is not only *larger* but also displays many distinct characteristics indicative of a *dysfunctional* WAT. This is reflected by a state of chronic low-grade inflammation, fibrosis, altered adipokine secretions, and hypoxic and insulin resistant adipocytes with an increased basal lipolysis, which “leak” lipids out into the circulation⁷⁹. These adverse features are believed to be the link between excessive adiposity and metabolic diseases, and understanding the underlying factors disrupting the function of a healthy WAT is thus of crucial importance.

1.4.2 Metabolic diseases in the absence of obesity

Despite the close association between obesity and metabolic disorders, excessive fat mass is not required to develop these complications. Perhaps the most convincing support for this comes from people suffering from lipodystrophy, a heterogenous disorder characterized by partial or complete loss of WAT. Metabolic impairments otherwise mostly observed in overweight and obese individuals, such as insulin resistance, T2D, dyslipidemia, and hepatic steatosis, are often also manifested in these patients⁸⁰, and similar observations have been made in different mouse models of lipodystrophy^{81,82}. Importantly, increasing the adiposity of these lipoatrophic mice by WAT transplantation from a healthy donor leads to complete or partial reversal of their metabolic complications⁸³. Conversely, surgical removal of scWAT markedly aggravates high fat diet (HFD)-induced increases in fasting plasma insulin and leptin levels⁸⁴. As a less extreme example, metabolic disorders are present in a substantial number of non-obese individuals; T2D prevalence among overweight and lean individuals in the U.S. has been reported to be 13% and 7%, respectively⁸⁵, and data from a German registry showed that 8% of people with T2D are lean⁸⁶.

1.4.3 Adipose tissue hyperplasia preserves metabolic health

The metabolic impairments displayed by lipodystrophic individuals reflect the crucial role of WAT to safely store excess energy. If it fails to do so, lipids will instead increase in circulation and cause lipotoxicity in other organs, which can result in fatty liver, pancreatic β -cell failure, heart disease, and insulin resistance in e.g. skeletal muscle and liver⁸⁷. Although lipodystrophy is an extreme manifestation of lipid storage

inability, all individuals seem to have a finite capacity for proper WAT expansion, which becomes increasingly evident with greater adiposity.

Storage capacity of the WAT is highly influenced by its means of expansion. The WAT can expand either through increased *size* (hypertrophy) or *number* (hyperplasia) of adipocytes. Hypertrophic expansion is coupled with some challenges. As it becomes larger, the adipocyte is exposed to increasingly higher mechanical stress as its surface presses against the surrounding ECM⁸⁸. To relieve this stress, ECM remodeling by degradation and reorganization of its components is crucial to allow for more space for the expanding cells. However, production, rather than degradation and reorganization, of ECM components is increased in obesity⁸⁹, ultimately causing fibrosis. Accordingly, deletion of the ECM component collagen VI allows for larger adipocytes, concomitant with reduced WAT inflammation, improved fibrotic profile, and improved metabolic health in obese mice⁸⁹. Further, oxygen requirement becomes greater with increased size, but the vascularization of the obese WAT is generally poor, as shown by lower vascular density⁹⁰. Thus, the expanding adipocyte eventually becomes hypoxic. As a consequence, the hypoxic signal hypoxia-inducible factor 1 α (HIF-1 α) is increased in the obese WAT⁹¹. HIF-1 α has pro-angiogenic properties in other contexts⁹² but does not seem to be able to induce angiogenesis in the obese WAT⁹³. Instead, it induces inflammation and systemic insulin resistance⁹⁴, and it increases the production of ECM components, thus contributing to the fibrosis seen in the obese WAT⁹³. Since fibrosis causes stiffness of the tissue, the mechanical stress of the adipocytes becomes further augmented. These stressors likely also explain why large adipocytes secrete more inflammatory factors, even when adjusted for cell surface volume⁹⁵. Indeed, hypoxia activates inflammatory signaling pathways in adipocytes *in vitro*. They also secrete more leptin⁹⁶ but less adiponectin⁹⁷.

Conversely, hyperplastic expansion through differentiation of progenitors into mature adipocytes is a process linked to a more extensive structural remodeling of the tissue and is associated with concomitant angiogenesis and ECM remodeling⁹⁸⁻¹⁰¹. Thus, hyperplastic expansion preserves WAT function by protecting the adipocytes from the mechanical and hypoxic stress coupled to excessive enlargement by shifting lipid storage away from mature adipocytes into newly formed ones and by maintaining vascular and structural integrity. Many interventions that stimulate hyperplastic expansion have proven beneficial for metabolic health despite unaltered or even increased (subcutaneous) WAT mass, in both mice^{69,102-105} and humans¹⁰⁶.

Human observational studies show strong links between the metabolic syndrome and a hypertrophic WAT. Adipocyte size has repeatedly been positively correlated to markers of metabolic disease, independently of BMI or total body fat^{96,107-111}. Similarly, non-obese diabetics have larger adipocytes in the scWAT than BMI-matched healthy individuals¹¹². In line with this finding, genetic predisposition to T2D is associated with a larger adipocyte size in both lean¹¹³ and non-obese¹¹⁴ individuals. Vice versa,

metabolically healthy obese subjects have smaller adipocytes than metabolically unhealthy obese subjects¹¹¹.

Thus, during prolonged overnutrition, mature adipocytes become increasingly stressed due to excessive enlargement. If not resolved through hyperplastic expansion, these stressors eventually lead to hypoxia, fibrosis, necrosis, and metabolic dysfunction (Figure 1). For reasons unknown, the rate of healthy WAT remodeling in obesity generally appears insufficient to avoid the dysfunctions caused by hypertrophic adipocytes. Understanding the mechanisms regulating adipogenesis could thus be useful in order to find new targets for the treatment of obesity-associated metabolic disease.

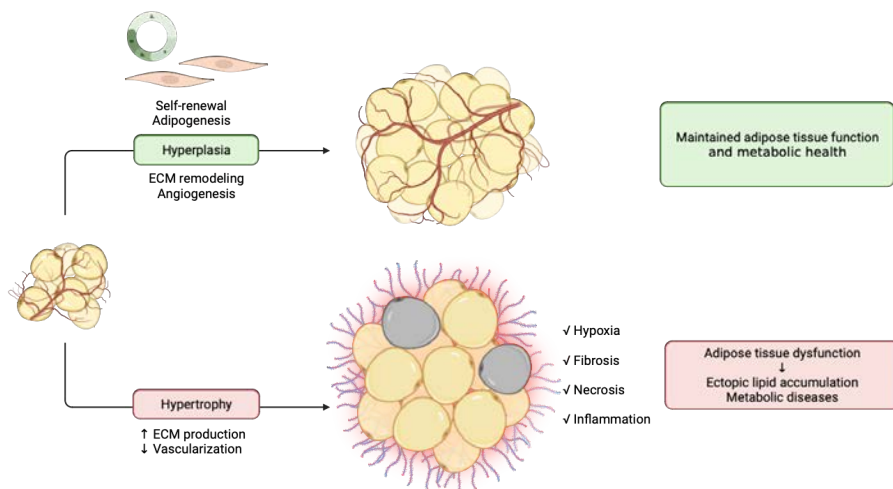


Figure 1. WAT expansion through hyperplasia and hypertrophy, as explained in the text. Created with BioRender.com

1.4.4 Depot- and sex-specific aspects

Several observational studies have highlighted the importance of *distribution*, rather than *total mass*, of body fat for metabolic health. Body fat is distributed in different WAT depots throughout the body. The majority is stored in the subcutaneous WAT (scWAT), which is located immediately beneath the skin all over the body, although it is generally largest in the abdominal and gluteofemoral (thighs and buttocks) regions. The visceral WAT (vWAT) is located more internally, between the organs within the abdominal cavity. Both abdominal scWAT and vWAT mass correlate positively to metabolic complications¹¹⁵⁻¹¹⁷, but the correlation is stronger for the latter^{117,118}. Conversely, leg/peripheral (subcutaneous) fat mass has either been inversely or not at all associated to metabolic disorders^{115,116,119}. In line with this, indicators of abdominal fat (e.g. waist circumference) are stronger predictors of metabolic syndrome than BMI¹²⁰⁻¹²². The reason for these depot-specific associations to

metabolic diseases is not entirely understood, but they mirror the metabolically and inflammatory dysfunctional features of abdominal, especially vWAT, depots. Compared to abdominal scWAT, vWAT has a more pro-inflammatory profile^{123,124}, lower adiponectin production¹²⁵, and higher basal lipolytic rate¹²⁶. The unique vWAT property of being portally drained, and thus delivering lipids and cytokines directly to the liver, may contribute to more adverse outcomes¹²⁷. Another, not mutually exclusive, theory is that increased vWAT mass merely reflects a relative inability of the scWAT to expand and safely store lipids, which causes ectopic lipid accumulation (and increased vWAT mass) independently of vWAT-mediated effects^{128,129}. The more beneficial metabolic status associated to gluteofemoral compared to abdominal scWAT is not well understood. Gluteofemoral lipid flux is less influenced by acute feeding status than abdominal scWAT, reflected by lower uptake of meal-derived lipids and lower FFA release in the postabsorptive state¹³⁰. However, no such regional difference is observed for uptake of recycled cholesterol and FFAs, and the gluteofemoral depot has therefore been proposed to act as a secondary, more long-term deposit of the energy that other depots cannot handle short-term¹³⁰. In line with this, femoral WAT may be better adapted for healthy expansion, as it exhibits a higher degree of hyperplasia than abdominal scWAT^{131,132}, and it has a larger abundance of adipocyte progenitors¹³³. Additionally, lower IL-6 secretion from this depot has been observed¹³⁴.

Notably, women preferentially store body fat in gluteofemoral regions, a trait that has been attributed to estrogens¹³⁵, whereas men tend to accumulate abdominal fat. This sexual dimorphism likely partly explains why women generally have a more favorable metabolic phenotype than men, who develop T2D at a younger age and lower BMI than women¹³⁶, despite women, on average, having 10% more body fat for a given BMI¹³⁷. Even in obesity, women appear more protected, as the proportion of obese women who are metabolically healthy is higher than among obese men^{138,139}. These sex differences are also reflected in animal models; male mice fed HFD display a more pro-inflammatory WAT phenotype^{140,141} and develop glucose intolerance and liver steatosis to a higher degree than females¹⁴⁰. The relative protection against HFD-induced ectopic lipid accumulation in female mice can be mimicked in males by administering estrogen, which enhances insulin-mediated suppression of adipocyte lipolysis through decreased WAT inflammation¹⁴². How many of these sex differences can be attributed to greater gluteofemoral lipid storage in women, and whether the inflammatory status of the obese WAT differs between sexes in humans, is not well understood.

1.5 Hypertrophic and hyperplastic adipose tissue expansion *in vivo*

Under healthy and energetically balanced conditions, the WAT is a highly dynamic organ, with continuous lipid flux and adipocyte turnover. Adipocyte size and number remain stable through a balanced rate of lipogenesis and lipolysis on the one hand,

and apoptosis and adipogenesis on the other hand. Under caloric excess, this equilibrium is shifted to favor lipid accumulation in the WAT through an increase in size and/or number of adipocytes. Since the latter is linked to preserved metabolic health, many studies have aimed to determine the degree to which these two distinct processes contribute to overall expansion of the tissue, and how they may differ in different depots.

1.5.1 Mice

To track adipogenesis of murine WAT, different pulse-chase models have been utilized, which are based on the labeling of either adipocytes or progenitors (Figure 2). The former can be selectively labeled in transgenic mouse models expressing an inducible reporter (e.g. *lacZ* or a fluorescent protein) under control of the adiponectin promoter^{8,143,144}. Treatment with an inducer (usually doxycycline) permanently labels all existing adipocytes, but subsequently formed fat cells will be unlabeled and thus possible to distinguish. Alternatively, progenitors have been labeled through similar strategies but different promoters (most often *Pdgfrb*), thereby enabling lineage tracing^{105,145}, or with thymidine analogs¹⁴⁶⁻¹⁴⁹, which are only incorporated during proliferation and thus not in post-mitotic adipocytes. Adipocytes stemming from labeled progenitors can then be detected. The thymidine analog labeling has also been used to measure progenitor proliferation, which has uncovered rapid responses to overfeeding, and all models have provided valuable insights on the mode of WAT expansion and how it differs between different depots.

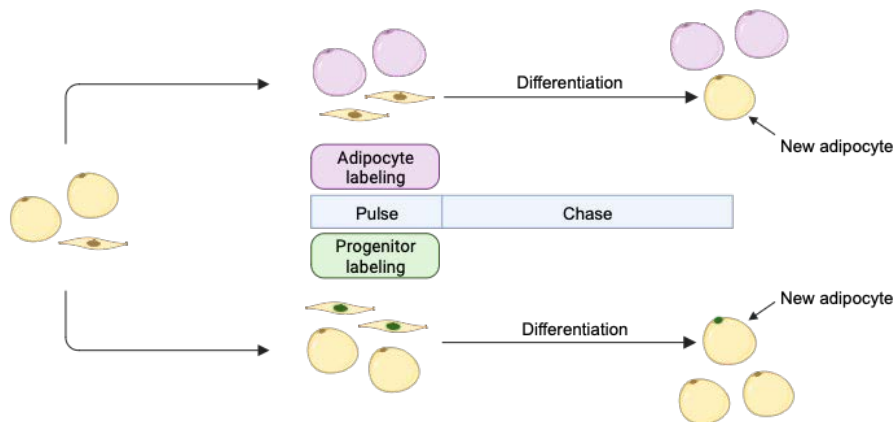


Figure 2. Two general strategies to track adipogenesis *in vivo*, as explained in the text. Created with BioRender.com

At the onset of HFD, adipocytes in both scWAT and vWAT markedly increase in size, with essentially no new fat cell formation^{143,147}, likely partly because adipogenesis is a much slower process than lipogenesis. However, after this initial hypertrophic phase that seems to last at least a month, the two depots appear to expand in strikingly

different ways. Most studies can measure new adipocytes in the vWAT after around 7-8 weeks of HFD^{143,145-147,149}. Despite the initial absence of new adipocyte formation, the progenitors of the vWAT respond very rapidly to HFD; their proliferation rate is increased already after one day of HFD, peaks after 3 days, and returns to baseline levels after 5 days, where it stays for at least 5 weeks¹⁴⁷. The fate for many of these progenitors is clearly differentiation; several weeks later, there is a significant increase in new adipocytes stemming from proliferated precursors, and the rapidly responding precursors can no longer be detected in the progenitor fraction¹⁴⁶, suggesting either they have all differentiated, or some have undergone apoptosis. Stopping HFD after the initial intense proliferation phase results in fewer new adipocytes¹⁴⁶, suggesting these rapidly responding progenitors require additional cues in order to proceed to differentiate. Interestingly, after those 7 weeks, the proliferated precursors cannot be detected in the progenitor fraction either¹⁴⁶, suggesting they undergo apoptosis in the absence of additional cues. This is noteworthy, because if these mice receive a second short-term HFD intervention several weeks later, no induction in progenitor proliferation is observed¹⁴⁶, suggesting that just a short period of overfeeding depletes, or alters function of, the self-renewing adipocyte precursor pool from the vWAT for at least several weeks. Similar observations have been made in scWAT after long-term treatment with thiazolidinediones (TZDs), which initially stimulates adipogenesis but eventually impairs self-renewal and adipogenic properties of progenitors¹⁵⁰.

Data on scWAT expansion are somewhat more varying. Many studies, lasting up to 3 months, were unable to detect progenitor proliferation or new adipocyte formation in response to HFD^{143,147,149}, but others have reported a significant increase exceeding that of the vWAT¹⁴⁸. In this case, adipogenesis was tracked much later during the course of HFD (from day 50), which could indicate that hyperplastic expansion begins later in scWAT. The depot differences seem largely determined by distinctions in microenvironment, not intrinsic differences between adipocyte precursors, as shown by transplantation experiments¹⁵¹. Importantly, sex differences have been reported regarding these depot-specific observations; whereas vWAT expands by hyperplasia in response to HFD in both sexes, the scWAT of females, but not males, also expands by hyperplasia¹⁵¹. This was demonstrated by a rapid increased progenitor proliferation, similar to that seen for vWAT, and formation of new adipocytes, and has been directly attributed to estrogen¹⁵¹. Of note, the majority of studies tracking adipogenesis have been performed in male mice.

An important caveat regarding these observations is that the vWAT investigated in almost all murine studies is the perigonadal WAT (gWAT), also referred to as epididymal WAT (epiWAT) in males. This depot is very tiny in humans, where omental, mesenteric and perirenal fat are the dominating visceral depots¹⁵². The gWAT differs from other murine visceral fat pads as well as from the major human vWAT depots, and it has therefore been termed a “peri-visceral” depot¹⁵³. For instance, it does not drain to the portal vein¹⁵², and its immune cell composition and expansion mode differs

from that of murine mesenteric vWAT¹⁵⁴. Thus, it can be questioned whether gWAT-specific observations can be translated to vWAT in humans.

1.5.2 Humans

In lean, healthy individuals, all major human depots (vWAT and abdominal and femoral scWAT) expand in mass upon 8 weeks of overfeeding^{131,155-157}. By measuring adipocyte size and number before and after overfeeding in these individuals, it has been demonstrated that scWAT of the gluteal, abdominal, and triceps regions expand by hypertrophy^{131,158}, while the femoral scWAT expands by hyperplasia¹³¹. In line with this, plotting abdominal scWAT or vWAT mass against adipocyte size reveals a positive association that is stronger at lower fat mass values and partially plateaus at higher WAT mass⁹, indicating that hypertrophy is the predominant expansion mode during early weight gain, but that hyperplasia may increase at later stages. Indeed, observational data has shown that the variation in vWAT mass between individuals primarily is determined by variations in adipocyte number, suggesting this depot mainly expands by hyperplasia¹⁵⁹. An elegant study using carbon dating to assess age, and thereby turnover rate, of adipocytes from abdominal scWAT found that, although fat cell number is higher in obese individuals, it seems to be mainly determined during childhood and adolescence, after which it remains at a constant level, suggesting that adipogenesis does not play an important role for expansion of this depot during adult obesity⁹. A caveat is that the obese subjects in this study had early-onset obesity; it is thus possible that adipogenesis plays a role at later stages of obesity.

1.6 Regulation of adipogenesis

1.6.1 Transcriptional regulation

Adipogenesis occurs in two main steps. First, a multipotent progenitor becomes a preadipocyte by committing to the adipocyte lineage, thereby losing its capacity to differentiate into other cell types¹⁶⁰, but without major changes in cell morphology and with maintained ability to self-renew. Early important positive regulators of the commitment step include the Smads and zinc finger protein 423 (Zfp423), which are activated by bone-morphogenetic proteins (BMPs)¹⁶¹.

The second step of adipogenesis is the terminal differentiation, in which a committed, fibroblast-like preadipocyte differentiates into a round mature adipocyte with a unilocular lipid droplet, expressing adiponectin (*ADIPOQ*) and several genes involved in storage, handling, and metabolism of lipids, such as *PLIN1*, *FASN*, *LIPE* (encoding HSL), and fatty acid-binding protein 4 (*FABP4*). Terminal differentiation is propelled by two waves of transcription factors¹⁶². Upon adipogenic induction *in vitro*, the first wave is activated, including cAMP response element-binding protein (CREB),

glucocorticoid receptor (GR), CCAAT/enhancer-binding protein β (C/EBP β), C/EBP δ , Krüppel-like factors (KLFs), and signal transducer and activator of transcription 5a (STAT5a)¹⁶³. CREB, GR and STAT5a are activated within minutes, whereas the rest are transcriptionally upregulated (largely by the two former) within hours^{164,165}. These in turn upregulate the second wave of transcription factors, where the main players are PPAR γ and C/EBP α , which largely drive the remainder of the adipogenic program by orchestrating global transcriptional changes, activating a plethora of genes involved in mature adipocyte function¹⁶⁶. Already a few hours after adipogenic induction, the chromatin is extensively rearranged¹⁶³ and many of the first-wave transcription factors co-localize in transcriptional hotspots¹⁶³, reflecting an intimate cooperation in gene regulation between these early factors. These hotspots associate to early expressed adipogenic genes, but they also occupy many later PPAR γ -target genes without inducing their expression and thus seem to be enabling access to these regions by the second wave¹⁶³. Interestingly, C/EBP β binds to closed chromatin in many of these regions prior to adipogenic induction and thus seems to recruit the remaining first-wave transcription factors to these hotspots¹⁶³. During the first day or so upon adipogenic induction *in vitro*, in concomitance with the first wave of transcription factors, growth-arrested 3T3-L1 cells undergo at least one round of cell division termed mitotic clonal expansion¹⁶⁷⁻¹⁷⁰. Data is conflicting or lacking on whether this step is required for subsequent differentiation and to which extent it occurs in human progenitors and *in vivo*^{168,170,171}, but it is possible that the chromatin rearrangement associated with the S phase is important to enable transcriptional access to adipogenic genes.

C/EBP β and C/EBP δ

Both C/EBP β and C/EBP δ are important for *in vitro* differentiation¹⁷²⁻¹⁷⁷, although the former seems to be more indispensable¹⁷⁶. Ectopic expression of either factor stimulates adipogenesis, although C/EBP δ requires the standard adipogenic cocktail to do so¹⁷⁷. Deletion of both factors in mice impairs *in vivo* WAT development¹⁷⁶. All C/EBPs bind as dimers to the CCAAT box motif on target gene promoters. This can be in the form of homodimers or heterodimers with other C/EBPs (or other non-C/EBP transcription factors)¹⁷⁸. The promoters of C/EBP α and PPAR γ contain the CCAAT box motif, and C/EBP β and C/EBP δ can directly upregulate expression of both^{179,180}. C/EBP β and C/EBP δ are rapidly upregulated upon adipogenic induction, but their expression declines already after one or a few days^{177,181,182}, reflecting their crucial role during early but not late adipogenesis. In fact, C/EBP β overexpression during the late differentiation stage downregulates the adipogenic genes that it activates during the early stage¹⁸³. Interestingly, despite their rapid upregulation, there is a significant lag time of about half a day before C/EBP β and C/EBP δ acquire DNA-binding activity, coinciding with the entry into S phase during the mitotic clonal expansion, which both have been implicated to be regulators of¹⁷²⁻¹⁷⁵. This delay is partly attributed to the requirement of C/EBP β hyperphosphorylation for DNA-binding activity; immediately

after its expression, C/EBP β is phosphorylated by the mitogen-activated protein kinase (MAPK) Erk1/2, which primes it for subsequent hyperphosphorylation, and thereby transactivation activity, by glycogen synthase kinase 3 β (GSK3 β)¹⁸⁴. However, GSK3 β does not enter the nucleus (where C/EBP β resides) until the onset of the S phase. This lag time is thought to partly act to delay the expression of PPAR γ and C/EBP α until the mitotic clonal expansion phase is over, since these transcription factors are anti-mitotic¹⁸⁴.

PPAR γ and C/EBP α

The nuclear hormone receptor PPAR γ is a ligand-activated transcription factor considered the master regulator of adipogenesis, as its activity is both required and sufficient for *in vivo* WAT formation and *in vitro* adipogenesis, even in non-committed fibroblasts¹⁸⁵⁻¹⁸⁷. It regulates transcriptional activity by binding to PPAR response elements and induces expression of the second major adipogenic regulator, C/EBP α . PPAR γ and C/EBP α are upregulated later than C/EBP β and C/EBP δ , and their expression and transactivation activity remains elevated in the mature adipocyte. PPAR γ and C/EBP α promote the expression of each other¹⁸⁰, as well as a large number of genes involved in maturation and adipocyte function, including *ADIPOQ*, *PLIN1*, *FABP4*, and *LIPE*¹⁸⁸. They often work in concert with each other; in fact, 91% of PPAR γ -bound DNA regions of differentiated 3T3-L1 adipocytes also contain C/EBP motifs¹⁸⁸, and about 40-60% of these PPAR γ -bound regions have been found to also be occupied by C/EBP α in both murine and human adipocytes^{188,189}.

Wnt/ β -catenin signaling

There are also negative regulators of adipogenesis, of which the most well-recognized involve Wnt/ β -catenin signaling¹⁹⁰⁻¹⁹². The Wnts are a large family of proteins that bind to cell surface receptors called Frizzled receptors. Although some Wnts can stimulate adipogenesis, most have been described as anti-adipogenic. Progenitors can produce Wnts, and this system is generally believed to be controlled in an auto- or paracrine manner^{193,194}. Active Wnt signaling can suppress both the commitment and subsequent step of adipogenesis. When Wnt signaling is inactive, β -catenin is continuously phosphorylated by a multiprotein complex, which includes GSK3 β . This leads to subsequent ubiquitination and proteasome degradation. When Wnts bind to the Frizzled receptor, the multiprotein complex is recruited to it, thus preventing β -catenin phosphorylation and allowing it to enter the nucleus, where it interacts with transcription factors to transactivate genes involved in stem cell maintenance and suppress expression of adipogenic genes, including C/EBP α and PPAR γ ¹⁹³. For instance, β -catenin promotes expression of axis inhibition protein 2 (Axin2), which binds to GSK3 β in the cytoplasm, thus preventing it from entering the nucleus to phosphorylate C/EBP β ¹⁹⁵.

1.6.2 Adipogenic signals *in vitro*

The adipogenic cocktail used to differentiate progenitors *in vitro* contains components that activate critical adipogenic transcription factors. In addition to a PPAR γ agonist, the classical adipogenic inducers used *in vitro* are dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and insulin. The regulation of adipogenic transcription factors by these components are summarized in Figure 3.

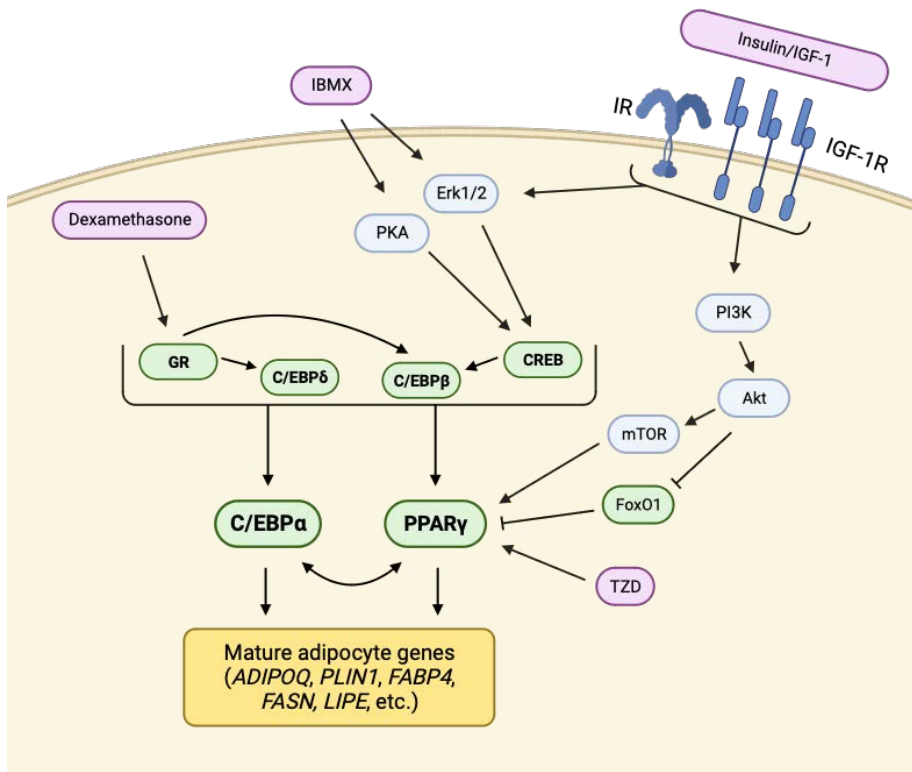


Figure 3. Major effects mediated by adipogenic inducers *in vitro*, as described in the text. Purple boxes: *in vitro* adipogenic inducers; blue boxes: kinases; green boxes: transcription factors. Created with BioRender.com.

Dexamethasone

Dexamethasone is a synthetic glucocorticoid. Like other steroid hormones, glucocorticoids are believed to passively diffuse through the cell membrane into the cytosol¹⁹⁶, where it binds to, and activates, GR, a nuclear receptor. Upon ligand binding, GR translocates to the nucleus and regulates the expression of several target genes by binding to glucocorticoid response elements (GREs) in their promoters. These include C/EBP δ , C/EBP α , C/EBP β , and PPAR γ ^{181,197}.

IBMX

IBMX is a broad-spectrum phosphodiesterase inhibitor and thus increases intracellular cAMP levels, ultimately leading to CREB activation. CREB binds to cAMP response

elements (CREs) in promoter regions on its target genes, which include C/EBP β , PPAR γ , C/EBP α , FABP4, and FASN¹⁹⁸⁻²⁰⁰. cAMP is known to activate PKA, which can directly activate CREB through phosphorylation at S133²⁰¹. PKA activation is required for optimal differentiation²⁰², and inhibition blocks *in vitro* adipogenesis and IBMX-induced PPAR γ activation²⁰³. However, the view that CREB would be activated by PKA in response to IBMX has been challenged. Data suggests that, although crucial for adipogenesis, the major function of PKA does not involve CREB activation, but rather regulation of insulin signaling-repressing factors²⁰⁴. Instead, Erk1/2 was shown to be activated by IBMX and required for cAMP-induced activation of CREB²⁰⁴. This indicates that IBMX promotes adipogenesis both by PKA activation and through separate Erk1/2-mediated CREB activation.

PPAR- γ ligands

To be able to drive the adipogenic program, PPAR γ needs to be activated by direct interaction with a ligand. *In vitro* cultures generally use the TZDs rosiglitazone or pioglitazone as synthetic PPAR γ ligands. In addition, the less specific cyclooxygenase inhibitor indomethacin, which is a PPAR γ ligand at high concentrations, is also sometimes used. The biological PPAR γ ligand(s) has not been discovered. Fatty acids, their derivatives, and eicosanoids have been identified as ligands²⁰⁵, but their physiological relevance for *in vivo* adipogenesis is unclear due to their low affinity and/or low levels in the WAT. 3T3-L1 cells have been shown to produce a PPAR γ ligand during early adipogenesis downstream of cAMP²⁰⁶, in line with data indicating that C/EBP β (which is upregulated by cAMP) is involved in the production of endogenous PPAR γ ligands, since an exogenous ligand is required for adipogenesis only when C/EBP β is inhibited²⁰⁷.

Insulin and IGF-1

Many of the insulin signaling mediators, including IRSs²⁰⁸, PI3K²⁰⁹, Akt²¹⁰, and mammalian target of rapamycin (mTOR)^{209,210}, have been attributed pro-adipogenic effects. Due to the known role of insulin in also promoting lipogenesis (and inhibiting lipolysis), such observations may sometimes be conflicted by an increased lipid accumulation due to adipogenesis-independent effects. Nonetheless, many mediators downstream of insulin can influence adipogenic transcriptional regulators. In addition to activating CREB through Erk1/2 signaling²¹¹, insulin could also promote PPAR γ activity in various ways. For instance, mTOR is required for PPAR γ transactivation activity in a manner unrelated to ligand production²¹². Additionally, insulin inhibits FoxO1 through PI3K-Akt-mediated phosphorylation. Although FoxO1 has a complex role in adipogenesis, with both inhibitory and stimulatory effects depending on the differentiation phase²¹³, it has been shown to inhibit cell cycle genes²¹⁴ as well as activity and expression of PPAR γ ^{215,216}, which is in line with its demonstrated reduced activity during the mitotic clonal expansion phase and during the initial PPAR γ upregulation^{213,214}.

Since insulin levels are elevated in response to a meal and in insulin resistance, it makes biological sense that the hormone would promote healthy energy storage by stimulating hyperplastic WAT expansion. However, its actions on preadipocytes *in vivo* can be questioned. Insulin binds IR with high affinity or insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) with lower affinity²¹⁷, and preadipocytes express very low levels of the former but high levels of the latter^{217,218}. It is currently unclear which receptor mediates the adipogenic effects of insulin *in vitro*; both IR and IGF-1R have been implicated^{217,219}. IGF-1 and insulin can both be used to stimulate *in vitro* adipogenesis, but due to the different affinities and expression levels of the receptors, supraphysiological doses of insulin are required, whereas biological levels of IGF-1 are sufficient²¹⁷, indicating that the latter is a more plausible candidate as a biological inducer of adipogenesis *in vivo*.

1.7 Adipose tissue immune cells and inflammation: physiological and pathological aspects

The hallmark of the obese WAT is its increased inflammatory profile, giving rise to a state of chronic low-grade inflammation, both locally and systemically. This obesity-associated WAT inflammation is strongly linked to metabolic diseases²²⁰ and is manifested by increased expression and secretion of pro-inflammatory factors^{221,222} as well as a higher proportion of resident immune cells with a pro-inflammatory phenotype²²³. The immune cells themselves are a major contributor to the inflammatory state, but progenitors and mature adipocytes also display a more pro-inflammatory profile in obesity^{224,225}.

1.7.1 Immune cell infiltration during obesity development

ATM populations are significantly altered in the obese WAT. In addition to a higher total number of ATMs, the relative ratio of pro- and anti-inflammatory ATMs is shifted in favor of the former²². Pro-inflammatory ATMs have been reported to be enriched in the WAT already after one week of HFD²²⁶, concomitant with reduced levels of monocytes in circulation²²⁶. This may be preceded by, and dependent on, CD8⁺ T cell infiltration³¹. Obesity-induced ATM enrichment is often mostly attributed to infiltration of circulating monocytes^{19,227}, but local proliferation of ATMs also occurs^{228,229}. Circulating monocytes and T cells are recruited to the WAT by specific chemokines that bind to distinct chemokine receptors on their cell surface, mainly C-C and CXC chemokine receptors (CCRs and CXCRs, respectively). Production of chemokines that attract pro-inflammatory immune cells are elevated in the obese WAT. MCP-1 is among the better studied in this regard; its levels are highly elevated in the obese WAT, with preadipocytes appearing as a major source^{230,231}, and attracts circulating monocytes, which express the MCP-1 receptor CCR2²³². Additionally, MCP-1 can also directly promote proliferation of local ATMs²²⁸. This obesity-associated pro-inflammatory immune cell infiltration into the WAT is strongly linked to metabolic impairments²⁵, and blocking of the MCP-1/CCR2 system in obese mice reduces ATM

numbers, concomitant with improved metabolic health^{232,233}. Similarly, depletion of pro-inflammatory ATMs once obesity is established improves metabolic and inflammatory markers (at least short-term)²³⁴.

1.7.2 Lipid handling by adipose tissue macrophages in lean and obese WAT

The rapid HFD-induced infiltration of pro-inflammatory ATMs long before obesity is developed suggests that it is a physiological response that relates to energy handling in the WAT. This likely, at least partly, relates to the lipid handling role that has been demonstrated for ATMs in both lean and obese WAT. ATMs are highly sensitive to nutritional cues. They accumulate in the WAT rapidly in response to increased lipolysis, e.g. during prolonged fasting, and appear to act as a local lipid buffer by taking up fatty acids released from adipocytes, thus preventing excessively elevated circulating lipids²³⁵. Conversely, a lipid-handling ATM subtype that appears to be involved in postprandial cholesterol recycling is rapidly activated in the WAT of lean, healthy mice in response to overnight HFD²³⁶. It takes up chylomicron remnants (the potentially harmful lipoprotein particles that remain after adipocytes have taken up fatty acids from chylomicrons) and transfer the cholesterol to less harmful high-density lipoprotein (HDL) particles²³⁶. In obesity, the majority of ATMs are located in what is known as crown-like structures, which consist of several, often lipid-laden ATMs clustering together around a dead adipocyte to scavenge debris, mainly lipid droplets, from the dead adipocytes they are surrounding^{223,237}. In fact, single-cell data has observed an increased expression of lipid-handling genes in ATMs of obese mice at the expense of macrophage-specific genes¹⁵. In parallel, adipocytes show a reduced expression of lipid-handling genes²³⁸, suggesting ATMs somewhat compensate for the adipocyte dysfunction in obesity. Single-cell sequencing identified LAMs as an ATM subtype highly enriched in obesity, closely associated to crown-like structures and expressing many genes involved in lipid metabolism¹⁹. Deletion of its essential signature gene, the lipid receptor *Trem2*, aggravated adipocyte hypertrophy, impaired glucose tolerance and increased circulating lipids in obese, but not lean, mice¹⁹. Similar observations have been made by silencing lipoprotein lipase expression specifically in vWAT ATMs in obese, but not lean mice²³⁹. Overall, this suggests that ATMs have a crucial function in handling excess lipids to protect both adipocytes and peripheral organs from lipid overload and toxicity.

1.7.3 Local inflammatory signals

Pro-inflammatory factors are known to have a direct impact on the function and dysfunction of adipocytes and progenitors. Cytokines like IL-6, TNF- α , IL-1 β , and IFN- γ have been shown to impair insulin sensitivity²⁴⁰⁻²⁴⁹, promote lipolysis²⁵⁰⁻²⁵² and/or inhibit adipogenesis²⁵³⁻²⁵⁹ *in vitro*.

Many of these inflammatory factors signal via nuclear factor κ -light-chain-enhancer of activated B cells (NF κ B). In canonical NF κ B signaling (shown in Figure 4), inhibitor of

κ B (I κ B) kinase β (IKK β) is activated by phosphorylation through cytokine or toll-like receptor (TLR) signaling. Activated IKK β phosphorylates I κ B, which marks it for rapid degradation. In the basal state, I κ B sequesters the NF κ B complex (p65 and p50) in the cytosol. I κ B degradation allows NF κ B to enter the nucleus and transactivate its many pro-inflammatory target genes²⁶⁰. Activated NF κ B not only propagates inflammation, but also directly alters the function of the cells by redistributing transcriptional co-factors from super-enhancer-associated cell identity genes, such as PPAR γ and C/EBP α in adipocytes (and likely preadipocytes) to the enhancers regulating pro-inflammatory genes²⁶¹.

Despite the strong link between WAT inflammation and dysfunction as well as the vast *in vitro* data pointing to inflammatory factors directly causing WAT dysfunction, *in vivo* murine studies have shown highly varying and somewhat conflicting results regarding the impact of local inflammatory signals on WAT function and systemic metabolic health. Silencing TNF- α or the pro-inflammatory cytokine osteopontin specifically in vWAT-resident ATMs in already obese *ob/ob* mice improves glucose tolerance²⁶². Further, adipocyte-specific loss of TLR- and IL-1 signaling attenuates HFD-induced weight gain and insulin resistance²⁶³. On the other hand, adipocyte-specific blocking of major inflammatory pathways leads to an impaired capacity for healthy, hyperplastic WAT remodeling, concomitant with systemic glucose intolerance and hepatic steatosis²⁶⁴, as well as systemic insulin resistance²⁶⁵, under both chow- and HFD-fed conditions. Similarly, adipocyte-specific knockout of IKK β impairs healthy remodeling of vWAT, systemic glucose tolerance and insulin sensitivity²⁶⁶. Curiously, this deletion causes increased ATM infiltration and inflammation in the WAT²⁶⁶. Adipocyte-specific deletion of IL-6 has no effect on glucose tolerance in obese mice²⁶⁷. Inflammatory signals in progenitors also seem to be important, as deletion of IKK β specifically in the white adipose lineage reduces vWAT mass and impairs adipogenesis²⁶⁸. This is in line with data showing that LPS injection directly into the WAT or adjacent lymph node stimulates local formation of new fat cells^{264,269,270}. Taken together, these observations highlight the multifactorial role of inflammation and immune cells in WAT function. Obesity-associated chronic low-grade WAT inflammation clearly seems to aggravate metabolic dysfunction, but it is also evident that some inflammatory signals play an important role in maintaining tissue homeostasis. Likely, these are more transient inflammatory surges in response to increased stress, prompting adaptive reactions aimed at relieving the strain and return to a healthy equilibrium. Deciphering these different roles, and the immunomodulatory factors mediating them, will be crucial to combat the comorbidities of obesity.

1.7.3.1 IL-1 β

IL-1 β is a powerful pro-inflammatory cytokine expressed by various immune cells, predominantly macrophages²⁷¹. It is produced as an inactive precursor (pro-IL-1 β) in response to e.g. TLR or cytokine receptor signaling and must be proteolytically cleaved by caspase 1 to become bioactive²⁷². Caspase 1 is activated by the NLRP3 inflammasome, which itself can be activated by damage- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively)²⁷². IL-1 β secretion²⁷³ and IL-1 β -producing ATMs¹⁸ are elevated in the obese WAT. As previously mentioned, *in vitro* studies have shown that it can inhibit adipogenesis of preadipocytes²⁷³ and induce insulin resistance²⁴⁴ and lipolysis²⁵¹ in adipocytes. IL-1 β signals through the IL-1 receptor type 1 (IL-1R1), which contains the intracellular Toll-IL-1R (TIR) domain that is nearly identical to that of all TLRs²⁷⁴. IL-1 α and IL-1Ra also binds to IL-1R1 but the latter does not activate signaling and thereby acts as an antagonist, hindering binding of IL-1 β /IL-1 α ²⁷⁴. Canonical IL-1R1 signaling is mediated through the NF κ B pathway and the three MAPKs Erk1/2, p38 and c-Jun N-terminal kinase (JNK) (Figure 4)^{271,275}. Activated MAPKs directly or indirectly activate several transcription factors, which in turn can transactivate a plethora of genes involved in inflammation, proliferation, differentiation, apoptosis, and metabolism. Early signaling events involve the myeloid differentiation primary response 88 (MyD88) adapter protein and different IL-1R-associated kinases (IRAKs)^{271,275}. In obese mice, IRAK2 and IRAK3 have been shown to translocate to the adipocyte mitochondria, where they reduce oxidative phosphorylation/fatty acid oxidation and increase *de novo* lipogenesis, respectively, and adipocyte-specific deletion of either factor attenuates HFD-induced obesity and insulin resistance^{263,276}. Whether IL-1R1 or TLR signaling (or both) are mediating these actions *in vivo* remains unknown. Further, little attention has been paid to the possible physiological role IL-1 β may play in the WAT; in lean, healthy mice, IL-1 β is upregulated postprandially in macrophages resident in WAT, but not other tissues²⁷⁷, suggesting it is a response to energy influx. Given its described important role in postprandial systemic glucose homeostasis²⁷⁸, there is reason to suspect that it might also have a physiological role in local energy handling.

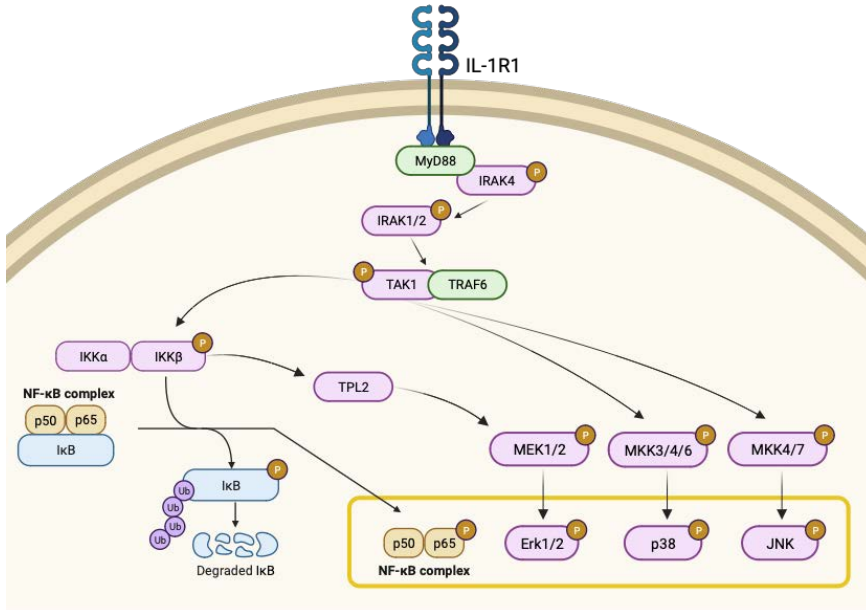


Figure 4. Activation of NFκB, p38, JNK, and Erk1/2 through IL-1R1 signaling. Created with BioRender.com.

1.7.3.2 IL-10

IL-10 is a pleiotropic cytokine well known for its immunosuppressive actions. It is produced by many different immune cells, including macrophages, T cells, and dendritic cells. IL-10 signals through the IL-10 receptor (IL-10R), of which the ligand-binding subunit is IL-10R1²⁷⁹. Many of its effects are exerted through the Jak1/tyrosine kinase 2 (Tyk2)/STAT3 pathway²⁷⁹. STAT3 is a transcription factor that directly upregulates several anti-inflammatory genes, including *IL1RN* (the gene encoding IL-1Ra) and suppression of cytokine signaling 3 (*SOCS3*), an inhibitor of MAPK and NFκB signaling that mediates the suppressive effect of IL-10 on e.g. TNF-α and IL-6 production by LPS-treated macrophages²⁸⁰. Due to its potent anti-inflammatory properties, IL-10 has repeatedly been attributed a protective role against obesity-associated metabolic diseases. For instance, it counteracts inflammation-induced insulin resistance in muscle and liver^{281,282}, and protects against atherosclerosis partly by suppressing the inflammatory process in plaques²⁸³. Its role in the WAT is somewhat more controversial and has been difficult to elucidate since the cytokine appears to have different local roles in mice and humans. Murine studies have shown that IL-10 worsens HFD-induced insulin resistance and obesity by directly suppressing the thermogenic program in adipocytes^{36,284,285}. In contrast, human adipocytes are metabolically unresponsive to IL-10, likely due to very low expression of IL-10R1²⁸⁶,

which is not the case for murine adipocytes²⁸⁴. Thus, findings in mice regarding the local role of IL-10 in the obese WAT may not be applicable to humans, in which studies have been limited. WAT IL-10 production is elevated in obese, compared to non-obese, healthy women²⁸⁶, in which it is linked to an insulin resistant phenotype²⁸⁶, but it is associated to a healthier cardiovascular profile in obese children²⁸⁷. Based on IL-10R1 expression pattern, immune cells appear to be the predominant targets of IL-10 in human scWAT²⁸⁶. Interestingly, the major source of local IL-10 appears to be a pro-inflammatory subtype of ATMs that is enriched in the obese WAT²⁸⁶. Thus, it is likely part of a negative feedback regulation that may act to counteract obesity-associated local inflammation.

1.7.3.3 CCL18

CCL18 is one of the lesser studied chemokines, in part because a corresponding protein in rodents is lacking, greatly complicating *in vivo* studies. It is a known chemoattractant of various immune cells, including T and B lymphocytes and dendritic cells²⁸⁸. In WAT, it is highly expressed by ATMs and correlates to M2 markers²⁸⁹. scWAT CCL18 production correlates positively with BMI²⁹⁰ and has been associated to T2D²⁹¹ and metabolic risk factors for both T2D and CVDs²⁸⁹. However, no mechanistic studies have been carried out regarding the role of CCL18 in the WAT and its link to cardiometabolic diseases.

2 RESEARCH AIMS

This thesis aimed to study the interplay between WAT-resident immune cells and local metabolic processes. More specifically, we wanted to investigate how pro- or anti-inflammatory factors secreted by local immune cells may influence, or be influenced by, the expansion and metabolic function of WAT and how this relates to metabolic health.

Study I: IL-1 β promotes adipogenesis by directly targeting adipocyte precursors

Background: The adverse role of WAT inflammation in the context of obesity and metabolic disease is well understood, but much less is known about its physiological function. In lean, healthy mice, the IL-1 β system is upregulated postprandially in macrophages resident in WAT, but not in other tissues, suggesting IL-1 β is part of a healthy, biological response to WAT energy influx.

Aim: To investigate a physiological role of IL-1 β in WAT expansion and/or metabolic function.

Study II: Adipose tissue specific CCL18 associates with cardiometabolic diseases in non-obese individuals implicating CD4⁺ T cells

Background: A subset of non-obese subjects suffers from cardiometabolic diseases, but the role of WAT inflammatory status in the pathology of these individuals has not been thoroughly investigated.

Aim: To elucidate the link between WAT inflammatory status, adipocyte metabolism and CVD and/or T2D in non-obese individuals by identifying WAT-derived inflammatory mediators associated with cardiometabolic diseases and investigating their potential effect on adipocyte metabolism.

Study III: Sex-specific regulation of IL-10 production in human adipose tissue in obesity

Background: Obese women are protected from T2D to a greater extent than men, which may partly be explained by sex differences in WAT inflammation. The anti-inflammatory cytokine IL-10 was previously shown to be increased in WAT of obese women and linked to insulin resistance, but whether this pertains to men is unknown.

Aim: To compare the influence of obesity and T2D on WAT IL-10 production in men and women.

3 METHODOLOGICAL CONSIDERATIONS

3.1 *In vitro* models

In vitro cell cultures have been the primary experimental model used in this thesis. Such systems have some major limitations. The cells used may be biologically different from the actual cell of interest in the body, although this can be partly overcome by using primary cells instead of immortalized cell lines. Of perhaps greater concern is the fact that the *in vitro* environment is very distinct from the *in vivo* milieu, lacking the complex network of signals from all other cells and tissues in the body, thereby making interpretations about *in vivo* effects difficult. However, this shortcoming is also an important advantage; *in vitro* models allow experiments to be conducted in a clean, controlled environment and thus facilitate investigations of detailed mechanisms. As such, they are very valuable when combined with other data, such as *in vivo* mouse models or human observational data.

3.1.1 Human adipose-derived stem cells

Studies I-III, and predominantly **study I**, employed an *in vitro* cell model of human adipose-derived stem cells (hASCs) induced to differentiate into mature adipocytes. These mesenchymal stem cells, derived from the scWAT SVF of a 16-year-old male donor, were cryopreserved and expanded for at least 12 passages before adipogenic induction, and used until passage 22. Thus, these are considered pseudo-primary cells, as they are biologically much closer to WAT-resident progenitors than an immortalized cell line, but the expansion (and possibly cryopreservation) can alter adipogenic capacity and overall phenotype of hASCs²⁹². The advantage of expanding cells is that it enables many more experiments to be conducted, including ones that demand a high cell number, from a single donation, which is desirable both for reduced inter-donor variability and from an ethical perspective.

In addition to using passaged hASCs, in **study I**, we confirmed our main finding in true primary progenitors from human and murine WAT, isolated by FACS or magnetic bead sorting, respectively, and immediately induced to differentiate. This allowed us to use expanded cells for the majority of the experiments, while still being confident that our main results were not due to intrinsically different characteristics of the expanded hASCs. In **study I**, hASCs were primarily used to study early events in the adipogenic process. Before adipogenic induction, these cells are multipotent and thus have the capacity to differentiate into osteocytes, myocytes and chondrocytes^{293,294}. They therefore allow the studies of early events that take place before or during adipogenic commitment.

Study II investigated effects on adipocytes differentiated from hASCs, thus using them as a mature adipocyte model. Such a strategy of using *in vitro* differentiated

adipocytes, rather than primary ones directly isolated from WAT, is commonly employed due to the several challenges associated with the latter cells. Mainly, primary mature adipocytes are notoriously fragile; they must be used freshly after isolation since they will burst by freezing and are difficult to keep intact in culture for more than one or a few days. Thus, repeated experiments requiring higher yields of cells will demand a frequent access to relatively large, fresh WAT samples. The major disadvantage of using *in vitro* differentiated adipocytes is that they are biologically different from primary fat cells in many ways, likely mainly because of differences in the signals they receive throughout differentiation. For instance, primary adipocytes are unilocular, whereas *in vitro* differentiated adipocytes have several, smaller lipid droplets. Since lipid droplets are central to the major processes of adipocytes, this morphological disparity may reflect a functional difference between the two cell models. Further, *in vitro* differentiated adipocytes are collected before they accumulate enough lipids to detach from the bottom of the plate. Thus, it might be questioned whether they are truly mature adipocytes, or cells in the late differentiation stage. Regardless, they will always be of a much younger age than primary adipocytes, which may affect their function²⁹⁵.

3.1.2 Immune cell lines

In **study II**, cultures of Jurkat cells were used as an *in vitro* model of human T cells. This is an immortalized T lymphocyte cell line originally obtained from the peripheral blood of a boy with T cell leukemia, and is probably the most widely used model for *in vitro* studies on T cell activation and T cell receptor signaling. Different T cell subtype models can be induced by treating Jurkat cells with distinct anti- or pro-inflammatory factors. In our case, we only stimulated with CCL18 to investigate its effect on T cell activation.

THP-1 cells were used as a human macrophage model in **study III**. This is an immortalized monocyte cell line, isolated from the peripheral blood of a leukemia patient, which has been extensively used to study monocyte/macrophage function. Classically, and in our case, phorbol-12-myristate-13-acetate (PMA) is used to induce differentiation into macrophages. Different stimulatory agents can be used to induce subsequent polarization into distinct subtype models.

3.2 Animal models: mice with loss of IL-1 signaling

Study I included three different mouse models with genetic or pharmacologic blocking of IL-1R1-mediated signaling.

To examine the role of IL-1 signaling in mature adipocytes *in vivo*, mice with an adipocyte-specific knockout of *Il1r1* were used. For this purpose, mice with homozygous *loxP*-flanked *Il1r1* gene (*Il1r1^{FF}*) were bred with *Adipoq*-Cre mice (i.e. mice expressing Cre under the control of the *Adipoq* promoter). Since adipocytes are the only cells reported to express adiponectin, the *Adipoq*-Cre line is now more widely

accepted than the previously utilized *Ap2*-Cre line. *Ap2* (corresponding to the human *FABP4* gene) is expressed in several cell types in addition to adipocytes, including macrophages²⁹⁶, and has been shown to target cells in several other tissues^{297,298}. Consequently, the *Adipoq*-Cre line is much more adipocyte-specific than the *Ap2*-Cre line²⁹⁷⁻²⁹⁹. However, an important side note is that brown adipocytes also express *Adipoq*, complicating conclusions about effects specifically on white adipocytes.

We also used two mouse models, one genetic and one pharmacological, with a systemically blocked IL-1 signaling. While the use of such unspecific models makes it impossible to attribute results to loss of IL-1 signaling in a specific cell type, they can be very useful to combine with e.g. *in vitro* data when there are no available possibilities to selectively target the cell type of interest. As our genetic model, mice with whole-body knockout of *Il1r1* were used. Since IL-1 signaling in adipocyte progenitors were our main interest, a progenitor-specific knockout model would have been more suitable for our purposes. However, there is no known Cre line that selectively targets WAT-resident progenitors. The most utilized model for this purpose is the *Pdgfra*-Cre line. *Pdgfra* (encoding platelet-derived growth factor receptor α) is a mesenchymal stem cell marker and this line indeed targets most, if not all white adipocyte progenitors^{8,300}. However, *Pdgfra* is also expressed in many other cells/tissues, including brown adipocyte progenitors³⁰⁰, oligodendrocyte progenitors³⁰¹, testes³⁰² and heart³⁰³. Of perhaps even more relevance, the *Pdgfra*-Cre line has been shown to target some CD45⁺ WAT-resident cells⁸, making it a highly inappropriate model to study adipocyte progenitor-specific effects of IL-1 β , considering its potent inflammatory actions in many immune cells.

The permanent nature of the genetic ablation in standard whole-body KO mice makes it difficult to rule out that observations in adulthood are a result of loss of function during development (or even in earlier generations). Also, biological adaptations may have developed that can rescue or in other ways alter the phenotype of the mice. Although an inducible KO model could overcome these limitations, a faster and easier approach is to block the signal pharmacologically. Our pharmacological model consisted of WT mice which were given daily intraperitoneal injections of the human drug anakinra, a recombinant IL-1Ra. This drug has some important limitations. It needs to be administered daily since the half-life is only about 4-6 h in humans³⁰⁴ and 2 h in mice at the dose we used³⁰⁵. Further, since this is the human form of IL-1Ra, mice eventually develop antibodies against it, and we therefore limited the intervention period to two weeks. For these reasons, a better suited pharmacological option would probably have been a blocking antibody against IL-1 β , like the human drug canakinumab, which has a half-life of 26 days³⁰⁶. A murine analogue has been developed but is unfortunately not commercially available.

3.3 Assessment of adipogenesis

3.3.1 *In vitro*: lipid droplet quantification by high content screening

Several experiments in **study I** assessed adipogenic differentiation by quantifying lipid droplet accumulation. For this purpose, cells differentiated in 96-well plates were fixed and stained with fluorescent nuclear (Hoechst) and lipid droplet (Bodipy) dyes. The plate was scanned in an imaging platform and nuclei and lipid droplets were digitally quantified from the images using object identification and spot detection algorithms, respectively. Briefly, nuclei were identified based on pixel intensity, morphology, and size. Lipid droplets within a digitally defined cell surface mask with a set maximal distance from the nuclei were then identified based mainly on pixel intensity.

Lipid droplet accumulation per cell was calculated as the total area of lipid droplets in a well normalized by the total number of nuclei in the same well, thus only providing the average lipid droplet area of the cells. Measuring total accumulated lipids is a commonly employed strategy to assess degree of *in vitro* adipocyte differentiation, but this approach alone is incapable of confidently distinguishing between adipogenesis and lipogenesis, which are two closely intertwined processes. The latter is highly influenced by the former and starts taking place, with increasing rate, already during the early stage of differentiation. It can therefore be difficult to disentangle effects on adipogenesis and lipogenesis from each other. A somewhat more insightful strategy is to analyze lipid droplet accumulation at single-cell resolution, which was also performed in **study I**, to obtain the entire distribution of lipid droplet accumulation within each individual cell. This provides valuable information, such as percentage of differentiated cells and maximal lipid droplet accumulation of individual cells, which can give clues about which part of the process is affected. Regardless, lipid droplet quantification should always be complemented with additional measurements (e.g. mRNA levels and signaling events) to get an indication of which process is affected.

3.3.2 Observational: adipocyte size and morphology value

In **study I**, we used both adipocyte size and morphology value from clinical cohort data to analyze relationships between WAT morphology and IL-1-related genes. Adipocyte size is often used as an indicator of whether hyperplastic expansion is altered *in vivo*. However, WAT morphology is a very indirect and unreliable assessment of adipogenesis, as adipocyte size is dependent on many other things, such as the balance between lipogenesis and lipolysis in existing fat cells, and the rate of adipocyte death. A better approach has been to use a morphology value³⁰⁷; when body fat mass is plotted against adipocyte volume, it fits a curve-linear model (strong positive relationship at low fat mass that plateaus at higher fat mass)^{9,307}. The morphology value is defined as the amount of deviation from that curve, i.e. how much the mean adipocyte volume of an individual varies from what is expected based on their fat mass. Positive values indicate hypertrophy and negative values indicate

hyperplasia. The morphology value correlates strongly (negatively) with adipocyte number, but not with adipocyte size³⁰⁷, indicating that it is a reliable measurement for degree of hyperplasia.

3.3.3 *In vivo*: EdU-tracing

To directly measure new adipocyte formation *in vivo*, different tracking strategies have been employed. Probably the currently most accurate approach is to use an inducible transgenic model, in which adipocytes can be selectively and permanently labeled at a chosen time point^{8,143,144} (as described in the introduction). WAT is collected several weeks later, and tissue sections are imaged to measure the proportion of unlabeled, and thus newly formed, adipocytes.

In **study I**, we used another approach to assess new adipocyte formation, namely EdU-tracing (Figure 5). This method labels progenitors instead of adipocytes, and then tracks the differentiation of the labeled progenitors. The labeling step entails treating mice (through injections or drinking water) with EdU, a modified thymidine analog that incorporates into DNA during DNA synthesis, and thus only in proliferating cells. Adipocytes can therefore not be directly labeled with EdU, since they are postmitotic. Instead, any EdU⁺ progenitor that differentiate will become an EdU⁺ adipocyte, thus allowing the detection of newly formed fat cells. This approach, using either EdU¹⁴⁶, bromodeoxyuridine (BrdU)^{147,148} (also a thymidine analog) or a stable isotope-tagged thymidine¹⁴⁹ has been widely employed to track adipocyte formation. It has the advantage of not requiring transgenic mice, which can be expensive, time-consuming, and technically difficult to obtain if being used in combination with another transgenic model of interest. The incorporated EdU is stained in a copper-catalyzed reaction in which an alkyne in the EdU molecule covalently binds to an azide coupled to a fluorophore. Since EdU is localized exclusively in the DNA, analysis can be performed by flow cytometry of the isolated adipocyte nuclei (since primary adipocytes are too large and fragile to be run intact). This enables analysis of the entire adipocyte pool, rather than tissue sections from a small fraction of the WAT.

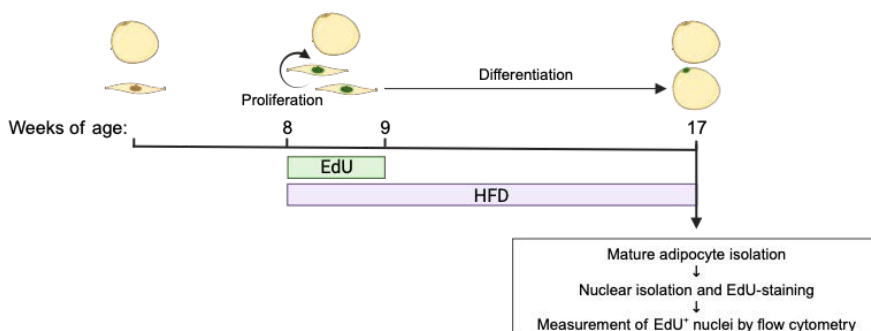


Figure 5. Schematic of EdU-tracing assay performed in **study I** to assess *in vivo* adipogenesis. Created with BioRender.com.

The major limitation with the EdU-tracing approach is that it only labels adipocytes arising from progenitors that proliferated at the time of EdU-treatment. During the first week of HFD, there is a marked increase in proliferating progenitors (at least in gWAT)¹⁴⁶⁻¹⁴⁸. If HFD continues, these progenitors go on to differentiate¹⁴⁶, but their proliferation rate declines to chow-fed levels already after five days and remains low for at least five weeks¹⁴⁷, suggesting that EdU treatment during the initial week likely catches all progenitors that undergo HFD-induced proliferation and subsequent differentiation. However, it cannot be ruled out that a subset of progenitors differentiate without immediate prior cell division, and if so, this assay would not detect them. Another disadvantage with using EdU is its potential toxicity^{308,309}, which may lead to underestimation of the degree of differentiation, or a stress response which could affect surrounding progenitors. This is reason to keep the dose and duration of EdU treatment as short as possible. We administered it through one injection every other day for a week. This summed up to a much lower EdU exposure, and thus lower risk for toxicity, than daily administration through drinking water (and has the added benefit of ensuring an identical dose to all mice) but has the likely disadvantage of reducing the amount of labeled cells and the signal intensity within each cell. Additionally, the EdU tracing approach is dependent on the notion that adipocytes do not undergo DNA synthesis, which has indeed been the consensus for a long time, since they are postmitotic. However, more recent data has shown that mature adipocytes can enter S-phase and incorporate EdU in what appears to be a postmitotic cell cycle, and this is closely linked to senescence and insulin resistance³¹⁰. Thus, adipocytes may be EdU⁺ due to senescence, rather than progenitor differentiation, and this assay should therefore always be interpreted with some caution, especially in insulin resistant mice. Despite these different limitations, the EdU-tracing assay appears to be a reliable method; although no proper comparisons have been made between transgenic and EdU-based tracking models, both strategies yield fairly comparable results, both in terms of the absolute proportion of new fat cells and the relative proportions between diets and depots¹⁴⁷.

3.4 Flow cytometry

Studies I-III performed analyses and/or isolation of cell populations by fluorescence-activated cell sorting (FACS), which is a fluorescence-based flow cytometry technique. The basic principle of flow cytometry is to run cells one at a time past a laser beam. Due to differences in size and granularity, distinct cell types cause a unique scattering signature of the laser light that runs through them, which enables analysis of different cell populations. With FACS, a vast amount of additional information can be gained by fluorescently labeling the cells before running them through one or several excitation laser beams. Fluorophore-conjugated antibodies are often used to stain cell surface markers known to be expressed by distinct cell populations. Since most FACS

instruments are equipped with several different laser beams and filters, many markers can be used in the same FACS run, thus providing a large amount of data from one run. When used properly, this is a highly specific and sensitive method. However, its accuracy is completely dependent on the use of appropriate staining controls, laser voltages, and fluorescence compensation and gating strategies.

3.5 Gene expression analyses

For gene expression analyses, mRNA levels were measured with two widely accepted methods: quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used in **studies I-III**, and RNA-seq in **study I**.

3.5.1 qRT-PCR

qRT-PCR is the golden standard to measure gene expression. Briefly, total RNA is extracted from cells and then reverse transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase. The cDNA is added to a qRT-PCR reaction mix, which also consists of short oligonucleotide primers specific to the mRNA of interest (one for the template DNA and one for its complementary strand), nucleotides and a *Taq* DNA polymerase to allow amplification of the target sequence. In addition, a fluorescent marker is used to quantify the amplicon. The reaction takes place in a thermocycler, which fluctuates between specific temperatures to allow DNA denaturation, primer annealing, and extension throughout several cycles, with each cycle yielding twice as many amplicons of interest (and thereby fluorescent signal) as the previous cycle. To control for variations in starting material and/or cDNA synthesis efficiency between samples, the expression value of the gene of interest is normalized to that of a gene which is stably expressed throughout the intervention, thereby reflecting the number of cells from which the cDNA originated. There are two assays used for qRT-PCR, which differ in the fluorescent chemistry utilized to yield a signal. The SYBR Green assay uses a dye that increases in fluorescence when bound to double-stranded DNA. Since this dye will bind to double-stranded DNA indiscriminatory, it is very important that the oligonucleotide primers are highly specific to the target sequence; if they bind to unspecific DNA sequences, each other (primer dimerization) or to themselves in hairpin structures, false positive signals will be generated. The TaqMan™ assay, on the other hand, uses a fluorophore-oligonucleotide construct that binds to a sequence within the target amplicon. This probe is bound to a fluorescent dye on the 5' end and a quencher on the 3' end. Due to their close proximity, the quencher blocks the fluorescence emitted by the dye as long as the probe construct is intact. During the extension phase, *Taq* DNA Polymerase cleaves the 3' end of the probe, thereby releasing the quencher and allowing the dye to emit its fluorescent signal. Since emission of the signal is dependent on the probe-fluorophore construct binding to the target cDNA, and not indiscriminatory double-stranded DNA binding, as in the SYBR Green assay, the risk of false positives is greatly reduced.

3.5.2 RNA-seq and downstream analyses

Transcriptomics provides data on all (or most) mRNA transcripts within a cell and is therefore a valuable tool to get a complete picture of the transcriptional events taking place in your system. Although less accurate compared to qRT-PCR, the loss in quality is made up for by the quantity of data provided. RNA-seq is now the golden standard technique for transcriptomics. There are different types of RNA-seq that differ between which type of RNA is measured, depths of sequencing, etc. but the entire process always includes three crucial steps: library preparation, sequencing, and data analysis. During the cDNA library preparation, mRNA is isolated from total RNA, fragmented, converted to cDNA, and ligated to adapters to enable binding to the flow cell during the sequencing process. The principle of the sequencing is the use of nucleotides coded with specific fluorescent dyes and reading of the fluorescent signal after the addition of each new nucleotide to the single-strand cDNA template, thus obtaining the sequence of all mRNA fragments originally in the sample. After quality control, the resulting reads are aligned to a reference genome, and counts for each gene are quantified and normalized. In addition to differential expression analyses, many downstream analytical tools can be utilized to obtain more information about the transcriptional profile of your samples. To identify cellular processes altered by IL-1 β in **study I**, we performed gene set enrichment analysis (GSEA)³¹¹. A gene set is a pre-defined group of genes involved in the same cellular process, and GSEA identifies gene sets in which the genes are differently expressed between two groups. Additionally, to predict which transcription factors were driving the IL-1 β -regulated changes in gene expression, we performed an integrated motif activity response analysis (ISMARA)³¹², which infers transcription factor activity in the RNA-seq data based on predictions of transcription factor binding sites in proximal promoters.

3.6 Protein measurements: immunoassays

Measuring mRNA levels is necessary to investigate transcriptional regulation. However, to be biologically relevant, a change in expression of a protein-coding gene needs to lead to a change in the amount of protein encoded by that gene. The rate of mRNA translation and protein degradation can sometimes highly influence the levels of a protein independently of its mRNA levels. Further, there are many post-translational modifications, such as phosphorylation, influencing the activity of a protein that may be important to measure. Additionally, many pro- and anti-inflammatory factors are secreted to exert auto-, para-, or endocrine functions. In those cases, it is the amount of secreted protein that is the relevant measurement. For these different reasons, measuring protein levels is often an important addition or substitute to mRNA measurements.

3.6.1 Intracellular proteins: Western blot

Study I employed Western blots to analyze IL-1 β -mediated regulation of early adipogenic transcription factors, by measuring total protein levels of C/EBP δ and C/EBP β , as well as activation of CREB (i.e. phosphorylation at Ser133). Western blot is a widely used technique for protein measurements. The method was invented in 1981, and although new technology has made the entire process faster than it used to be, the principle remains the same. Briefly, proteins (usually from cell lysates) are denatured and loaded onto a polyacrylamide gel in a buffer containing SDS, which gives all proteins the same degree of negative charge in relation to their molecular weight. When current is run through the gel, the proteins migrate through it. The dense acrylamide matrix obstructs the migration of the proteins in a degree proportional to their size, with smaller proteins being able to migrate faster than large ones, thus allowing separation by size. The proteins in the gel are then transferred to a membrane by electrophoresis, and the membrane is incubated with a primary antibody targeting the protein of interest, followed by a secondary antibody, which is often enzymatically or fluorescently labeled, thus allowing detection by chemiluminescence or fluorescence. Western blotting is a relatively tedious process and can traditionally only yield information on one target protein at a time. Further, it can only provide relative quantification between the samples on the same membrane, thereby limiting the number of samples that can be directly compared.

3.6.2 Secreted proteins: single- and multi-plex immunoassays

In **studies II and III**, we used three different immunoassay techniques to measure pro- and anti-inflammatory proteins in media from WAT explants and cell cultures, as well as from serum samples. Generally, the enzyme-linked immunosorbent assay (ELISA) was used to measure single proteins of interest and the Luminex or Meso Scale Discovery (MSD) techniques were used in multi-plex assays. As all of these techniques are much more sensitive than Western blots, they are generally used to measure secreted/circulating proteins, whose abundance is often lower than intracellular proteins. They also have the added advantage of allowing for analysis of many more samples at once (the assays are generally performed in a 96-well plate), and since a standard curve of known analyte concentrations is run in parallel, they allow for absolute quantification of the protein of interest.

ELISA is probably the most common single-plex technique to measure secreted proteins. There are a few different types of ELISAs with roughly the same general principle. Briefly, the analyte of interest, which is immobilized at the bottom of a coated plate along with all other proteins in the solution, is targeted with a specific antibody. An enzyme, conjugated to either the primary or a secondary antibody, synthesizes a colored reaction product upon addition of a specific substrate, and the optical density in each well can be measured in a standard microplate reader. ELISAs can generally only measure a single protein of interest. To measure several factors at once, we

therefore used the multi-plex immunoassays developed by Luminex® and Meso Scale Discovery® (MSD), which, in contrast to ELISA, are not enzyme-linked. The Luminex technology uses color-coded beads. Each bead is dyed with a known concentration of a red or infrared fluorophore and coupled to a specific capture antibody targeting one of the analytes, which is then again targeted with a biotinylated detection antibody, along with a streptavidin-phycoerythrin (PE) reporter. The samples are analyzed in a flow-based instrument, which uses a red laser to determine the bead color (i.e. *which* analyte), and a green laser to measure the PE signal (i.e. *amount* of analyte). The MSD system uses electrodes instead of beads, and SULFO-TAG-labeled detection antibodies instead of the biotin-streptavidin-PE reporter system. The bottom of each well contains several electrode spots (up to 10) at known locations, and each separate spot is coated with capture antibodies for one unique analyte. During measurement, electricity is applied to one spot at a time, which causes the SULFO-TAG connected to that electrode to emit light, thus allowing quantification of the analyte associated to that spot. Luminex and MSD techniques provide comparable relative quantification. However, the former is more precise and therefore better suited when the absolute concentrations are important, or when subtle differences between groups are expected. On the other hand, the MSD system is more sensitive and is therefore very useful for analytes of low abundance or when the starting material is sparse. This can also make it a better option than ELISA, even when only a single analyte is of interest.

3.7 Lipolysis measurements

Lipolysis is the enzymatic breakdown of TGs, which results in the release of free fatty acids and glycerol. In **study II**, lipolysis of *in vitro* differentiated adipocytes was assessed by measuring glycerol release into the media. Glycerol is a more reliable measurement than fatty acids, since the latter can be re-esterified into TGs³¹³, whereas adipocytes are unable to recycle the free glycerol³¹⁴ (they instead synthesize glycerol-3-phosphate through glycolytic pathways). Thus, glycerol is accumulated in the media in a manner proportional to the lipolytic rate of the adipocytes. However, an important caveat is that glycerol can also be released by adipocytes through glycolytic pathways³¹⁵. During the lipolytic intervention, cells are incubated in media containing albumin, which is important due to its fatty acid-sequestering properties. Released free fatty acids can otherwise be taken up by adipocytes again and inhibit lipolysis³¹⁶. Glycerol in the media is measured through coupled enzymatic reactions involving glycerol kinase and glycerol phosphate, whereby hydrogen peroxide is produced as a byproduct from free glycerol, and a horseradish peroxidase-catalyzed reaction between hydrogen peroxide and Amplex™ UltraRed generates a brightly fluorescent reaction product.

3.8 Clinical cohorts

All studies of this thesis, and primarily **studies II and III**, utilized previously obtained data from several clinical cohorts with well-characterized subjects. Data included systemic measurements, such as BMI, body fat percent, and insulin sensitivity, as well as local characterization of the scWAT (obtained through needle biopsy), including global mRNA expression, adipocyte size, and lipolysis. Further, frozen scWAT explant media and serum from these individuals were used in **studies II and III** to measure levels of cytokines and other factors. In addition, SVF cells obtained from scWAT pieces from individuals undergoing cosmetic plastic surgery were used for flow cytometry and/or FACS in **studies I-III**.

3.9 Ethical considerations

This thesis included human data and samples, as well as studies performed on mice, and therefore had some important ethical aspects to consider. All human samples and data were obtained in accordance with The Declaration of Helsinki guidelines and approved by regional committees, and all participants provided oral and written consent. Cosmetic surgery samples were obtained from individuals who would have undergone the surgery regardless and thus added no extra physical risk. Subcutaneous needle biopsies can be uncomfortable, and there is a risk for infection, but they are generally very safe. Data related to personal identity was kept coded and secret at all times.

All mouse studies were ethically approved by the relevant local authorities. The translatability of animal models to humans can sometimes be questioned, but it is currently the only feasible model to study *in vivo* interactions and it is therefore an essential part of research. Nonetheless, mouse studies were always carefully considered and planned with the three Rs in mind (replacement, reduction, refinement) in order to reduce the number of mice and unnecessary harm. We performed the majority of experiments on *in vitro* cells and only used mice when *in vivo* investigations were deemed necessary.

4 RESULTS

Study I: IL-1 β promotes adipogenesis by directly targeting adipocyte precursors

IL-1 β was previously shown to be upregulated postprandially in macrophages from WAT, but not other tissues²⁷⁷, suggesting it is involved in local energy handling. Therefore, in study I, we investigated a metabolic role of IL-1 β in the WAT by exploring different *in vivo* and *in vitro* models with blocked or activated IL-1 signaling, complemented by human observational data.

Adipocyte-specific deletion of *Il1r1* (*Il1r1*^{AKO}) had essentially no long-term effects on body weight, adiposity, WAT morphology, or local or systemic insulin sensitivity in neither chow- nor HFD-fed mice. In contrast, mice with whole-body KO of *Il1r1* (*Il1r1*^{KO}) displayed reduced body weight, fat pad mass and adipocyte size, despite unaltered food intake, and pharmacological IL-1 blockage in WT mice by anakinra treatment during the first two weeks of HFD led to similar results. This suggests that IL-1 signaling in cells other than adipocytes, possibly other tissue-resident cells, influence WAT lipid storage. Measurement of *Il1r1* expression in different cell fractions of murine WAT revealed very low mRNA levels in adipocytes, in line with the unaltered phenotype displayed by the *Il1r1*^{AKO} mice and identified the highest expression in CD45⁺ SVF cells, of which progenitors make up the largest proportion. A more detailed analysis of human scWAT-resident cell populations isolated by FACS confirmed that progenitor cells indeed have the highest expression of *IL1R1*, and mature adipocytes among the lowest levels. Therefore, we hypothesized that IL-1 β may be involved in WAT expansion by regulating differentiation of adipocyte progenitors. To investigate this, we performed *in vivo* EdU tracing experiments, which revealed a markedly impaired formation of new adipocytes in *Il1r1*^{KO} mice in response to HFD, in both scWAT and gWAT, whereas no difference was seen in chow-fed mice, suggesting IL-1 β is important for hyperplastic WAT expansion in response to caloric excess. Curiously, pharmacological IL-1 blockage with anakinra led to increased adipocyte formation in gWAT and no effect in scWAT.

The involvement of IL-1 β in adipocyte differentiation was further supported in a clinical cohort, in which scWAT expression of *IL1RN* (the gene encoding IL-1Ra) correlated positively to both adipocyte size (independently of BMI or body fat percent) and the WAT morphology value³⁰⁷, which reflects how much the adipocyte size of an individual deviates from what is expected based on their body fat percent, with positive and negative values indicating hypertrophy and hyperplasia, respectively. This links increased IL-1 antagonism to hypertrophic adipocytes, supporting an adipogenesis-promoting role of IL-1 β also in humans.

A direct adipogenic action of IL-1 β on progenitors was confirmed in two different *in vitro* systems of hASCs, in which IL-1 β treatment during differentiation dose-dependently increased lipid droplet accumulation and expression of genes involved in adipogenesis and mature adipocyte function. This was blocked by co-treatment of recombinant IL-1Ra. Image analysis at single-cell resolution revealed a marked increase in the proportion of differentiated cells, suggesting that IL-1 β might push more cells towards the adipogenic program. Conversely, treatment with other pro-inflammatory factors known to act in the WAT (IFN- γ , IL-6, LPS, MCP-1, TNF- α) had no stimulatory effect on adipogenesis, suggesting this is not a general property shared among the major inflammatory mediators, but relatively unique to IL-1 β .

RNA-seq performed at different stages of differentiation showed genes involved in adipogenesis and lipid handling upregulated by IL-1 β during the early, but not late stage of differentiation. In line with this, a time course of *IL1R1* expression in differentiating hASCs showed a rapid upregulation upon adipogenic induction that started to decline after about 2 days and reached very low levels at the late stage of differentiation. This suggested a role of IL-1 β predominantly during early adipogenesis. Indeed, treatment during distinct differentiation phases revealed that IL-1 β promotes adipogenesis exclusively during the early stage. Conversely, inflammatory gene expression was induced by IL-1 β regardless of differentiation stage.

The chronically elevated inflammation in the obese WAT is strongly linked to metabolic dysfunction and impaired adipogenesis, whereas the transient rise in IL-1 β after a meal²⁷⁷ is an important physiological regulator of glucose homeostasis²⁷⁸. To investigate whether these distinct situations have different impacts on adipogenesis, we compared acute and chronic IL-1 β treatments *in vitro*. Treatment with transient pulses (2 h) from start of adipogenic induction stimulated adipogenesis more potently than continuous exposure during early differentiation. Conversely, neither acute nor continuous IL-1 β treatment was able to stimulate adipogenesis in hASCs that had been chronically exposed to the cytokine prior to adipogenic induction, suggesting these cells had become resistant to IL-1 β . Interestingly, the inflammatory induction by IL-1 β was aggravated in these pre-treated cells, suggesting the desensitization is specific to the adipogenic signal, and not caused by e.g. receptor downregulation. To investigate whether acute inflammatory signals in general can promote adipogenesis, we treated cells transiently with different inflammatory factors. TNF- α stimulated adipogenesis, although to a much smaller degree than IL-1 β , whereas no effect was observed for IFN- γ , IL-6, LPS, or MCP-1.

To gain clues about which adipogenic signals are induced by IL-1 β , *in vitro* experiments in suboptimal differentiation media were performed. IL-1 β could completely replace IBMX, but not any other cocktail component, as an adipogenic

inducer. IBMX is a broad-spectrum phosphodiesterase inhibitor, which ultimately causes CREB activation through elevated cAMP levels¹⁹⁸. During early adipogenesis, CREB is known to upregulate C/EBP β ^{181,182}, but it has also been shown to bind to the promoter region of C/EBP δ ¹⁸². We found that IL-1 β potently activated CREB and upregulated both C/EBP δ and C/EBP β , although the former more rapidly than the latter, and these effects were more pronounced by IL-1 β than by IBMX. Additionally, we confirmed through ChIP that C/EBP δ and C/EBP β were enriched near *PPARG* and *ADIPOQ* promoters in response to IL-1 β , strongly suggesting that the IL-1 β -induced upregulation also leads to an enhanced activation of the adipogenic program.

Finally, to gain support for our hypothesis that postprandially elevated IL-1 β may act to stimulate adipogenesis, we analyzed publicly available microarray data from human scWAT obtained before and after a meal³¹⁷ and found that IL-1 β was upregulated postprandially, in line with murine data²⁷⁷. Intriguingly, the same observation was made for *CEBPD* and *CEBPB*, and their change in expression compared to baseline correlated positively to the change of *IL1B* expression, supporting our hypothesis that postprandial IL-1 β promotes adipogenesis through upregulation of *CEBPD* and *CEBPB*.

In summary, we showed that IL-1 signaling is of minor importance in mature adipocytes and instead identified their precursors as the major WAT target of IL-1 β , in which it acts to stimulate the early stages of adipogenesis. Due to the distinct actions seen for acute and chronic treatment, we propose that the adipogenic stimulation of IL-1 β takes place during its transient, postprandial surge²⁷⁷, and but these beneficial effects are lost during the chronically elevated IL-1 β levels seen in obesity (Figure 6).

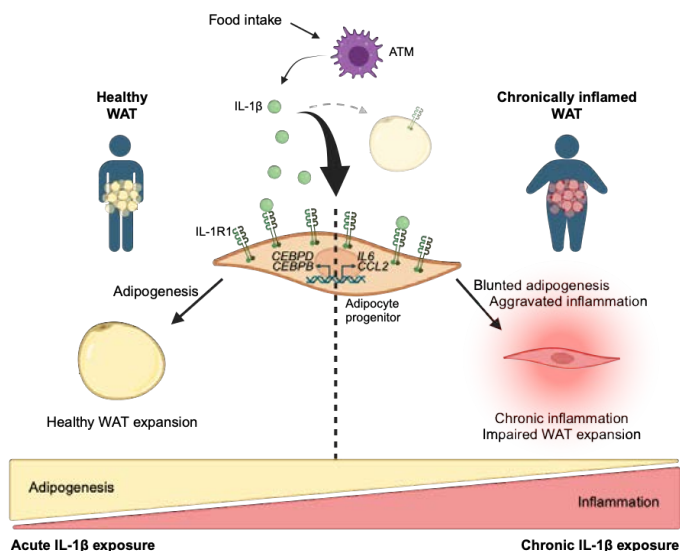


Figure 6. Graphical abstract for study I. Created with BioRender.com.

Study II: Adipose tissue specific CCL18 associates with cardiometabolic diseases in non-obese individuals implicating CD4⁺ T cells

Obesity-associated inflammation is strongly linked to cardiometabolic diseases. However, a subset of non-obese individuals also suffers from these complications, and the role of WAT inflammatory status in this context is not known. This was investigated in study II by employing several clinical cohorts combined with an *in vitro* model of T cell-adipocyte crosstalk.

WAT explant media from subjects with T2D, CVD, T2D+CVD, or healthy controls (all non-obese) was screened for 17 pro- and anti-inflammatory secreted proteins. The only factors that were elevated in all three disease groups were plasminogen activator inhibitor-1 (PAI-1) and CCL18, of which the latter showed the highest relative increases compared to healthy controls. CCL18 is a known chemoattractant of various immune cells. It was previously shown to be highly expressed by ATMs and correlated to M2 markers²⁸⁹. Analysis of both published single-cell RNA-seq data⁵ and bulk transcriptomics data of FACS-sorted cell populations³¹⁸ confirmed that CCL18 is mainly expressed by ATMs and was found to be enriched in M2 and LAM populations. We therefore analyzed immune cell populations in the WAT of these subjects to investigate whether potential source and/or target cells of the chemokine were altered. Compared to healthy controls, M1 macrophages were more abundant in T2D and T2D+CVD groups, but not in the CVD group, whereas the M2 macrophage population was not increased in any group, suggesting that the elevated CCL18 secretion was not caused by an increased infiltration of the cells producing it. The only immune cell population that was increased in all three disease groups was the CD4⁺ T cells (although it did not reach statistical significance in the CVD group; $p = 0.078$). Notably, WAT CCL18 secretion and CD4⁺ T cell proportion correlated positively in these individuals, and this relationship was further validated in three additional cohorts, where WAT expression of *CD4* and *CCL18* were positively associated. Since CCL18 is a known chemoattractant of T cells, we hypothesized that CCL18 was driving the increase in CD4⁺ T cells. Expression analysis of the reported CCL18 receptors (*CCR8*, *CCR6*, *PITPNM3*, *GPER1*, and *GPR3*) in WAT-resident cell populations supported this theory; of all cell types and receptors analyzed, the highest mRNA levels were found for *CCR6* and *CCR8* on CD4⁺ T cells, suggesting that these cells are the predominant target of CCL18 in the WAT and that the chemokine acts through *CCR6* and *CCR8* on these cells.

Increased basal lipolysis contributes to elevated circulating lipids⁵² and is therefore strongly linked to both T2D and CVD. Thus, we wanted to investigate whether the increased WAT CCL18 and CD4⁺ T cells in the cardiometabolic groups were linked to adipocyte lipolysis. Basal lipolysis of WAT explants was positively correlated with CCL18 secretion. This was not observed for lipolysis of isolated adipocytes, suggesting that the link between CCL18 and adipocyte lipolysis is dependent on non-

adipocyte cells, like CD4⁺ T cells. In line with this, treatment with recombinant CCL18 directly on adipocytes *in vitro* had no effect on lipolysis, whereas conditioned media from Jurkat T cells treated with CCL18 stimulated lipolysis of the same adipocytes. This strongly suggests that CCL18 somehow activates T cells to release lipolytic agents.

To gain clues about which factor(s) in the conditioned media from CCL18-treated T cells that stimulate lipolysis, we used scWAT-derived CD4⁺ T cells from individuals with T2D and healthy controls (all non-obese) to measure expression of ten cytokines known to be produced by CD4⁺ T cells. Five of the genes were undetectable, and among the remaining five, the genes encoding IFN- γ and TGF- β 1 were significantly upregulated in subjects with T2D. T cells were found to be the predominant expressers of both cytokines in human scWAT. Both IFN- γ and TGF- β 1 were elevated in the conditioned media from CCL18-treated T cells and recombinant proteins stimulated lipolysis when directly added to adipocytes. Importantly, inhibiting IFN- γ using a blocking antibody completely abolished the lipolytic effect of the conditioned media, whereas inhibiting TGF- β 1 showed a strong trend towards attenuated lipolytic effect ($p = 0.0504$). Using previously published single-cell RNA-seq data, we identified tissue-resident memory (TRM) and cytotoxic CD4⁺ T cells as co-expressing IFN- γ and TGF- β 1, indicating that these subpopulations might be responsible for the CCL18-mediated elevated lipolysis *in vivo*.

In summary, we showed that WAT of non-obese subjects with T2D and/or CVD secretes elevated levels of CCL18, which recruits and activates CD4⁺ T cells to stimulate basal adipocyte lipolysis through IFN- γ and TGF- β 1 (Figure 7).

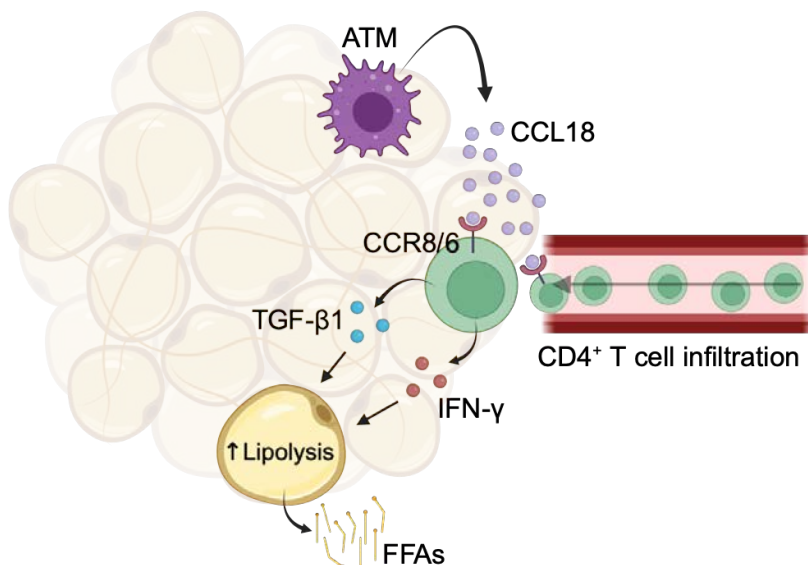


Figure 7. Graphical abstract for study II. Created with BioRender.com

Study III: Sex-specific regulation of IL-10 production in human adipose tissue in obesity

Men generally develop metabolic diseases at a lower BMI than women¹³⁶, despite women, on average, having a larger fat mass (10%) for a given BMI¹³⁷. In study III, we investigated whether sex differences in WAT inflammatory status may account for this. IL-10 was previously shown to be elevated in WAT of obese women and linked to insulin resistance, but whether this pertains to men was not known.

To gain insights on potential sex differences in WAT IL-10 production and its regulation by obesity and metabolic diseases, we measured IL-10 levels in media from scWAT explants from men and women belonging to one of the following three groups: 1) non-obese, healthy, 2) non-obese with T2D, or 3) obese with T2D. Obese women with T2D had elevated IL-10 secretion compared to the corresponding male group and all other female groups, whereas no differences were seen between any of the male groups. Non-obese women with T2D showed similar levels as non-obese healthy women, suggesting that obesity, rather than T2D drives the increased IL-10 secretion in women.

Previous studies have shown that WAT IL-10 is mainly expressed by M1 ATMs²⁸⁶. To determine whether these cells were the source of the elevated IL-10 levels in obese women with T2D, we established a protocol to stain intracellular IL-10 and analyzed the M1 population by FACS. Similar to the pattern observed for WAT IL-10 secretion and expression, the proportion of IL-10⁺ M1 ATMs was increased in obese women with T2D compared to both the corresponding male group and non-obese, healthy women, whereas no difference was observed between the male groups. This strongly suggests that the elevated IL-10 in these women originates from M1 ATMs and is due to an enrichment of the cells producing it.

We next wanted to investigate whether the difference in IL-10 production between men and women may be linked to sex-related hormones. In obese women, scWAT *IL10* expression correlated positively, albeit weakly, to circulating estrone (E1) and free E1, but not to estradiol (E2) or other hormones linked to the gonadotropin axis. This relationship did not seem to reflect a direct regulation of estrogens on IL-10 production, since *in vitro* treatment with recombinant E1 or E2 had no effect on *IL10* expression on neither THP-1 nor SVF cells.

In summary, we demonstrated a seemingly obesity-driven increase in levels of both IL-10 and IL-10-producing macrophages in women, but not men, with T2D. Although circulating E1 and WAT *IL10* expression correlated positively, *in vitro* models suggested that this was likely not due to a direct action of E1 on IL-10 production (Figure 8).

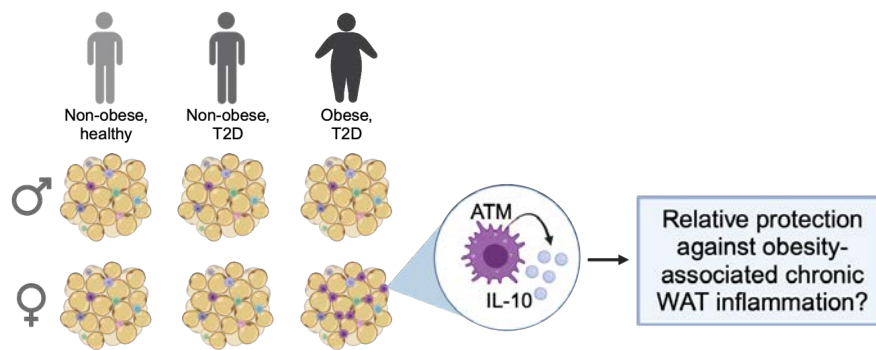


Figure 8. Graphical abstract for study III. Created with BioRender.com.

5 DISCUSSION

Study I

In study I, we showed that IL-1 β is of minor importance to mature adipocytes but instead targets adipocyte precursors in the WAT to stimulate adipogenesis. Transient treatment had a more potent adipogenic effect, whereas prolonged exposure induced resistance to the adipogenic stimulation and instead aggravated inflammation. Thus, we propose that acute elevations of IL-1 β , as seen postprandially^{277,278}, has a physiological role in allowing WAT to adapt in response to caloric excess. However, in the obese, chronically inflamed WAT, IL-1 β may have lost this ability and instead induces an exacerbated inflammatory response by progenitors. This might help explain why hyperplastic expansion often appears to be insufficient in obese individuals and is in line with studies showing an enrichment of progenitors with a pro-inflammatory profile in the obese WAT^{225,319}. This may even reflect a state of senescence, as senescent progenitors are increased in the WAT of individuals with T2D and linked to both reduced adipogenic capacity as well as increased inflammation³²⁰.

Previous *in vitro* studies have shown that IL-1 β directly influences metabolism and inflammation of adipocytes^{251,263,276,321,322}, including inducing insulin resistance^{244,245,322}. However, mice with adipocyte-specific loss of IL-1R1 showed essentially no indications of altered local or systemic metabolism, suggesting that IL-1 signaling in adipocytes is not a driver of this *in vivo*, possibly due to their low IL-1R1 expression which might render adipocytes unresponsive to IL-1 β at physiological doses. To the best of our knowledge, our study is the first to investigate a specific role of IL-1R1 signaling on mature adipocytes *in vivo*. Contradictory to our results, previous publications have reported reduced fat mass, adipocyte size, and improved local and systemic metabolism in mice with adipocyte-specific deletion of MyD88 and IRAK2²⁶³, the signaling proteins immediately downstream of IL-1R1. Notably, these and many other mediators are shared between IL-1R1 and TLR signaling. At least TLR4 and TLR2 are functional on adipocytes³²³⁻³²⁵ and can be activated by many different ligands. Thus, these mouse models have lost the ability to respond to a much broader range of stimuli than mice with IL-1R1 deletion, making conclusions about specific receptors and ligands difficult. Overall, our study strongly suggests that loss of IL-1 signaling *alone* in adipocytes does not significantly alter metabolism. However, whether IL-1R1 and TLR signaling can partially replace one another, whereby a loss of the former in our *Il1r1*^{AKO} mice was compensated by the latter, remains to be elucidated.

To track adipogenesis *in vivo*, we performed EdU-tracing assays, which revealed an impaired adipocyte formation in HFD-, but not chow-fed *Il1r1*^{KO} mice, suggesting that

IL-1 β stimulates adipogenesis as an initial response to caloric excess, but it does not seem to be involved in the “basal” differentiation that takes place in the absence of WAT expansion¹⁴⁶, as part of normal adipocyte turnover^{307,326}. From this assay, it is impossible to dissect whether IL-1 signaling promotes fat cell formation by stimulating HFD-induced proliferation or subsequent differentiation (or both). Measuring EdU⁺ progenitors after the initial week of HFD would have helped us dissect these different processes, but regardless, IL-1 clearly seems to contribute to hyperplastic WAT expansion *in vivo*. Curiously, anakinra treatment had no effect on adipocyte formation in scWAT and stimulated it in gWAT. This might reflect a true difference between transient IL-1 inhibition in adulthood and an inborn, chronic lack of IL-1 signaling, which may warrant further investigations. However, due to the nature of the intervention (daily injections with IL-1Ra or saline for the first two weeks, in addition to EdU injections every other day for the first week), these mice may have experienced increased stress and inflammation that affected the results, which is supported by the very mild and similar body weight increase in saline- and IL-1Ra-treated mice during the injection period. This is especially relevant, since the *Il1r1*^{KO} data suggests that IL-1 β only is important for adipogenesis in the context of caloric excess, which anakinra-treated mice appear to not have been in during the EdU-labeling period. Further, the short half-life of anakinra reported in mice (2 h at our used dose)³⁰⁵ makes it questionable whether one injection per day was enough to block IL-1-stimulated adipogenesis, especially considering the potential relevance of the postprandial rise and the long feeding window of mice.

Although reduced adipogenesis is generally reflected by a hypertrophic WAT, we observed unaltered (gWAT) or decreased (scWAT) adipocyte size in HFD-fed *Il1r1*^{KO} mice. Likely, ubiquitous deletion of *Il1r1* led to decreased fat mass partly due to reasons independent of impaired adipogenesis, since we also observed it in chow-fed mice, in which adipocyte formation was normal. Thus, the absence of WAT hypertrophy despite impaired adipogenesis was likely due to a decreased overall lipid accumulation that was unrelated to reduced fat cell formation, thus rescuing the hypertrophic phenotype that would otherwise be expected from impairments in adipogenesis. In further support of this, gWAT of HFD-fed mice showed the largest impairment in adipogenesis and was the only depot with no decrease in adipocyte size. Reduced lipid accumulation unrelated to impaired adipogenesis may have been the result of *Il1r1* deletion in other tissues. For instance, IL-1 β has a physiological role in stimulating postprandial insulin secretion²⁷⁸. Ubiquitous loss of IL-1 signaling might therefore lead to a decrease in insulin and its downstream lipogenic and anti-lipolytic actions, and thereby reduced lipid accumulation. Of note, other studies on *Il1r1*^{KO} mice have shown varying results, with unaltered³²⁷ or increased fat mass^{327,328}, and unaltered³²⁹, decreased³²⁹ or increased adipocyte size^{329,330}, and deletion of IL-1Ra curiously causes reduced WAT mass and adipocyte size^{331,332}, similar to our *Il1r1*^{KO} mice. These contradictory observations likely reflect the multifaceted role of IL-1 in

energy homeostasis throughout the body, including leptin secretion and signaling^{333,334}, insulin secretion and glucose homeostasis²⁷⁸, energy expenditure³³¹, and postprandial fatigue³³⁵, and that interfering with this complex system may have varying results depending on factors such as the genetic background, age, sex, diet, and infectious status of the mice, as well as whether littermate mice were used as controls.

Unfortunately, we did not use a progenitor-specific knockout model and therefore cannot rule out that the attenuation in adipogenesis was influenced by loss of IL-1 signaling in non-progenitor cells. Although progenitor-targeted models have been used, mainly *Pdgfra* promoter-driven knockout lines, neither this nor other used models are exclusive to WAT-resident progenitors^{8,300-303}. Instead, we extensively confirmed a direct adipogenic action of IL-1 β on progenitors using two different human *in vitro* models. The exclusivity of this action to the early differentiation stage is consistent with the lack of phenotype displayed by the *Il1r1*^{AKO} mice, whose *Il1r1* deletion is under control of the *Adipoq* promoter, which is not active in the initial stages of differentiation. It is also in line with C/EBP δ and C/EBP β being the mediators of IL-1 β , as the adipogenic action of these transcription factors only takes place during the early stage of differentiation. In fact, overexpression of C/EBP β LAP (the adipogenic isoform) downregulates genes related to adipogenesis and mature adipocyte function during the late differentiation stage¹⁸³.

Our findings are somewhat in contrast to previous studies, which have shown impaired *in vitro* adipogenesis by IL-1 β in WAT models^{256,273,336}, whereas a pro-adipogenic effect has been demonstrated in dermal³³⁷ and orbital^{338,339} fibroblasts in the context of wound healing and Grave's ophthalmopathy, respectively. Our study clearly demonstrated that the adipogenic effect of IL-1 β is highly dependent on differentiation stage and exposure time. *In vitro* adipogenesis studies on IL-1 β are generally carried out by treating the cells throughout the entire course of differentiation. When we employed such stimulation (13 days of IL-1 β treatment in differentiating hASCs), there was no increase in lipid accumulation, and *PPARG* expression was downregulated. With this treatment setup, any potential stimulation of adipogenesis during the early stage is likely counteracted by an opposite action during the later stage (either due to chronic or late treatment). This switch in actions by IL-1 β may appear at somewhat different stages in different cell models. Additionally, since IBMX and IL-1 β employ similar adipogenic pathways, the IL-1 β -induced signal may have been masked by IBMX in some studies. For instance, if IBMX maximally activates CREB, adding IL-1 β might not have an additional stimulatory effect, and later opposing actions might then become more pronounced. We observed a more potent stimulation by IL-1 β than IBMX, possibly explaining why IL-1 β could promote adipogenesis even in the presence of IBMX, but this likely differs between cell models and IBMX concentrations.

A recurring observation in our *in vitro* investigations was a clear distinction between the adipogenic and inflammatory effects caused by IL-1 β . The latter displayed no discrimination between differentiation stages, and was exacerbated by chronic treatment, in stark contrast to the adipogenic stimulation. This raises the question of whether these two responses are mediated via entirely separate signaling arms, or if their pathways are somehow connected. Other pro-inflammatory factors did not have similar effects on differentiation as IL-1 β , suggesting its adipogenic stimulation is not mediated through classical inflammatory pathways, although it might be via a pathway shared between IL-1 β and TNF- α , since acute treatment with the latter cytokine also promoted differentiation mildly. Our data point to IL-1 β promoting adipogenesis via CREB-mediated *CEBPD* induction. In other contexts, both CREB and C/EBP δ have been shown to be intimately involved in immune responses^{340,341}. C/EBP δ can both induce and be induced by several pro-inflammatory factors³⁴⁰, including IL-1 β and TNF- α ³⁴². Similarly, CREB can be activated by many pro-inflammatory cytokines through p38 and Erk1/2 pathways³⁴³. The role of cytokines as inducers of these signals in adipogenesis have not been thoroughly studied, but it is known that Erk is important for adipogenesis^{344,345}, whereas p38 has been attributed both pro- and anti-adipogenic effects^{346,347}. Notably, a recent study on dermal fibroblasts demonstrated an adipogenic effect of IL-1 β via CREB-activation dependent on p38 and NF κ B pathways³³⁷. Taken together, this points to an intimate but complex interplay between inflammatory and adipogenic intracellular signals, which remains to be fully elucidated.

It is widely accepted that the obesity-associated chronic low-grade WAT inflammation has many pathological features that are detrimental to local and systemic metabolic health. However, studies have also shown that both progenitor- and adipocyte-specific inflammation is required for healthy, hyperplastic WAT remodeling and insulin sensitivity^{264-266,268,348}, likely reflecting the physiological importance of transient WAT inflammation. Indeed, our own data point to acute IL-1 β promoting adipogenesis over inflammation, and vice versa. Adipogenesis is a progressive and sequential process, and acute IL-1 β exposure was clearly sufficient to stimulate early regulators, which could then propel the adipogenic program even after its removal. This is in line with the described delayed adipogenic activity of C/EBP δ and C/EBP β ; although their expression is rapidly induced upon adipogenic induction, there is a lag time before they acquire DNA-binding activity, which coincides with the S-phase of the cell cycle^{167,184,349}. Thus, the rapid induction of *CEBPD* by IL-1 β is likely enough to relay the adipogenic program several hours later, long after the cytokine is removed. The slight reduction in adipogenic promotion when continuing IL-1 β treatment past 2 hours may have the same underlying mechanism as the pre-treatment-induced desensitization to the adipogenic effects of IL-1 β ; given the aggravated inflammatory gene induction in both prolonged treatment conditions, NF κ B was likely more activated, which may have counteracted any pro-adipogenic signal induced by IL-1 β .

For instance, NF κ B has been shown to redirect co-factors shared by adipocyte-specific and inflammatory genes to the latter²⁶¹. Removing IL-1 β after 2 hours likely also turned off NF κ B activation, and thus enabled C/EBP δ and C/EBP β to relay the adipogenic program with less obstruction once they acquired DNA-binding activity. However, this hypothesis remains to be elucidated, as we did not investigate the underlying mechanism of the desensitization induced by prolonged treatment. Taken together, our results highlight that a pro-inflammatory stimulus can have markedly different effects depending on the duration of the signal, possibly reflecting distinct roles of acute and chronic inflammation in metabolic function of the WAT.

Due to the potent adipogenic effect by acute IL-1 β treatment, we propose that the transient postprandial upregulation of IL-1 β reported in murine ATMs previously²⁷⁷ and in whole scWAT in humans according to our analysis of publicly available data³¹⁷, is a physiological response to energy influx which serves to promote healthy, hyperplastic WAT expansion. This raises the question of whether it would be beneficial and/or feasible to promote adipogenesis after every meal. It is possible that this response is involved in regulating adipocyte turnover, and thus acts to maintain, rather than increase adipocyte number. In that case, postprandial adipogenic induction might be feasible due to the relatively high rate of adipocyte turnover (8% annually in humans). However, this is less likely (at least in mice) since we did not observe an altered rate of adipocyte formation in chow-fed *Il1r1*^{KO} mice. Based on our *in vitro* experiments in suboptimal differentiation media, IL-1 β is not able to initiate the adipogenic program on its own but requires e.g. insulin signaling and activation of GR and PPAR γ (at least in the *in vitro* setting). Therefore, it is likely that the postprandial rise in IL-1 β is only able to stimulate adipogenesis when other mediators are simultaneously present, and that may happen less frequently.

Study II

In study II, we aimed to identify WAT-derived pro- or anti-inflammatory factors that may be involved in cardiometabolic disease progression in the absence of obesity and identified a CCL18-driven accumulation and activation of CD4⁺ T cells secreting IFN- γ and TGF- β 1, which in turn stimulated basal lipolysis of adipocytes.

An important aspect of this study is that it highlights a pathological process occurring in non-obese individuals and thus provides some possible mechanistic insights on why a subset of individuals develop cardiometabolic diseases in the absence of obesity. It was previously shown that WAT CCL18 production is elevated in T2D²⁹¹ and associated to metabolic risk factors for both T2D and CVDs in obese individuals²⁸⁹. Further, scWAT CCL18 expression is higher in obese compared to lean^{350,351} and non-obese³⁵² individuals and correlates positively to BMI²⁹⁰. In parallel, CD4⁺ T cells³⁵³, IFN- γ ³⁵³ and TGF- β 1³⁵⁴ are all elevated in the obese WAT. Therefore, it is likely that

our study reflects a sequence of events that also occurs in obesity, perhaps even to a greater extent. However, this was not investigated by the present study. Instead, our study uncovers an inflammation-mediated crosstalk between immune cells and adipocytes in the WAT, which takes place even in the absence of obesity.

The cause of elevated CCL18 secretion was not investigated in this study. Non-WAT studies have shown expression of the chemokine in macrophages³⁵⁵, dendritic cells³⁵⁵⁻³⁵⁷, monocytes³⁵⁵, eosinophils³⁵⁸, and neutrophils³⁵⁹. We and others²⁸⁹ found that ATMs are the predominant source of this chemokine in the WAT, and the single-cell sequencing data analysis pointed to both M2 and LAM populations. Since neither M1 nor M2 macrophage populations were enriched in all cardiometabolic groups (the latter not in any), the elevated CCL18 secretion was likely not due to an increased infiltration or polarization of these cells but may instead have been a result of increased production by individual cells. However, we cannot rule out that a subset of CCL18-secreting macrophages was enriched in the WAT, since we did not investigate additional macrophage populations, such as LAMs. This subtype is intriguing, as its abundance in the tissue, unlike M2 macrophages, is increased in obesity, with *CCL18* among its signature genes¹⁹. LAMs may thus be an important source of the elevated CCL18 levels in the obese WAT, and possibly also in our non-obese cardiometabolic groups. The regulation of CCL18 production has never been studied in the WAT, but *in vitro* studies in other contexts have shown upregulation in macrophages by estrogen receptor α ligands³⁶⁰, IL-4^{356,361,362}, IL-6³⁶¹, IL-10^{362,363}, IL-13³⁶², and granulocyte-macrophage colony-stimulating factor (GM-CSF)³⁶⁴, all of which are present in the WAT, and many of which are linked to metabolic impairments. Future studies are needed to investigate the factor(s) driving the cardiometabolic disease-associated elevation in WAT CCL18.

CCL18 is a known chemoattractant of T cells, including CD4⁺ T cells^{355,356,365-367}, and was thus likely driving the infiltration of these cells into the WAT of cardiometabolic groups. This is in line with CCR8 being identified as one of the two CCL18 receptors highly expressed by WAT-resident CD4⁺ T cells, since this receptor has been shown to mediate CCL18-induced chemotaxis of Th1, Th2 and CD8⁺ T cells^{366,368}. Further, we found that stimulating T cells with CCL18 alone promoted secretion of IFN- γ and TGF- β 1, suggesting CCL18 both recruits CD4⁺ T cells and activates them into a specific phenotype. This latter action is somewhat unconventional for a chemokine, although it is in line with the many functions beyond chemotaxis demonstrated for CCL18³⁶⁹. IFN- γ and TGF- β 1 are described to be secreted by Th1³⁷⁰ and Tregs^{371,372}, respectively. Thus, CCL18 might stimulate polarization into these two different subtypes, or it may induce a subtype not currently recognized as secreting both factors, such as cytotoxic or TRM CD4⁺ T cells, as alluded to from our single-cell transcriptomics analysis. Unfortunately, we did not include markers of CD4⁺ subtypes in our FACS panel.

Basal, but not stimulated fat pad lipolysis was positively correlated to CCL18 secretion. This is important, because only basal lipolysis is a significant contributor to circulating lipid levels⁵², which in turn can contribute to the development of both CVD and T2D³⁷³. The lack of correlation observed for lipolysis of isolated adipocytes (rather than fat pad) may reflect a requirement of factors derived from non-adipocytes, such as CD4⁺ T cell-derived IFN- γ or TGF- β 1, for the lipolytic induction to take place. We attributed the lipolytic action of CCL18-stimulated T cells to these cytokines. Both IFN- γ and TGF- β 1 are elevated in the WAT in obesity and strongly implicated in the metabolic diseases that accompany it³⁷⁴. The lipolytic action of IFN- γ is well established^{251,375,376} and has been at least partly attributed to prostaglandin synthesis²⁵¹ and decreased activity and expression of lipoprotein lipase³⁷⁵. Much less is known about the direct role of TGF- β 1 on lipid metabolism of adipocytes. Opposite effects have been described for two other TGF- β -like ligands; Activin A reduces lipolysis in 3T3-L1 adipocytes³⁷⁷, and signaling of ALK7, a type I TGF- β receptor, has been linked to reduced lipolysis both *in vivo* and *in vitro*, partly through downregulation of HSL and ATGL^{378,379}. Neither Activin A nor TGF- β 1 binds to ALK7, but they all canonically act through SMAD2/3 signaling, although SMAD-independent signaling also occurs³⁸⁰, which may explain the distinct effects on lipolysis. Further investigations are warranted to elucidate the mechanism of TGF- β 1-induced lipolysis.

Curiously, even though we identified IFN- γ as one of the secreted factors by T cells in response to CCL18, its secretion from WAT explants was not significantly elevated in the cardiometabolic disease groups, although all groups showed a small trend towards increased levels. Most of the measured proteins were measured with the Luminex platform, which is less accurate at low concentrations than the MSD system^{381,382}, and it is therefore possible that we, for some low-abundance factors like IFN- γ , missed subtle differences between groups that would have been caught with a more sensitive method.

Finally, due to the relatively small size of the cardiometabolic disease cohort (15-18 individuals per group), we cannot confidently conclude that all our results would hold true in a larger cohort. Measurements like lipolysis regulation in different patient groups may require larger cohorts.

Study III

In study III, we reported an increased IL-10 production in the WAT of obese women with T2D compared to both the corresponding male group and non-obese women, whereas no differences were observed between the male groups. This was concomitant with an enrichment of IL-10-producing M1 macrophages in the WAT of the same group of women. Although WAT *IL10* expression correlated positively to circulating E1, *in vitro* experiments excluded a direct effect of the hormone on

macrophage IL-10 production. Taken together, this points to an obesity-driven stimulation of IL-10 production, which is exclusive to women. Since circulating levels were not regulated in the same manner, which is in line with data implicating the spleen as the major source of circulating IL-10³⁸³, this sex-specific elevation of IL-10 likely acts locally in the WAT and may be a contributing factor to the sexual dimorphism displayed in obesity-associated inflammation and its comorbidities.

Although sexual dimorphism in IL-10-mediated immune responses have been displayed for colitis³⁸⁴, lung infection³⁸⁵, and traumatic brain injury³⁸⁶, we are, to the best of our knowledge, the first to report it in the WAT and in the context of chronic metabolic diseases. The elevated WAT IL-10 production in women with T2D who were obese, but not non-obese, suggests that WAT IL-10 production is obesity-, rather than T2D-associated. However, since we did not include obese, healthy women in the cohort for comparison, it cannot be ruled out that it is regulated by a combination of both. In fact, positive correlations between WAT IL-10 secretion and markers of insulin resistance independent of adiposity in non-obese and obese individuals have been observed by a previous study, although both men and women were included²⁸⁶. Elevated WAT *IL10* expression in insulin resistant compared to insulin sensitive obese women was also found²⁸⁶, suggesting that IL-10 production in women is linked to metabolic impairments at least in obesity. Both obesity and metabolic dysfunction are intimately linked to WAT inflammation, to which IL-10 production is also strongly associated. For instance, WAT/adipocyte IL-10 production is directly promoted by pro-inflammatory cytokines^{286,387} and its expression can be induced by NF κ B³⁸⁸, which is in line with our observed positive and BMI-independent correlation between WAT secretion of inflammatory factors and IL-10. Further, in human WAT, IL-10 is highly secreted by the pro-inflammatory M1 ATM subtype²⁸⁶, and we confirmed that a higher proportion of these cells were producing IL-10 exclusively in obese women with T2D, strongly pointing to these cells as the source of their elevated IL-10 secretion. However, we cannot rule out that other WAT-resident cells, such as B and T cell subtypes, have also contributed, as described in mice^{285,389}. Thus, considering the well-established anti-inflammatory role of IL-10, its production may be enhanced in women to attenuate the chronic inflammation seen in the obese and/or metabolically dysfunctional WAT, which may partly explain why females have a healthier metabolic phenotype and milder inflammatory WAT profile than males^{136,140,141}. However, why this regulation appears to be different in men and women, and which mediators, inflammatory or not, are responsible for this sexual dimorphism, remains to be elucidated.

One possible contributor to the increased IL-10 production in women could be sex hormones. We found that circulating E1, but not other measured hormones, correlated significantly (positively), albeit weakly, to WAT *IL10* expression. WAT produces estrogens, including E1, in both pre- and postmenopausal women^{390,391}, and is the predominant site of estrogen production in postmenopausal women³⁹². The role of

estrogens in obesity-associated diseases is complex. The preferential adiposity in subcutaneous gluteofemoral regions in women, which is linked to preserved metabolic health, has been attributed to estrogens¹³⁵, likely partly because it promotes adipocyte hyperplasia in subcutaneous depots, although it has the same effect in visceral depots^{151,393}, and its production is higher in the latter in women^{390,391}. Further, estrogens have been linked to increased risk of breast cancer³⁹⁴ and pulmonary arterial hypertension³⁹⁵, but also seems to protect against inflammation and contribute to insulin sensitivity in muscle, WAT and liver³⁹⁶. In mice, the protection against HFD-induced ectopic lipid accumulation in females can be mimicked in males by administering estrogen, which enhances insulin-mediated suppression of adipocyte lipolysis through decreased WAT inflammation¹⁴². The positive correlation between E1 and *IL10* is in line with data showing that estrogen stimulates IL-10 levels, both in circulation *in vivo*^{397,398} and secreted from T cells *in vitro*³⁹⁹⁻⁴⁰¹. However, we excluded a direct effect of E1 on macrophage IL-10 production, although we cannot rule out an indirect stimulation *in vivo*. On the other hand, the anti-inflammatory properties of IL-10 can indirectly suppress estrogen production, as inflammatory cytokines upregulate aromatase, which catalyzes the conversion of androgens to estrogens in WAT⁴⁰². We only had access to scWAT in this cohort, and since vWAT is the predominant producer of E1^{390,391}, circulating levels may be more influenced by, and thereby correlate differently to, IL-10 production in the latter depot. Further, it is possible that our observed correlation was confounded by e.g. inflammatory factors, since they are expected to increase E1 production⁴⁰², and they correlated to IL-10 in our cohort.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis explored three ATM-derived adipokines (IL-1 β , CCL18, and IL-10) and their role in WAT under distinct metabolic contexts, using *in vitro* cell systems, *in vivo* mouse models, and clinical cohorts. These studies have provided valuable insights on the intricate connections between WAT-resident immune cells and processes related to expansion and metabolic function of the tissue in both health and disease.

In **study I**, we reported a direct pro-adipogenic action of acute IL-1 β exposure on adipocyte precursors, which is believed to be an ATM-mediated physiological response to energy influx and touched upon a possible switch to a more adverse role in obesity-associated chronic inflammation. **Study II** discovered a crosstalk between ATMs, CD4⁺ T cells and mature adipocytes mediated through CCL18 and IFN- γ /TGF- β 1 that leads to increased lipolysis and thus likely contributes to the development of cardiometabolic diseases in the absence of obesity. In **study III**, we demonstrated a seemingly obesity-driven elevation in ATM IL-10 production exclusively in women that may act to attenuate obesity-associated chronic inflammation and thereby protect against its comorbidities.

The main research regarding WAT inflammation has been focused on obesity-associated, chronic, pathological aspects, whereas acute, physiological signals have been gaining less attention. **Study I** highlighted a clear distinction between acute and chronic IL-1 β , which may hold true for many inflammatory mediators acting on different cells in the WAT. As described in the introduction, ATMs respond rapidly to nutritional cues to activate intrinsic lipid handling properties, but our study suggests that their postprandial crosstalk with other resident cells should also be explored. The importance of acute inflammatory signals may also explain why therapeutic strategies aimed to directly block inflammation have largely proven unsuccessful at treating metabolic diseases. In the case of IL-1 β , IL-1 blocking drugs have been explored against metabolic diseases, e.g. T2D⁴⁰³⁻⁴⁰⁵. Their major effect seems to be to improve pancreatic β -cell function, but no improvements in systemic insulin sensitivity have been found⁴⁰³⁻⁴⁰⁷. Therapeutic strategies that are able to differently target acute and chronic actions may be more successful in combating obesity-associated diseases.

The role of adiposity in cardiometabolic diseases have, for obvious reasons, been mainly studied in obese individuals, and not much is known about how/whether inflammatory status of the WAT may contribute to these complications in non-obese individuals. **Study II** discovered a crosstalk between immune cells and adipocytes occurring in non-obese individuals that results in an adverse metabolic status of the WAT, thus highlighting a potentially pathological role of WAT-resident immune cells even in the absence of excessive adiposity. This emphasizes the need for further

characterization of WAT from non-obese, metabolically unhealthy individuals to identify other potential local alterations in cytokine production and how it may contribute to metabolic diseases. This might be key to understanding the etiology of metabolic diseases in these individuals, and whether it differs from that of obese individuals. In the case of CCL18, elucidating the upstream events leading to its increased production will be important to pinpoint initiating alterations that may eventually cause or aggravate cardiometabolic diseases in the absence of obesity.

Although the sexual dimorphism displayed for metabolic diseases and adiposity is well recognized, less has been explored regarding differences in WAT inflammatory status between obese men and women. **Study III** identified a sex difference in IL-10 production and IL-10-producing ATM proportion in obesity, but due to the mainly observational nature of this study, it left many questions open regarding the sex-specific regulation of IL-10 production and the potential role it plays in metabolic diseases. Nonetheless, the study highlighted that the inflammatory status of scWAT is regulated by obesity differently in men and women, opening up for further, more detailed explorations on sex differences in inflammatory modulators and immune cell phenotypes. Single-cell technologies could provide valuable, in-depth insights on immune cell subtypes regulated differently in obese men and women. Identifying the potential role IL-10 and any other factor discovered in the future play in local and systemic metabolic health and the sex-specific mechanism behind their production will be key to understanding the underlying factors protecting women from metabolic diseases.

Still a lot remains to be elucidated about the intricate crosstalk between WAT-resident cells and its impact on local and systemic metabolism. This thesis has highlighted certain aspects and contexts of WAT inflammation that have been somewhat overlooked, namely 1) distinction between acute and chronic WAT inflammation in general, and the role of postprandial WAT inflammation in particular, 2) pathological WAT inflammation in the absence of obesity, and 3) sex-specific regulation of local cytokine production. Future studies should carefully consider these aspects.

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