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CHARACTERIZATION OF ADIPOSE FACTORS REGULATED BY BODY WEIGHT

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ABSTRACT

White adipose tissue (WAT) constitutes our most expandable tissue and largest endocrine organ secreting hundreds of polypeptides collectively termed adipokines. Changes in WAT mass induce alterations in adipocyte secretion and function, which are linked to disturbed whole-body metabolism. Although the mechanisms controlling this are not clear they are dependent on changes in gene expression, a complex process which is regulated at several levels. Results in recent years have highlighted the role of small non-coding RNA molecules termed microRNAs (miRNAs), which regulate gene expression via post-transcriptional mechanisms. The aim of this thesis was to characterize global gene expression levels and describe novel miRNAs and adipokines controlling the function of human WAT in conditions with pathological increases or decreases in WAT mass. Obesity and cancer cachexia were selected as two models since they are both clinically relevant and characterized by involuntary changes in WAT mass.

In **Study I**, expressional analyses were performed in subcutaneous WAT from cancer patients with or without cachexia and obese versus non-obese subjects. In total, 425 transcripts were found to be regulated in cancer cachexia. Pathway analyses based on this set of genes revealed that processes involving extracellular matrix, actin cytoskeleton and focal adhesion were significantly downregulated, whereas fatty acid metabolism was upregulated comparing cachectic with weight-stable cancer subjects. Furthermore, by overlapping these results with microarray data from an obesity study, many transcripts were found to be reciprocally regulated comparing the two conditions. This suggests that WAT gene expression in cancer cachexia and obesity are regulated by similar, albeit opposing, mechanisms. In **Study II**, the focus was on the family of fibroblast growth factors (FGFs), members of which have recently been implicated in the development of obesity and insulin resistance. A retrospective analysis of global gene expression data identified several FGFs (FGF1/2/7/9/13/18) to be expressed in WAT. However, only one, FGF1, was actively secreted from WAT and predominantly so from the adipocyte fraction. Moreover, FGF1 release was increased in obese compared to non-obese subjects, but was not normalized by weight loss. Although the clinical significance of these findings is not yet clear, it can be hypothesized that FGF1 may play a role in WAT growth, possibly by promoting fat cell proliferation and/or differentiation. In **Study III**, we identified adipose miRNAs regulated in obesity. Out of eleven miRNAs regulated by changes in body fat mass, ten controlled the production of the pro-inflammatory chemoattractant chemokine (C-C motif) ligand 2 (CCL2) when overexpressed in fat cells and for two, miR-126 and -193b, signaling circuits were defined. In **Study IV**, a novel adipokine, semaphorin 3C (SEMA3C), was identified by combining transcriptome and secretome data. Detailed studies focusing on SEMA3C revealed that this factor was secreted from adipocytes and induced the expression of extracellular matrix and matricellular genes in preadipocytes. Furthermore, SEMA3C mRNA levels correlated with interstitial fibrosis and insulin resistance in WAT derived from subjects with a wide range in BMI.

In summary, the results presented in this thesis have delineated transcriptional alterations in WAT in two clinically relevant conditions, obesity and cancer cachexia.

This has allowed the identification of novel adipokines and microRNAs with potential pathophysiological importance. These findings form the basis for further studies aiming at understanding the central role of WAT in disorders associated with metabolic complications.

LIST OF PUBLICATIONS

I. Dahlman I, **Mejhert N**, Linder K, Agustsson T, Mutch DM, Kulyte A, Isaksson B, Permert J, Petrovic N, Nedergaard J, Sjölin E, Brodin D, Clement K, Dahlman-Wright K, Rydén M, Arner P. Adipose tissue pathways involved in weight loss of cancer cachexia. Br J Cancer. 2010;102:1541-8.

II. **Mejhert N**, Galitzky J, Pettersson AT, Bambace C, Blomqvist L, Bouloumié A, Frayn KN, Dahlman I, Arner P, Rydén M. Mapping of the fibroblast growth factors in human white adipose tissue. J Clin Endocrinol Metab. 2010;95:2451-7.

III. Arner E*, **Mejhert N***, Kulyté A, Balwierz PJ, Pachkov M, Cormont M, Lorente-Cebrián S, Ehrlund A, Laurencikiene J, Hedén P, Dahlman-Wright K, Tanti JF, Hayashizaki Y, Rydén M, Dahlman I, van Nimwegen E, Daub CO, Arner P. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. Diabetes. 2012;61:1986-93.

IV. **Mejhert N**, Wilfling F, Esteve D, Galitzky J, Pellegrinelli V, Kolditz C-I., Viguerie N, Tordjman J, Näslund E, Trayhurn P, Lacasa D, Dahlman I, Stich V, Lång P, Langin D, Bouloumié A, Clément K, Rydén M. Semaphorin 3C is a novel adipokine linked to insulin resistance and fibrosis deposition in human adipose tissue. Submitted.

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CONTENTS

LIST OF ABBREVIATIONS

BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HNF4	Hepatic nuclear factor 4
IL6	Interleukin 6
miRNA	MicroRNA
RT-PCR	Real-time polymerase chain reaction
SEMA3	Class 3 semaphorin
TF	Transcription factor
TG	Triglyceride
TNFα	Tumor necrosis factor α
UTR	Untranslated region
WAT	White adipose tissue

1 INTRODUCTION

White adipose tissue (WAT) is a remarkably plastic organ that can alter considerably in size and constitution within and between individuals. In order to enable rapid changes in fat mass upon energy oversupply/deprivation, the tissue is constantly remodeled. This process includes both changes in the structure/composition of WAT as well as adipocyte metabolism. In all papers included herein, two conditions, obesity and/or cancer cachexia, that affect WAT function were studied using gene expression profiling (a method described in section 3.1.1 Transcriptional profiling) and candidate transcripts were chosen for in-depth analyses using several other techniques. Since important inter-species differences (e.g. comparing human and mice) in terms of WAT metabolism exist and the presented studies are combining clinical as well as basic research, the following sections summarize areas relevant for the understanding of human WAT biology from a clinical and molecular perspective. However, this is not intended to be a full overview of all aspects in this field, since there are a number of publically available reviews covering these topics (1–3). Instead, only aspects important for interpreting the main findings presented in each study will be discussed below.

1.1 CANCER CACHEXIA

Cachexia is a multifactorial wasting syndrome observed in several different clinical settings, which is associated with poor prognosis and aggravated risk of developing complications in response to therapeutic interventions. Although no consensus definition exists, cachexia is generally defined as an involuntary weight loss exceeding 5 % in the last three months or 10 % in the last six months, and is often associated with symptoms such as fatigue, weakness, decreased appetite, reduced physical activity and loss of lean body mass (primarily muscle) as well as WAT. In oncology, it is estimated that approximately 50 % of all cancer patients suffer from cachexia, although the frequency varies depending on the location of the tumor (e.g. upper gastrointestinal cancer patients have a prevalence up to 80%) (4). The exact mechanism(s) causing cachexia in cancer is not known, but it has been suggested that factors derived from the tumor or the body's response to the tumor (host-response) may be of importance. For instance, it is well established that circulating pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin 1 and interleukin 6 (IL6), are elevated in cachexia and it is believed that these, may play a role in promoting weight loss (2). There are no effective treatment options for cachexia and current strategies aim at stimulating appetite, providing nutrient-rich food and inhibiting cytokine production. However, ensuring adequate caloric intake and/or reducing inflammation have failed to reverse the wasting process.

1.2 OBESITY

In adults, the most commonly used clinical definition of obesity is based on body mass index (BMI), which is calculated as the quotient between an individual's weight (in kilo grams) and the square of the height (in meters). Normal weight is defined as BMI values between 18.5-24.9, overweight as 25.0-29.9 and obesity as values $\geq$30 kg/m^2 (5). High BMI levels are associated with increased risk for developing adverse metabolic effects on blood pressure, insulin sensitivity and circulating cholesterol as

well as triglyceride levels, which in turn are linked to cardiovascular disease and type 2 diabetes. Although current BMI ranges are used world-wide, they have primarily been validated in Caucasian European populations. However, it is important to emphasize that other (in particular certain Asian) populations may develop metabolic complications at considerably lower BMI values, which has raised the debate whether the BMI classification system should be different for specific ethnic groups (6).

According to the World Health Organization, the prevalence of obesity has more than doubled between 1980 and 2008 and it is estimated that at least 2.8 million people die each year as a result of being overweight or obese (5). The rapid escalation in prevalence is thought to be explained by life style factors primarily determined by increased energy intake and reduced energy expenditure (due to a sedentary life style). It is important to stress that although genetic factors certainly affect energy balance, it is not likely that any single gene explains the accelerated obesity incidence observed in the last decades. There are no effective and at the same time completely safe ways to pharmaceutically treat obesity or prevent it from causing co-morbidities. At present, sustained weight loss achieved via bariatric surgery, dietary restrictions and/or increased physical activity seems to be the only option that efficiently reverses the metabolic consequences of obesity (7,8).

1.3 WHITE ADIPOSE TISSUE

White adipose tissue is a heterogeneous organ consisting of a mixed population of cells, including fat cells (adipocytes), leucocytes, fibroblasts, adipocyte progenitor and endothelial cells. There are several WAT depots in the body and the main functions of the tissue are to store and release energy, thermally insulate and mechanically protect the body. In addition, work from the last two decades have made it clear that WAT is an active endocrine organ, which secretes a vast number of factors with local and/or systemic effects on several processes including lipid and glucose metabolism (further discussed in section 1.5 Adipokines).

1.3.1 Adipocytes

Adipocytes, which are the most abundant cell type within WAT, are specialized in storing energy in the form of triglycerides (TGs). Fat cells are morphologically characterized by a single, large lipid droplet, which constitutes >95% of the cell volume. A thin layer of cytoplasm containing a stretched-out nucleus is observed in one pole of the cell (Figure 1). Stored TGs originate primarily from dietary intake, but they can also be produced *de novo* from non-lipid substrates within WAT (discussed in detail in (9)). Adipocyte lipid metabolism, i.e. synthesis, re-esterification, hydrolysis and oxidation of lipids, is tightly regulated by hormones, exercise and nutritional status. When the body needs energy, TGs are hydrolyzed by three distinct lipases into non-esterified fatty acids and glycerol in an enzymatic process termed lipolysis. The majority of the non-esterified fatty acids are then released from the fat cells into the circulation, bound to albumin and transported to target tissues for subsequent oxidation. In addition to being energy substrates, fatty acids can also function as signaling molecules, constituents of plasma membranes or be re-esterified into TGs (10).

1.3.2 Adipose tissue morphology

In adults, it has recently been shown that approximately 10 % of the adipocyte population is renewed annually independent of body mass status and that the turnover is dependent on the generation and death rate of the fat cells (11). A restricted production of adipocytes leads to adipose hypertrophy, a phenotype characterized by fewer and larger adipocytes. This is in contrast to adipose hyperplasia where the generation rate is high, leading to many, but smaller fat cells (12). It is well-established that adipose hypertrophy correlates with a pernicious metabolic profile (12,13).

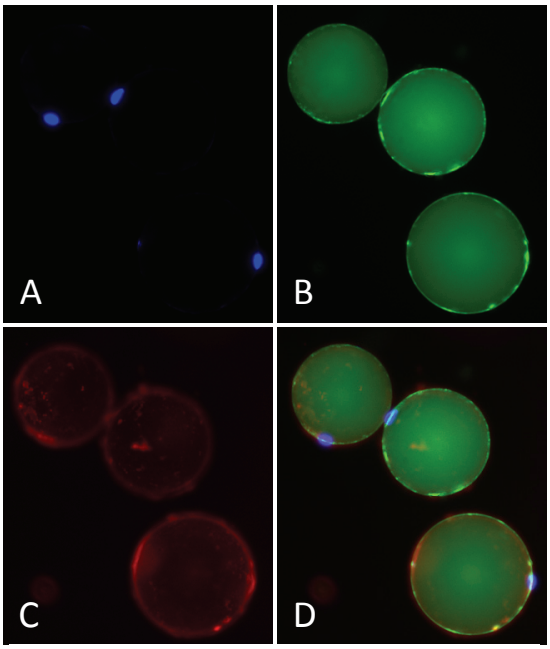

Figure 1. Isolated fat cells stained for **A)** nuclei, **B)** lipids and **C)** cytoplasm. In **D)** an overlay image is presented.

1.4 PATHOPHYSIOLOGICAL ALTERATIONS IN WHITE ADIPOSE TISSUE FUNCTION

1.4.1 Comparison of obesity and cancer cachexia

Comparisons of obesity and involuntary weight loss (cancer cachexia), have identified two main processes to be reciprocally regulated in WAT, namely lipid catabolism and adipocyte size. Thus, while lipid oxidation is increased in cancer cachexia, spontaneous (non-hormone-stimulated) lipolysis and adipocyte size are decreased (14,15). In contrast, WAT inflammation and adipocyte insulin sensitivity seem to be specifically regulated, since both pathways are altered in obesity, but not in cachexia (16). While previous findings have shown that the expression of selected genes in WAT is reciprocally regulated comparing the two conditions (14,15), no systematic assessment had been conducted at the initiation of Study I. In fact, the effects of cancer cachexia on

WAT global gene expression had not been studied previously and this was therefore assessed in Study I.

1.4.2 Gene expression alterations

The transcription of a gene to a functional product, i.e. protein- or non-coding RNA, is controlled at several different levels. In brief, the regulatory cascade is initiated by a signal, e.g. ligand-receptor interaction, that directly or indirectly (through several steps) modulates the activity of a group of proteins named transcription factors (TFs), which bind to DNA in a sequence-specific manner and either promote or block transcription of DNA to RNA (17). It is important to stress that several other processes, e.g. histone modifications and DNA methylation, are essential in order to enable the transcriptional control of TFs. Moreover, post-transcriptional modifications affect RNA abundance and during the last decades a group small of non-coding RNAs, termed microRNAs (miRNAs), have been shown to be important in this process. A miRNA is a low-molecular weight RNA (~22 nucleotides) that bind to complementary sequences in the 3' untranslated region (UTR) of a transcripts leading to gene silencing by translational inhibition or target degradation. The interaction between target mRNAs and miRNAs are mediated by the multimeric RNA-induced silencing complex, which is composed of several different proteins (18).

Genome-wide transcriptional profiling (a method described in 3.11 Transcriptional profiling) has identified both mRNAs and miRNAs to be affected by weight alterations in human WAT (19–22). Although the relative importance of each regulated transcript and the causal relationship between its expression and phenotype is often difficult to define, it is clear that genes important for ECM composition, immune response and adipocyte metabolism are altered in obesity (19). Compared to protein-coding mRNAs, miRNAs have been considerably less studied in the context of WAT function and obesity. This is probably due to the fact that this research area is quite young (miRNAs were discovered in the 90's). However, a couple of recent studies have identified that several miRNAs, of which some are regulated in obesity, control adipogenesis (23).

1.4.3 Inflammation

Inflammation is a closely regulated protective response classically characterized by pain, heat, redness and swelling, that aims to remove harmful stimuli such as pathogens, irritants and/or damaged cells. The inflammatory response is usually triggered by immune cells, e.g. granulocytes, lymphocytes, macrophages and dendritic cells, stationed within the tissue and depending on onset, duration and outcome, inflammation can be classified as acute or chronic. Obesity is associated with a chronic low-grade inflammation that affects several aspects of WAT function. Increased levels of pro-inflammatory cytokines, such as TNFα and IL6, within WAT have been shown to augment adipocyte lipolysis as well as decrease insulin sensitivity and thereby possibly link obesity with its associated comorbidities (24). For more information regarding the initiation of inflammation see section 1.5.2 Chemokine (C-C motif) ligand 2.

1.4.4 Extracellular matrix composition

The extracellular matrix (ECM) is the non-cellular fraction of an organ that not only provides a structural network important for tissue integrity, but also modulates cell function. It is composed of glycosaminoglycans (carbohydrate polymers) and fibrous proteins, which vary qualitatively and quantitatively between tissues. The pericellular matrix, i.e. the subcompartment of the ECM that is closest to the cells, contains bioactive molecules, collectively termed matricellular factors, which do not contribute directly to the organization of the tissue, but affect several properties, e.g. proliferation, differentiation and motility, of the residing cells (25). Similarly to adipocytes, the ECM is constantly remodeled and the total turnover is regulated by the balance between production and degradation.

Adipose tissue is histologically considered to be a loose connective tissue rich in collagens and fibronectin. Within WAT, the ECM provides structural support for adipocytes and it has also been shown to be essential for adipogenesis (described in detail in (26)). However, in obesity, ECM over production results in interstitial fibrosis, which is believed to reduce oxygenation (also termed hypoxia), vascularization, adipogenesis and insulin sensitivity (26,27). Hypoxia has, in turn, been proposed to increase the expression of several ECM genes, thereby facilitating the development of a vicious cycle (28). It is not known which cells within WAT that contributes the most to ECM production, but it has recently been shown that adipocyte precursor cells (preadipocytes) secrete high levels of collagens and fibronectin (29).

1.5 ADIPOKINES

In order to allow a healthy expansion/loss of WAT mass, the cells present within the tissue need to communicate closely with each other (3). Results in recent years have demonstrated that WAT secretes polypeptides, termed adipokines, which mainly act within the tissue through auto- and/or paracrine mechanisms, and thereby allow the cells to cross-talk. There are distinct classes of adipokines, which control different processes such as cell proliferation, adipogenesis, vascularization, inflammatory response and WAT structural composition (24). Since several hundred (>600) of adipokines have been described to date, only the ones central for this thesis will be presented below.

1.5.1 Fibroblast growth factors

Fibroblast growth factors (FGFs) belong to a family of structurally related proteins that, in mammals, comprises 22 members (FGF1-23; where FGF15 is the murine orthogue of human FGF19), which are important for many developmental processes, including cell proliferation and differentiation. Most FGFs are secreted proteins that signal through interactions with cell surface tyrosine kinase receptors named fibroblast growth receptor 1-4 (FGFR1-4) and the co-factors/-receptors heparin (FGF1-10) or klotho (FGF19/21/23). Ligand-receptor interactions lead to dimerization, phosphorylation and activation of downstream signaling pathways including Akt, p38, and p44/42. However, FGF11-14, also known as FGF homologous factors or intracellular FGFs, form a distinct subfamily which is not secreted and therefore mediate their effects in an FGFR-independent manner (30).

The expression of FGFs/FGFRs is tightly regulated, resulting in a unique ligand/receptor signature in different tissues. In WAT research, interest for the FGF-system grew when it was shown that injections of Matrigel (a gelatinous protein mixture resembling the extracellular environment), supplemented with FGF2, induced fat pad formation in mice (31). This was followed by a study performed in humans, where microarrays were used to map the presence of several FGFs and FGFRs in different fractions and depots of WAT (32). In total, five FGFs (FGF1/2/7/9/18) and two receptors (FGFR1/2) were detected, although their respective expression profile varied between depots and tissue fractions. It should be noted that not all family members were represented on the microarray platform and that further studies were needed in order to complete the mapping. Interestingly, while recent publications have highlighted the importance of FGF1 for adipose tissue function (33–35), conflicting data regarding the role of FGF2 have emerged (36). FGF1 knockout mice fed a high fat diet develop an aggressive diabetic phenotype with WAT inflammation and abnormal adipocyte size distribution, a phenotype which is not reversed by weight loss (33). Other FGFs have also spurred significant interest in the metabolic field in recent years. In particular, FGF21 was shown to ameliorate hyperglycemia in primate models of diabetes, possibly via effects on peripheral insulin sensitivity (37). This and subsequent work from other renowned groups suggested that FGF21 could even constitute anti-diabetic agent in a clinical setting. However, recent studies have shown that obese and diabetic subjects have considerably higher circulating FGF21 levels than normal weight or non-diabetic individuals, suggesting that the relationship between FGF21 and insulin sensitivity may be more complicated than previously thought (discussed in detail in (38)).

In Study II, we aimed at systematically characterizing the presence of FGFs both at the mRNA and protein level in human WAT and how their expression was affected in obesity. However, since our study did not functionally evaluate the role of the identified FGFs in WAT, this remains to be studied.

1.5.2 Chemokine (C-C motif) ligand 2

Macrophages are versatile cells that are stationed in several tissues where they, in addition to respond to pathogens, remove apoptotic/necrotic cellular debris and thereby contribute to maintaining tissue function. In obesity, it has been shown that monocyte/macrophage infiltration into WAT is augmented and this is believed to be an important step in the development of obesity-associated inflammation. The exact mechanisms triggering this event are not known, but it is well established that monocytes are guided by chemokines in a gradient-dependent manner and that the production of several of these factors are increased in obese WAT (39,40).

Chemokines are structurally related proteins that can be subdivided into two classes, homeostatic and inflammatory, depending on their main function (for more information see (41)). Chemokine (C-C motif) ligand 2 (CCL2), also termed monocyte chemoattractant protein 1, is a member of the latter subgroup and has been intensively studied in the context of obesity and diabetes (39,42). Both adipocytes and stroma-vascular cells produce CCL2 and it is actively secreted, i.e. in a time-dependent

manner, from both WAT and isolated fat cells (40,43). Circulating levels of CCL2 are increased in both obese and type 2 diabetic subjects and its expression in WAT is closely correlated with the number of macrophages residing within the tissue (44). Furthermore, in murine *in vivo* studies it has been shown that CCL2 knockout mice fed a high-fat diet have reduced macrophage accumulation and improved insulin sensitivity compared to wild type littermates (45). Taken together, this suggests that CCL2 may be an important link between obesity, inflammation and insulin resistance. However, while the function and secretory pattern of CCL2 has been extensively characterized, fewer studies have focused on its expressional regulation. The aim of Study III was therefore to identify novel regulators of CCL2 in adipose tissue with particular focus on miRNAs.

1.5.3 Class 3 semaphorins

In vertebrates, semaphorins comprise five classes (3-7) out of which two (2 and 3) encode secreted molecules. While semaphorins were originally proposed to control axonal guidance in the nervous systems, they have also been shown to be important for non-neuronal tissues where they regulate diverse biological functions including bone homeostasis and cancer progression (46). There are seven class 3 semaphorins (SEMA3A-G), which signal through binding to multimeric receptor complexes consisting of neuropilins, plexins and cell adhesion molecules. To date, the receptor composition for each ligand has only been partially characterized, which is probably due to the fact that not all subunits have been identified yet and that several receptor components are shared between ligands. In general, it is believed that all SEMA3s, except SEMA3E, require neuropilins in order to bind to the receptor complex and that the plexins and cell adhesion molecules relay the intracellular signaling mechanics (47). Currently, only a few publications investigating the function of SEMA3C are available. Addition of recombinant SEMA3C protein has been shown to promote proliferation of murine glomerular endothelial cells (48) and $Sema3c^{-/-}$ mice die perinatally due to interruption of the aortic arch and improper septation of the cardiac outflow tract (49).

Not much is known regarding the potential functions of SEMA3s in human WAT. In fact, out of the seven class 3 members, only SEMA3A, detected in rat adipocytes (50), and SEMA3G, identified in a secretome study performed on conditioned media derived from primary human adipocytes (51), have been shown to be produced by WAT, although their role within the tissue have not yet been characterized. We were therefore eager to dissect if SEMA3C had any effects on WAT biology when we identified this class 3 member to be secreted by adipocytes (see Study IV).

2 AIMS

2.1 GENERAL AIM

In general, the present thesis aimed to systematically characterize the effects of weight alterations on WAT gene expression with particular focus on miRNAs and adipokines. Both conditions of weight gain (obesity) and voluntary (bariatric surgery or energy restriction) as well as involuntary (cancer cachexia) weight loss were used as a starting point and candidate factors identified to be regulated by at least one of these conditions were then selected for detailed studies.

2.2 STUDY-SPECIFIC AIMS

I. To compare WAT global transcriptional profiles between weight-stable and cachectic cancer subjects in order to identify pathways and genes altered by involuntary weight loss.

II. To map the expression, cellular source and regulation of FGFs in human WAT.

III. To identify adipose miRNAs regulated by obesity and determine how they control human WAT inflammation.

IV. To identify novel adipokines regulated by weight alterations and evaluate their possible role in human WAT function.

3 METHODOLOGICAL CONSIDERATIONS

The clinical cohorts, chemicals and techniques used in the present thesis are described in detail in each study. Although all the employed methods are well established, some of them are crucial for the understanding of the following sections and therefore briefly discussed below. The overall approach that has been applied to several of the studies included herein aimed to generate hypotheses using transcriptomic/proteomic platforms, validate the findings as well as map expression/secretion in WAT and functionally evaluate the effect of selected candidates in cell culture systems *in vitro*.

3.1 HYPOTHESIS GENERATION USING OMICS PLATFORMS

In the scientific world, the use of the suffixes "ome"/"omics" has increased dramatically (even more rapidly than the prevalence of obesity) during the last years. Today, several hundreds of "ome" words exist and they are generally used to highlight the totality of a specific type of data collected, e.g. the transcriptome is the set of all RNA molecules that exist in a given system and secretome all the secreted peptides/proteins.

3.1.1 Transcriptional profiling

A transcriptome is the entire repertoire of transcripts, including mRNA, miRNAs and other non-coding RNAs, present at a given time point. In contrast to the genome, it is highly variable and depends on cell type and environmental signals. There are several methods to assess genome-wide transcript abundance including DNA microarrays and sequencing based techniques. Herein, we have used the former platform in all the included studies since it is robust, relatively cheap and easily analyzed (streamlined handling and mature analysis strategies). In brief, microarrays consist of probes, which are complementary in sequence to the transcripts that are to be measured, immobilized on a solid surface. There are usually several probes for each gene and they are designed based on genome sequence. From each sample, total RNA is isolated, reverse transcribed, labeled and hybridized onto the microarrays, which are subsequently washed and scanned. Signal intensities obtained from the scanning are used as measures of expression for each transcript (52). Since microarrays are probe-based, a clear disadvantage with using this platform is that only known sequences can be detected. In addition, while sequence-based techniques are able to detect RNA editing events and allele-specific expression, this cannot be determined by microarrays.

3.1.2 Secretomics

Since alterations in mRNA expression do not necessarily affect corresponding protein levels, large-scale studies at the protein level, i.e. proteomics, are important. However, even though several new strategies have emerged (reviewed in (53)), current proteomic platforms lack somewhat in throughput, sensitivity and reproducibility. This is not only due to technical limitations, but also related to the extreme complexity of the proteome, which has been estimated to comprise close to one million protein products (if splicing variants and essential posttranslational modifications are included), which should be compared with the estimated 25 000 human genes (54). In Study IV, we set out to identify novel adipokines using mass spectrometry. Since the amino acid sequence

alone is not enough to predict if a protein is secreted, we collected and analyzed conditioned media from our samples. This approach, termed secretomics, enables the detection of hundreds of proteins in each experiment and has been widely applied in the quest for biomarkers. However, there are several caveats using this technique, including contamination of intracellular proteins that are derived from lysed cells, and careful validation experiments must therefore be performed.

3.2 VALIDATION EXPERIMENTS

Although both transcriptomics and secretomics are excellent tools for target discovery, variations between platforms, laboratories and users exist. In addition, increased throughput usually leads to decreased specificity and sensitivity. Therefore, it is important to validate omics-based findings with other techniques. In Study I-IV, we have confirmed and to some extent extended our results using real-time polymerase chain reaction (RT-PCR) and/or enzyme-linked immunosorbent assay (ELISA) and both methods are described (including relevant references) in each study.

3.3 FUNCTIONAL EVALUATION

In order to challenge the hypotheses generated by the omics data and validation experiments, functional evaluations need to be performed. There are several strategies enabling this step and most of them aim to perturb the expression/function of the candidate factor(s) *in vivo* or *in vitro* followed by phenotypical assessments. Since the omics data generated in Study I-IV are based on human samples and important species differences in terms of WAT biology exist, including variations in adipokine production (55) and lipolytic regulation (56), we have conducted all, except one (discussed below), of our functional evaluations in human cell systems.

3.3.1 Culture models

In comparison to *in vivo* models, *in vitro* experiments are less complex and allow functional mapping of specific candidates in well-characterized culture systems. Needless to say, due to the simplicity of these models, results obtained from *in vitro* studies must be interpreted with caution and cannot automatically be translated into a clinical situation.

Several human adipocyte cell culture models exist, e.g. isolated mature fat cells cultured *ex vivo* and precursor cells (mesenchymal stem cells or preadipocytes) differentiated into adipocytes *in vitro*, and there are different advantages/disadvantages with all systems (see Table I). Herein, we have performed our mechanistic studies in *in vitro* differentiated adipocytes, since this system is well-established in our laboratory and matched our requirements. Unfortunately, we are currently not able to transfect plasmids into this model system and 3T3-L1 cells, an immortalized murine cell line which can differentiate into adipocyte-like cells, were therefore used for these experiments. As described earlier, WAT contains several different cell types. Thus, we have not only performed our functional evaluations in adipocytes, but also in the monocyte/macrophage cell line THP1 (Study III) and WAT-derived endothelial cells (Study II and IV).

3.3.2 Perturbation experiments

For intracellular candidates, i.e. miRNAs (Study III), we have overexpressed/silenced their expression using mimic/inhibitor reagents and for secreted factors, i.e. FGFs (Study II) and SEMA3C (Study IV), recombinant proteins have been added to the cells. Endpoint measurements, e.g. assessments of effects on mRNA/protein levels, luciferase activity and cell function, have been performed at suitable time-points, as determined by careful optimizations, after the perturbations have been performed.

Table I

Advantages and disadvantages with different human cell culture systems

Cell culture model	Advantages	Disadvantages
Mature fat cells	*In vivo*-like Large amounts are easily obtained Pure population of cells	Fragile Difficult to handle and treat Large phenotypic variation between subjects
Mesenchymal stem cells	Expandable Pure population of cells	Difficult to isolate, culture and transfect
Preadipocytes	Well-established Fully characterized Easy to handle	Limited numbers Variation between subjects

4 RESULTS AND DISCUSSION

All results included in this thesis are presented and discussed in detail in each study. A short introduction to each project and a summary of the most important findings are presented and discussed below.

4.1 STUDY I

Our laboratory has previously identified two genes (hormone-sensitive lipase and cell death-inducing DFFA-like effector a) with increased mRNA levels in cachectic versus weight-stable cancer patients (14,15). Apart from evaluating their possible role in this condition, we also noticed that the expression of both factors was reciprocally regulated in cachexia compared to obesity. This encouraged us to assess global expression levels in weight-stable and cachectic cancer subjects and we hypothesized that the alterations in adipocyte metabolism that we observed in previous studies, i.e. increased non-hormone stimulated lipolysis and lipid oxidation, would be mirrored at the transcriptional level. We also assumed that the expression of selected genes and gene families would correlate with fat mass and be reciprocally regulated comparing obesity with cancer cachexia.

In total, 364 transcripts were decreased and 61 increased comparing cancer cachexia with cancer weight-stable subjects. In order to identify if the altered genes were enriched in specific cellular processes, pathway analyses were performed. Ontologies such as extracellular matrix, actin cytoskeleton and focal adhesion were significantly downregulated, whereas fatty acid metabolism, including electron transport, oxidative phosphorylation and mitochondrial genes, upregulated in cachexia. Furthermore, to assess if any TFs could possibly control the expression of genes within these pathways, promoter regions were scanned for over-represented motifs. This identified hepatic nuclear factor 4 (HNF4) as a potential regulator of ECM and cell adhesion genes. Interestingly, the mRNA expression of HNF4 was decreased in cancer cachexia versus weight-stable. Since electron transport and mitochondrial genes were upregulated according to the microarray analysis and we have previously reported that lipid oxidation is increased in cancer cachexia (15), we measured mitochondrial mass. Although one of the two proteins involved in oxidative phosphorylation was increased in cachexia, mitochondrial copy number was not. Altogether, these results indicate that the functional consequences of cachexia on adipocyte lipid catabolism are mirrored at the transcriptional level and that decreased fat mass leads to remodeling of the ECM compartment and cell adhesion as well as cytoskeletal genes. Regarding the relationship between mitochondrial mass and cachexia, the results were conflicting and no strong conclusion could be drawn.

In order to compare cachexia with obesity at the transcriptional level, we overlapped our microarray results with data from another study where global gene expression profiling had been performed on WAT from subjects with a wide range in BMI (40). Out of the 425 genes identified to be regulated by cachexia, 262 were present in the obesity study and 83 altered comparing obese with non-obese. Interestingly, all except one of the transcripts were regulated in a reciprocal fashion comparing the two conditions. This suggests that although a large proportion of the genes identified to be

dysregulated in obesity and cachexia are primarily related to changes in fat mass *per se*, condition-specific alterations do exist, a notion which is corroborated by the fact that some, but not all, aspects of adipocyte function are reciprocally affected comparing the two conditions.

4.2 STUDY II

At the initiation of study II, many laboratories had already highlighted the importance of the FGF-FGFR system for WAT biology. However, no systematic investigation focusing on the release of FGFs from human WAT had been performed. Therefore, we set out to map the secretion and cellular source of individual family members in WAT and if they were affected by body weight status. Since both FGF1 and FGF2 had previously been shown to exert functional effects on adipocytes, we hypothesized that at least one of these factors should be present in human WAT.

Retrospective analysis of published global expression data comparing obese and non-obese subjects (40) showed that out of the 15 FGFs that were present on the microarrays, six members (FGF1/2/7/9/13/18) were expressed in WAT. While FGF1 and FGF2 mRNA levels were regulated in obesity, the other ligands were virtually unaffected by alterations in fat mass. In order to map the presence, source and potential impact of obesity on the selected FGFs at the protein level, ELISAs were performed on conditioned media from intact and fractioned WAT. Since FGF13 is an intracellular protein and no ELISA kit was available for FGF18, these two ligands were not studied further. Out of the four remaining family members (FGF1/2/7/9), only FGF1 was secreted time-dependently from intact WAT and the major source of its secretion was from the fat cell fraction. In addition, FGF1 release was increased in obesity, but not reversed by weight-reduction induced by caloric restriction. Taken together these data indicate that out of the investigated FGFs, FGF1 may be of particular importance for WAT function. This conclusion is corroborated by a recent study were FGF1 knockout mice fed a high fat diet develop an aggressive diabetic phenotype with WAT inflammation and abnormal adipocyte size distribution, a phenotype which is not reversed by weight loss (33). However, the clinical relevance of this *in* vivo model is not clear, since our study show that FGF1 production in WAT is significantly increased among obese subjects. Instead, based on the finding that FGF1 promotes adipogenesis and that the secretion of this adipokine is not reversed by weight loss, it is more likely that FGF1 is important for the determination of fat cell number, a process which is accelerated in obese subjects and not normalized by weight reduction. However, this needs to be confirmed in future studies. Finally, it should be stressed that FGF members may affect WAT function not only via direct effects, but also indirectly by altering the expression of other FGFs. For instance, recent data suggest that FGF1 mediates its effects partly by downregulating the intracellular levels of FGF2, which in turn leads to reduced preadipocyte proliferation and increased expression of adipogenic genes (36).

4.3 STUDY III

The role of several miRNAs in inflammatory diseases has been established in recent years (57). However, in the field of adipose tissue and obesity, the primary focus has been to link individual miRNAs to adipogenesis. The aim of this study was therefore to identify miRNAs regulated by obesity and to assess if they control WAT inflammation.

Since CCL2 has been proposed to initiate inflammation by inducing leukocyte extravasation, we selected this chemokine as our endpoint.

A combination of global miRNA measurements and validations with RT-PCR identified eleven adipose miRNAs to be dysregulated in obesity. Out of these, ten were shown to regulate CCL2 secretion when over-expressed in *in vitro* differentiated adipocytes. However, only one miRNA (miR-126) was predicted to directly target the 3' UTR of CCL2, which was also confirmed by reporter assays. This suggested that the remaining miRNAs affected CCL2 secretion indirectly. Since TFs are well-established regulators of gene expression, we constructed a TF-centric network through which the miRNAs could exert their effects. The proposed framework allowed the identification of a novel signaling circuit in adipocytes where miR-193b controlled CCL2 secretion indirectly through effects on several TFs. Moreover, CCL2 is not only released by adipocytes, but also from other cell types present within WAT among which macrophages are particularly important. Therefore, in order to assess if miR-126 and -193b also affected CCL2 production in macrophages, we overexpressed these miRNAs individually in THP1 cells, a monocyte/macrophage cell line. Similarly to the results in adipocytes, both miRNAs clearly affected mRNA and secreted levels of CCL2. These results indicate that several miRNAs may be important regulators of WAT inflammation through their effects on CCL2 production. However, it is important to stress that only two circuits from miRNA to CCL2 were identified in this study and that future efforts are needed to clarify how the other eight miRNAs affect the production of this chemokine. It is likely that the framework constructed in this study was too simplistic and that other regulatory elements, such as DNA methylation and transcriptional co-factors, need to be included in order to complete the characterization.

4.4 STUDY IV

Although many laboratories have characterized the human adipokinome, the overlap between studies is rather poor and previously unknown secreted factors are discovered on a regular basis. Thus, the aim of study IV was to identify novel adipokines regulated by weight alterations and evaluate their possible functional role in WAT.

By jointly analyzing transcriptomics data from three recent studies, focusing on obesity and voluntary/involuntary weight loss, together with results from a secretomics screen based on conditioned media from different fraction of WAT, we identified six proteins to be secreted by adipocytes and regulated by alterations in fat mass. Out of these, only one factor, SEMA3C, had not previously been associated with WAT biology and was therefore further studied. The microarray results, showing that SEMA3C was increased in obesity and decreased by voluntary/involuntary weight loss, were confirmed and extended by RT-PCR and ELISA where SEMA3C mRNA and/or protein levels were also found to be upregulated during adipogenesis and positively correlated to WAT morphology as well as whole-body insulin resistance. While these data confirmed that SEMA3C is a true adipokine predominantly produced by adipocytes, it was still unclear what effects, if any, this factor had on WAT function. Since the vast majority of the hitherto discovered adipokines exert their effects within WAT and weight-alterations modify adipocyte metabolism, we hypothesized that SEMA3C might be involved in the regulation of these processes. However, addition of recombinant SEMA3C protein to *in*

vitro differentiated adipocytes had no effects on lipid catabolism or insulin sensitivity. Although the exact composition of the SEMA3C holoreceptor is not known, it is well-established that SEMA3C mediates its effects through a specific set of cell-surface receptors termed neuropilins and plexins. We therefore characterized the expression of these genes during adipogenesis and in different fractions of WAT. While the receptors had varying expression patterns comparing cell types present in WAT, all receptors, except one, were markedly decreased during adipocyte differentiation. This turned our focus to the preadipocytes, which in addition to differentiate into fat cells, recently have been shown to be an important source for the production of ECM components, a process which is strongly affected by weight-alterations and linked to insulin resistance. Recombinant SEMA3C protein affected both mRNA and protein levels of structural and matricellular ECM genes. Altogether, these results demonstrate that SEMA3C is a novel adipokine regulated by alterations in fat mass. In WAT, adipocyte-derived SEMA3C acts in a paracrine fashion to affect expression of genes important for ECM composition in preadipocytes. In order to determine the clinical relevance of these findings, SEMA3C mRNA levels were correlated with WAT fibrosis in a cohort comprising both obese and non-obese subjects. A significant positive association was observed further implicating SEMA3C in the modulation of ECM composition in WAT, which in turn may affect insulin sensitivity. However, it is important to stress that the link between these three elements, i.e. SEMA3C, WAT fibrosis and insulin resistance, needs to be tested further, preferably in murine *in vivo* models.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The studies included in this thesis, have mapped and compared global expression patterns in obesity and cancer cachexia, two clinically relevant conditions characterized by alterations in fat mass. Using this approach, numerous transcripts, including adipokines (FGF1 and SEMA3C) and miRNAs (miR-126 and -193b), have been identified and associated with pathophysiological alterations in WAT function, including interstitial fibrosis, inflammation and adipogenesis (Figure 2). Since the data presented herein are based on subcutaneous adipose tissue, results cannot be extrapolated to other depots. This is due to the fact that differences comparing WAT compartments in terms of structure and metabolic properties exist (58).

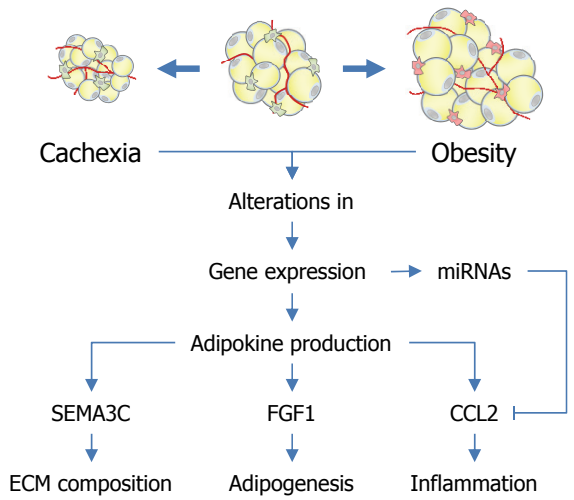

Figure 2. Summary depicting the main findings presented in the thesis.

Although having effects on different processes, both FGF1 and SEMA3C were predominantly secreted by adipocytes and had marked effects on preadipocyte function. Based on these findings, it is tempting to speculate that a distinct group of adipokines may constitute an important paracrine axis, which allows crosstalk between adipocytes and their precursor cells, and that disease-related modulations of this feedback loop could have detrimental effects on WAT function. In addition, compared to CCL2, the regulatory elements controlling SEMA3C and FGF1 production in adipocytes are poorly defined, but miRNAs could be involved. A quick search in publically available miRNA databases, such as miRWalk (59), show that out of the eleven adipose miRNAs that were identified in Study III to be downregulated in obesity, one was predicted to target SEMA3C and seven FGF1. Although these lists of potential target genes include many false positives and need to be confirmed with additional experiments, this suggests that several miRNAs could control FGF1 and SEMA3C production in obesity.

Altogether, the results presented in this thesis clearly show that there is a tight link between disease state, WAT function and gene expression. However, the causal

relationship between the three is not yet fully elucidated. Herein, we have partly addressed this by performing extensive studies on individual factors and thereby been able to map how they affect WAT function and possibly obesity and/or cancer cachexia. However, additional hundreds of candidates exist and a more systematic approach needs to be applied in order to fully understand how each altered transcript affects WAT biology. A very interesting possibility would be to combine transcriptomic-based results with functional RNAi screens where the function of thousands of genes can be assessed in parallel. While microarray data are produced easily nowadays, RNAi screens are still technically challenging to perform. For instance, the increase in throughput requires assays that are extremely accurate with low inter-individual variations within and between experiments. To date, there is only one publication that has systematically knocked down thousands of transcripts in human adipocytes (60). In that study, 7784 druggable genes were silenced and effects on adipogenesis and lipid accumulation were evaluated. Several factors, not previously associated with adipocyte biology, were identified indicating that this approach can be useful, especially when combined with clinically relevant data. I find this translational approach very promising and hope to pursue this line of research in the near future.

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