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THE ROLE OF THYROID STIMULATING HORMONE RECEPTOR AND NOVEL CANDIDATE GENES *FAM13A* AND *POM121C* IN ADIPOCYTE DYSFUNCTION

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The role of Thyroid Stimulating Hormone Receptor and Novel Candidate Genes *FAM13A* and *POM121C* in Adipocyte Dysfunction

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To my daughters, Freja, Nora and Idun

”*Sometimes me think, what is friend? And then me say, friend is someone to share the last cookie with”*

-Cookie Monster-

ABSTRACT

This thesis aimed to investigate the role of thyroid stimulating hormone receptors (TSHRs) in adipose tissue (AT) and their role in AT dysfunction and obesity-related metabolic complications. Furthermore, we aimed to functionally evaluate novel candidate genes associated with insulin resistance, a marker of dysfunctional adipose tissue.

Study I report that in a cohort of Swedish children with obesity, thyroid stimulating hormone was associated with the degree of obesity and metabolic risk markers such as fasting serum insulin levels and blood lipids.

In Study II, the TSHR in AT was functionally evaluated by investigating the effect of partially removing TSHRs from the adipocytes on body weight and body temperature, in animals on both normal and high-fat diets. Mice with reduced *TSHR*-expression gained weight at a faster rate than corresponding wild-type mice. Several genes central to adipogenesis and adipocyte function were down-regulated, in both white AT (WAT) and brown AT (BAT) in *TSHR* knockout mice.

Study III. From a genome-wide association study (GWAS) meta-analysis which identified SNPs for fasting insulin, we identified *FAM13A* and *POM121C* as novel candidate genes for obesity-related insulin resistance. Using expression quantitative trait (eQTL) analysis of SNPs associated with fasting insulin, we identified candidate genes for disease. Functional analysis of the candidate genes using siRNA knockdown in human mesenchymal stem cells revealed them to be important for lipolysis and adipogenesis and they might therefore be involved in the genetic control of insulin sensitivity.

Study IV, report that expression of the *TSHR* in human WAT is affected by weight change. We also report that *TSHR*-expression in WAT is associated with the expression of genes central to adipocyte functions such as lipolysis and insulin sensitivity. The results revealed that, independent of BMI, individuals with higher *TSHR* expression had a lower basal lipolysis rate and higher hormone stimulated lipolysis, suggesting that TSHRs in human WAT are involved in the regulation of adipocyte metabolism.

In summary, a reduction of *TSHR* led to dysfunctional AT regarding the regulation of adipocyte metabolism and adipogenesis. Our findings implicate that TSHRs have a regulatory role in both WAT and BAT, and thus having a role in the regulation of whole-body energy homeostasis. Furthermore, we identified two novel genes with potential regulatory roles in adipocyte lipolysis and adipogenesis, and might thereby be involved in the genetic control of systemic insulin sensitivity.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Den höga förekomsten av fetma har blivit ett hot mot folkhälsan. Hos vuxna med fetma ökar risken för en rad följdsjukdomar, bland annat diabetes typ 2, hjärt- och kärlsjukdomar och cancer. Fetma är en sjukdom som definieras av överflödig tillväxt av fettväven. Fettväven är inte bara en vävnad som lagrar energi, den har också viktiga endokrina funktioner eftersom fett utsöndrar hormoner och andra signaler, till både hjärnan och perifera vävnader, som i sin tur reglerar kroppens energijämvikt.

Fettväven delas in i vitt och brunt fett vilka har motsatta funktioner. Det vita fettet lagrar energi och vid ökat energibehov kan fettmolekyler brytas ner genom lipolys, vilket ger fria fettsyror som används som energisubstrat till andra vävnader. Vitt fett finns fördelat under huden, så kallat subkutant fett, och runt organen, invärtes fett. En ökning av mängden invärtes fett och subkutant bukfett, är associerat med fetmarelaterade följdsjukdomar. Brunt fett är en energiförbrukande vävnad som gör av med lagrad energi genom att producera värme och är därför ett intressant mål för fetmabehandling.

Det övergripande syftet med avhandlingen är att få bättre förståelse för de biologiska mekanismer som orsakar de metabola komplikationer som fetma medför. Specifikt har den tyroidea stimulerande hormonreceptorn (TSHR) studerats för att utröna vilken roll den utgör i fettväven. Nya underliggande gener till insulinresistens har även undersökts med syftet att identifiera gener som på verkar fettcellens funktion och där igenom systemisk insulinkänslighet.

Genom att identifiera underliggande mekanismer till fetmarelaterade metabola komplikationer kan nya behandlingssätt tas fram för fetma-relaterade sjukdomar.

I Studie I undersöktes associationer mellan TSH och metabola riskfaktorer hos svenska barn med fetma. Vi såg ett samband mellan TSH-nivåer och metabola riskfaktorer så som fasteinsulin och lipidnivåer i blodet.

I Studie II undersöktes funktionen av TSHR i en genetiskt modifierad musmodell. Vi såg att avsaknad av denna receptor påverkar fettvävens utveckling och funktioner som insulinkänslighet i vitt fett, och värmeproduktion i brunt fett.

Studie III identifierades två nya kandidatgener som verkar påverka fettcellens utveckling och funktion, och därmed systemisk insulinkänslighet. *FAM13A* och *POM121C* visade sig i studien ha en skyddande effekt mot metabol sjukdom och kan därmed vara intressanta mål för behandling.

Studie IV visade att genuttrycket av TSHR i fett hos människa, är associerat med BMI och även lipolys. Detta innebär att det vi såg i musmodellen kan även vara relevant i människa.

LIST OF SCIENTIFIC PAPERS

I. **Veroniqa Lundbäck**, Kerstin Ekbom, Emilia Hagman, Ingrid Dahlman and Claude Marcus
Thyroid-Stimulating Hormone, Degree of Obesity, and Metabolic Risk Markers in a Cohort of Swedish Children with Obesity. Hormone Research in Paediatrics 2017;88(2): 140–146.

II. **Veroniqa Lundbäck**, Agné Kulyté, Ingrid Dahlman and Claude Marcus
Adipose tissue-specific inactivation of thyroid stimulating hormone receptors in mice modifies body weight, body temperature and adipocyte-specific gene expression in brown and white adipocytes.
Manuscript

III. **Veroniqa Lundbäck,** Agné Kulyté, Rona J Strawbridge, Mikael Rydén, Peter Arner, Claude Marcus and Ingrid Dahlman
FAM13A and POM121C are candidate genes for fasting insulin: functional follow-up analysis of a genome-wide association study. Diabetologia 2018 May;61(5): 1112–1123.

IV. **Veroniqa Lundbäck**, Claude Marcus and Ingrid Dahlman
Thyroid stimulating hormone receptors in human white adipose tissue: association with BMI, adipose tissue phenotypes and gene expression.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADRB	Beta adrenergic receptor
AMPK	Adenosine monophosphate-activated protein kinase
AT	Adipose tissue
ATP	Adenosine triphosphate
ADIPOQ	Adiponectin
BAT	Brown adipose tissue
BMI	Body mass index
BMI SDS	Body mass index standard deviation score
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
eQTL	Expression quantitative trait locus
FA	Fatty acids
FABP4	Fatty acid binding protein 4
GLUT4	Glucose transporter 4
GWAS	Genome-wide association study
HSL	Hormone sensitive lipase
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
PPARγ	Peroxisome proliferator-activated receptor-gamma
qRT-PCR	Quantitative real-time polymerase chain reaction
siRNA	Small interfering ribonucleic acid
T2D	Type 2 diabetes
TG	Triglyceride
TNFα	Tumour necrosis factor alpha
TSH	Thyroid stimulating hormone

TSHR	Thyroid stimulating hormone receptor
T3	Triiodothyronine
T4	Thyroxine
UCP1	Uncoupling protein 1
WAT	White adipose tissue

1 INTRODUCTION

In this thesis I address the biological pathways in adipose tissue that are affected in obesity. In obesity, many molecular mechanisms become disturbed and contribute to the metabolic complications that follow.

In order to study obesity-related metabolic complications, and in particular the effects of the thyroid stimulating hormone receptor (TSHR) in obesity, biochemical data from children with obesity was investigated. To functionally examine the role of the TSHR in adipose tissue (AT), a transgenic mouse model was used in which the *TSHR* had been reduced. To further interpret the results observed in the mouse model, gene expression data from adult human AT was investigated with the *TSHR* and its association with the expression of AT-specific genes as the focus.

Furthermore, we investigated genetic determinants of insulin sensitivity. To find new candidate genes for insulin resistance (IR), a major risk marker for type 2 diabetes (T2D), we used results from a genome-wide association study (GWAS) together with human clinical data, adipose phenotypes and AT gene expression. The identified candidate genes for IR were then functionally evaluated in a cell-based model.

1.1 OBESITY

The prevalence of obesity has increased rapidly over the past few decades in both adults and children. It is a well-known fact that the obesity epidemic is now a worldwide health problem (1). Major contributing factors are the exposure to an obesogenic environment facilitating increased energy intake along with physical inactivity or sedentary behaviour.

Obesity is associated with several important health problems and an elevated risk of developing diseases such T2D, fatty liver, cardiovascular disease and cancer (2). Obesity and T2D in particular are closely linked through their association with the development of IR (an impaired cellular response to insulin) (3).

Why is it that our environment today, which provides sufficient food and security, is causing disease? Different theories have been presented in attempts to explain the origin of obesity. From an evolutionary perspective, genes favoring survival are selected for, and passed on in order to improve the survival of a species. According to the debated hypothesis of “thrifty genes” first presented by James V Neel in 1962, addressing the development of diabetes mellitus, our bodies are genetically programmed to store energy in order to cope with periods of limited food supply. Through selective

mechanisms and evolution, people with a beneficial genotype in times of need had a greater chance of survival. Genes favouring fat deposition are disadvantageous in today's society and would thus explain the excessive weight gain that leads to obesity (4, 5).

Other theories exist that contradict the "thrifty gene" hypothesis such as the "drifty genes" hypothesis proposed by the biologist John Speakman, in which genes favoring fat deposition are not selected for but are rather subjects to random drift due to the absence of selection by predators (6).

Although its origin is not fully understood, the development of obesity is primarily the result of an imbalance between energy intake and energy expenditure, and the combination of a high-energy diet and sedentary behaviour appears to be the major cause of obesity in westernized countries. Besides eating habits and food availability, obesity results from a combination of genetic, epigenetic, behavioural and environmental factors. The influence of each trait is highly variable with a relatively small effect size in cause and predisposition to obesity when considered individually. Obesity is therefore considered a complex disease, thus requiring a multidimensional approach to understanding its underlying mechanisms (7).

1.1.1 Body mass index

Obesity is characterised by an excessive adipose tissue (AT) accumulation and is associated with, and usually precedes, metabolic complications. The amount and distribution of AT as it expands are important factors for the complications or co-morbidities that follows obesity. Abdominal obesity is included as a risk factor in the metabolic syndrome and is associated with several obesity-related comorbidities and also considered a marker of dysfunctional AT (8, 9).

Body mass index (BMI) is a tool used to classify an individual's body weight in relation to height. BMI can be considered a proxy for body fatness and is the most widely used measurement to diagnose obesity. BMI is calculated by dividing an individual's weight in kilograms by their squared height in metres (kg/m^2), and is used to define an individual's weight status. BMI is not totally height independent, taller people have a higher BMI. BMI weight categories are listed in Table 1.1.

Table 1.1 Definition of weight status by BMI

Weight status	BMI (kg/m^2)
Underweight	<18.5
Normal weight	18.5–24.9
Overweight	25–29.9
Obese	>30
Morbidly obese	>40

The metabolic risk that follows obesity cannot be evaluated by BMI alone since it does not reveal how the AT is distributed in the body. Furthermore, BMI dose not reveal the phenotype of the fat cells (adipocytes) such as adipose tissue morphology, that is the size and number of fat cells. The metabolic risk in obesity depends on biological mechanisms affected by the excessive accumulation of AT as well as the phenotype of adipocytes.

1.1.2 BMI in children

Children are growing individuals and therefore have a continuously increasing BMI. The use of BMI as a measure of weight status during childhood is therefore complicated. To be able to compare weight status during childhood, BMI can be transformed into a BMI standard deviation score (BMI SDS), also called z-score, using reference populations of healthy children. These transformations are used to define cut-offs for obesity in children, and is a frequently used method in the clinical setting (10).

1.2 ADIPOSE TISSUE

AT is a heterogeneous organ consisting of several different cell types, and the interest in adipocytes has increased along with the obesity epidemic. The adipocyte is the body's largest cell type and makes up over 90% of the AT volume but only 20–40% of its cell numbers (11). Other cell types in the AT are endothelial cells, fibroblasts and immune cells which will not be addressed in this thesis.

The AT is an energy source for other organs and tissues and can be considered a buffer for changes in physiological energy demands. The ability of AT to expand and contract in response to an organism's energy status is a feature assigned to the adipocytes, and is unique compared to other tissues (12).

In healthy normal-weight adults, the adipose organ constitutes about 8–18% of body weight in males and 14–28% of body weight in females. In humans with morbid obesity, the adipose organ can expand up to 4 times and reach 60–70% of the body weight (13).

1.2.1 Adipose tissue in children

In the normal newborn the adipose tissue constitutes 11–16% of the body weight. At that time, the AT mass is mainly subcutaneous with hardly any intra-abdominal AT (14, 15). The formation of AT begins before birth. After birth, the AT mass increase rapidly by lipid accumulation (hypertrophic growth) and by mitotic activity in precursor cells (hyperplastic growth), thus there is an increase in both size and number of adipocytes. In infants, there is a marked increase in AT mass compared to the growth of the other tissues in the body. The AT mass is increasing about six times during the first year of life in comparison to the average increase in body weight being three-fold during the same time-period (14).

After the first year, the increase in AT mass slows down and there is only a slight absolute increase of the AT. After childhood and adolescence, the AT retains the ability to expand during adulthood in response to excessive nutritional intake (16, 17).

The AT is made up of white and brown depots (WAT and BAT). White and brown adipocytes have distinct characteristics and functions (18) as illustrated in Figure 1.1.

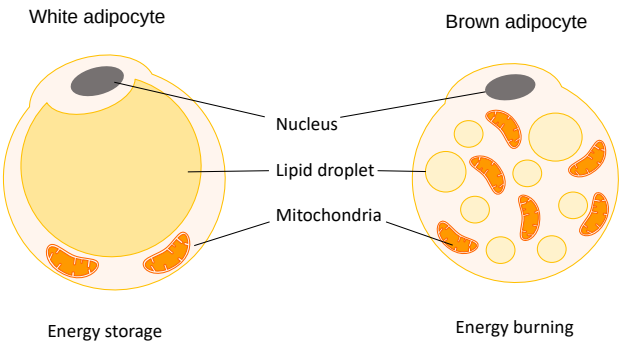


Figure 1.1. *Differences in characteristics and function of white and brown adipocytes.*

1.2.2 White adipose tissue

WAT is the largest fat depot where metabolic energy is stored in the form of triglycerides (TGs). White adipocytes contain a large single lipid droplet allowing fatty acid (FA) storage after nutrient intake. As energy demand rises, TGs within the lipid droplet are hydrolysed by adipose lipases, in sequential steps, forming free fatty acids (FFAs) and glycerol for the use by other tissues as energy substrates, a process known as lipolysis. FFAs are also important for the synthesis of phospholipids and lipoproteins and function as signal molecules.

Human WAT is located within specific depots that can roughly be divided into subcutaneous and visceral AT depots. About 80% of body fat is stored in subcutaneous depots and 20% in intra-abdominal, also known as visceral depots (19).

1.2.3 Brown adipose tissue

BAT is a much smaller part of the adipose organ and the site for non-shivering thermogenesis in mammals. Brown adipocytes are multilocular with many small lipid droplets. They are specialised cells with a high oxidative capacity due to their high content of mitochondria. The uncoupling protein 1 (UCP1) resides in the inner membrane of the mitochondria and is only expressed in brown adipocytes. UCP1, as the name implies, uncouples the respiratory chain from oxidative phosphorylation and enables the brown adipocytes to use stored energy to produce heat (20). The BAT is therefore an energy consuming AT depot. In mice and other small mammals, BAT-activity generates heat to enable the animal to adapt to cold ambient temperatures (20, 21).

1.2.3.1 Brown adipose tissue in children and adults

It has long been known that human infants possess BAT, with about 2-5 % of the body weight of infants being made up of BAT, which corresponds to approximately 150-250 grams (22, 23). However, BAT activity and content were believed to decline quickly and disappear in adulthood (24).

In 2007, it was demonstrated by using 18F-fluorodeoxyglucose combined positron emission tomography and computed tomography (18F PET-CT) that adult humans also possess active BAT (25). The findings were confirmed by several research groups, which also showed BAT activity to be associated with a beneficial metabolic phenotype (26-31).

1.2.3.2 Thermogenesis in brown adipose tissue

Thermogenesis is a key function of BAT. Upon stimulation, the sympathetic nerves innervating BAT release catecholamines acting on ß3-adrenergic receptors and lipolysis is induced leading to the production of FFAs. FAs directly activate UCP1 and thereby non-shivering thermogenesis (20). Thyroid hormones acting on thyroid hormone receptors on the brown adipocyte are involved in mediating the response in BAT to adrenergic stimulation (32) and the control of *UCP1* transcription (33). It has been reported that central infusion of thyroid hormone triiodothyronine (T3) increases the level of UCP1 in the cells. T3-induced hypothalamic AMPK inactivation leads to an

increased sympathetic tone in the nerves innervating BAT. Sympathetic stimulation is the physiological mechanism for BAT recruitment (20, 25). The binding of TSH to TSHRs on brown adipocytes is also involved in the formation of BAT probably by regulating *UCP1* and other factors involved in browning (34).

No mechanism other than UCP1 can mediate non-shivering thermogenesis, giving BAT an essential role in energy expenditure (35). Given its potential to stimulate energy expenditure and being associated with protection against obesity and metabolic comorbidities like T2D, BAT has become a potentially and interesting target in obesity treatment.

1.2.4 Adipogenesis

Adipose tissue expands by enlargement of existing mature adipocytes or the generation of new adipocytes by differentiation of precursor cells into mature adipocytes, a process known as adipogenesis (14, 36). Adipose precursor cells are present in the stromal vascular fraction of the AT (37).

Brown and white adipocytes require key transcription factors to promote differentiation. Peroxisome proliferator-activated receptor-gamma (PPARγ), a member of the PPAR family of transcription factors (TFs), is considered the master regulator of adipogenesis (38). CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors with key regulatory roles in adipogenesis (39).

Adipose tissue growth occurs through the accumulation of lipids and expansion of the adipocyte. During overnutrition and the development of obesity, the AT undergoes dynamic changes. When lipids are stored, pre-existing adipocytes expand and new adipocytes are produced by proliferation and differentiation of preadipocytes into mature adipocytes (36, 40, 41).

1.2.5 Adipose tissue morphology

Adipocyte morphology, that is the relationship between adipocyte size and number of adipocytes, is an important factor affecting obesity-related metabolic risk. The adipose tissue expands by increasing the volume and number of adipocytes causing distinct morphologies termed hyperplasia (many small adipocytes) or hypertrophy (few large adipocytes), (Figure 1.1). Adipose tissue morphology is independent of total body fat (42).

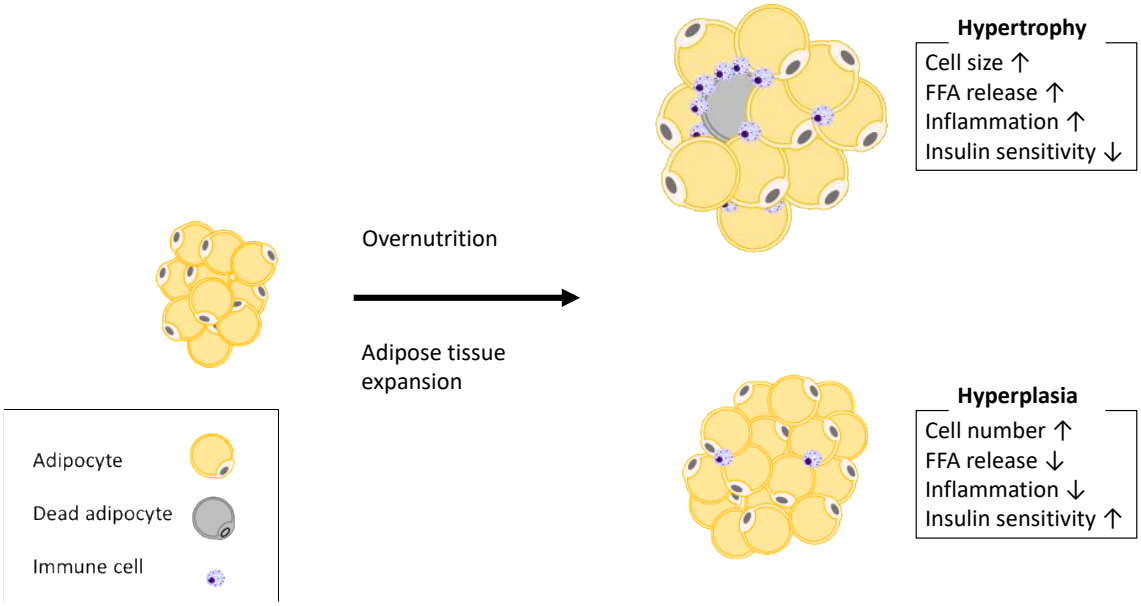


Figure 1.2. *Adipose tissue expansion in overnutrition. Hypertrophic AT is associated with impaired adipocyte function an increase in metabolic risk.*

The manner in which the AT expands can influence metabolic health. When the AT undergoes excessive expansion, it is followed by hypoxia, fibrosis and macrophage infiltration. The macrophages release a number of pro-inflammatory signal molecules which contribute to a detrimental metabolic phenotype. Hypertrophy is associated with IR (43) and T2D (44) independently of the degree of obesity. Increased adipocyte size correlates with impaired adipogenesis, due not to a lack of precursor cells but to the impaired ability to induce commitment of precursor cells to the adipose lineage and differentiation to mature adipocytes.

Reduced adipogenesis is seen in individuals with IR (42, 45). In BMI-matched individuals, IR-individuals had a larger proportion of hypertrophic adipocytes in SAT as compared to insulin sensitive individuals (46). Larger adipocytes blunted the ability of insulin to stimulate fat synthesis through glucose conversion into lipids (lipogenesis), which leads to an increased influx of FFA to the liver and systemic IR (47, 48).

The lipolytic activity in adipocytes is a determinant of insulin sensitivity and is altered in obesity. Hypertrophic adipose morphology is also shown to be positively correlated to total cholesterol and triglycerides (TGs). Visceral adipose morphology has been associated with metabolic disease and hypertrophic visceral fat is associated with higher plasma levels of lipids compared to hyperplastic VAT (49).

It has been shown that improving insulin signalling in adipocytes improves systemic metabolic homeostasis, implicating adipocyte IR as a promising target for treatment of obesity-related metabolic disease (50).

1.3 FUNCTIONS OF THE ADIPOCYTE

1.3.1 Lipolysis

Lipolysis is a complex process within the adipocyte, where enzymes (lipases) hydrolyse TGs into free fatty acids (FFAs) and glycerol) (51). FAs are energy-rich molecules and deliver fuels for peripheral tissues such as heart, liver and skeletal muscle, where they are oxidised to yield ATP. ATP is the energy-currency of our bodies, carrying the energy that is used in all the cells of the body (51).

Lipid turnover in AT, that is uptake and release of FAs, has a central role in energy metabolism and is closely related to body-fat mass. It is known that, in the obese state, basal lipolysis rate is increased but hormone-stimulated lipolysis is decreased (51), indicating that lipolysis has a central role in metabolic complications in obesity.

Lipolysis is tightly regulated by multiple hormonal and biochemical signals. Substances that regulates cyclic adenosine monophosphate (cAMP) levels in the adipocyte are direct regulators of lipolysis, whereby an increase in cAMP stimulates lipolysis and inhibition of lipolysis is associated with reduced levels of cAMP (52).

A large number of substances regulate cAMP levels and thus lipolysis. In adults, catecholamines have a pronounced and immediate lipolytic action, and the lipolytic action of catecholamine is mediated by β-adrenergic receptor subtypes: β1, β2, and β3. In human AT, β1 and β2 are most active whereas in rodents β3 is the most active adrenergic receptor. In both humans and rodents, β3-adrenergic receptor is active in BAT. A fourth receptor is present in human AT, α2A-adrenergic receptor, which inhibits lipolysis when bound to its ligand (51).

The β-adrenergic receptors are G-protein-coupled receptors that, when activated, trigger a signalling cascade in the cell (Figure 1.3). The binding of a ligand to its β-adrenergic receptor produces cAMP from adenosine triphosphate (ATP), catalysed by adenylyl cyclase (AC), increasing the cAMP level in the cell. This activates protein kinase A (PKA), which phosphorylates the lipid droplet proteins hormone sensitive lipase (HSL) encoded by *LIPE*, and perilipin encoded by *PLIN*, which in turn activates adipose triglyceride lipase (*ATGL*). The activation of cAMP-AC pathway is also involved in the regulation of gene transcription in the nucleus of genes involved in proliferation and inflammation (53, 54).

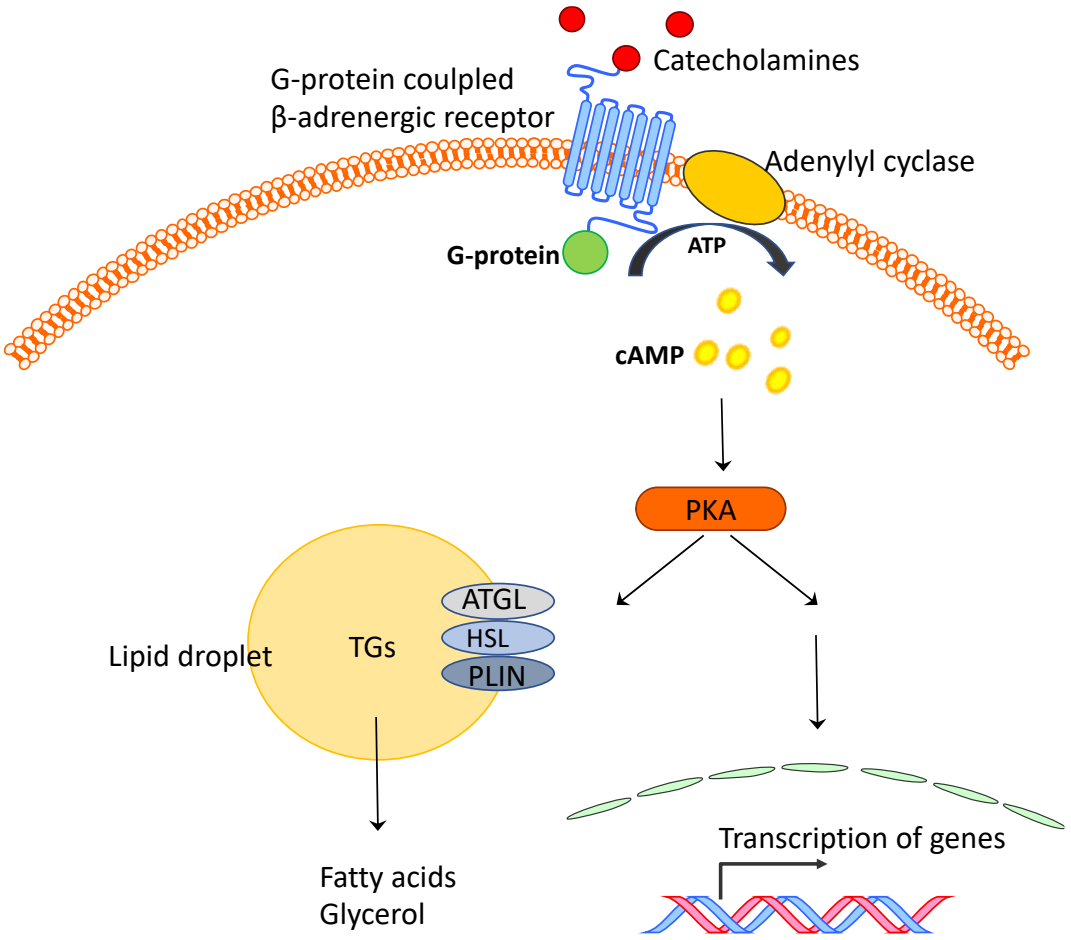


Figure 1.3. *Catecholamine-stimulated G-protein coupled receptor activation of the cAMP cascade.*

Adipose lipolysis is interesting due to its association with fat mass and its central role in energy homeostasis. A recent study by Arner et al (2018) (55) reported that subcutaneous fat cell lipolysis was linked to long-term weight gain in adult women as well as IR and the development of T2D. It is possible that variations in basal and hormone-stimulated lipolysis in adipocytes may affect the individual's predisposition to obesity.

Other substances that have more recently been shown to stimulate lipolysis are natriuretic peptides although they are thought to be involved in lipolysis under other conditions than obesity such as cancer cachexia (56, 57).

1.3.1.1 Regulation of lipolysis during childhood

In human newborns, thyroid stimulating hormone (TSH) is the dominating lipolytic hormone. (58, 59). As the child grows older, the lipolytic effect of TSH is reduced and catecholamines becomes the main lipolytic regulators (58, 60). TSH and catecholamines are ligands to G-protein coupled receptors and the TSH receptor is described in detail in section **1.4**.

1.3.2 Insulin action in adipocytes

Insulin is a major anabolic hormone in the body and regulates energy metabolism through the activation of glucose uptake in both adipose tissue and skeletal muscle. Insulin is a peptide released from the pancreatic beta cells in response to a rise in blood glucose level after a meal. When the insulin receptor is activated, glucose transporter type 4 (GLUT4), a protein encoded by the *SLC2A4* gene, is translocated to the cell surface and glucose is taken up by the cell (Figure 1.4) (61).

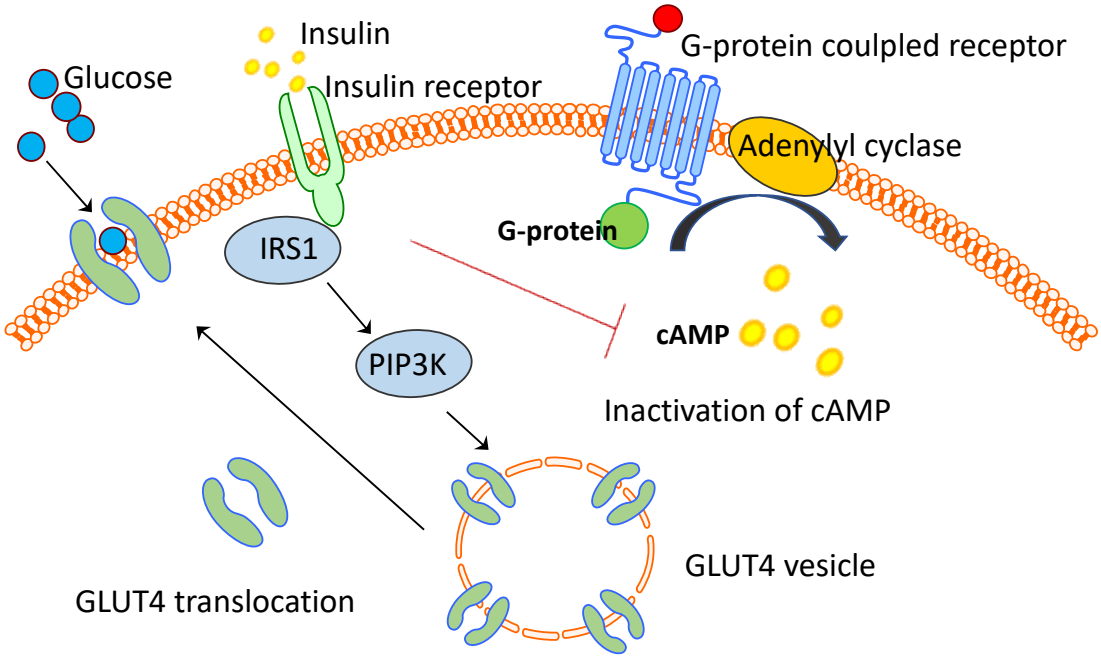


***Figure 1.4**. Insulin signalling and glucose uptake.*

Insulin also inhibits lipolysis by inactivation of cAMP in the adipocyte and stimulates lipogenesis, resulting in TG synthesis by glycerol esterification with FAs (62). Furthermore, insulin has a systemic effect on glucose levels by inhibiting gluconeogenesis in the liver (63).

In muscles and the liver, the intracellular accumulation of lipids (diacylglycerols) triggers the activation of protein kinases C followed by impairment of insulin signalling. This hypothesis accounts for one of the mechanisms behind the development of insulin resistance in obesity-related T2D (64).

1.3.3 Adipose tissue is an endocrine organ

The adipose tissue does not only store energy for future energy demands, it is also a dynamic endocrine organ with a major role in regulating whole-body energy homeostasis. Adipocytes are metabolically active cells that cross-talk with their close environment and peripheral tissues through endocrine and paracrine signalling. They release a large number of signalling molecules known as adipokines involved in the regulation of many biological functions such as appetite, inflammation, glucose and lipid metabolism and reproduction (65, 66).

1.3.3.1 Leptin

Leptin is an adipokine encoded by the *LEP* gene and is secreted into the circulation. It acts as a lipostatic, negative feedback signal (a signal that regulate energy intake and expenditure through signalling between the AT and the brain. Several studies have shown leptin to reduce food intake, body mass and body fat and also to increase energy expenditure and restore euglycemia (67-69). In humans, there is a strong positive association between circulating leptin levels and body-fat mass, which can be explained by larger adipocytes releasing more leptin than smaller ones (70). Leptin is now a well-studied adipokine and known as a regulator of energy intake and storage.

1.3.3.2 Adiponectin

Adiponectin is an adipokine produced by the AT and released into the blood. There is an inverse association between plasma levels of adiponectin and human fat mass, and reduced levels of adiponectin are associated with the development of T2D (71). In obesity, plasma levels of adiponectin are reduced and the administration of adiponectin in diet-induced obesity models has been shown to improve insulin sensitivity (72, 73). In addition to leptin and adiponectin, AT and adipocytes secrete several other adipokines and cytokines.

1.3.4 Adipose tissue inflammation

The immune system is closely linked to the metabolic system. It is well established that, in obesity, when the AT undergoes excessive expansion, inflammatory cells are infiltrated into the adipose tissue and inflammatory cytokines are released into the circulation (74). This results in a low-grade systemic inflammatory state which contributes to metabolic complications such as IR (75, 76). Three important proinflammatory secreted adipokines are investigated below.

1.3.4.1 TNF-alpha

Tumour necrosis factor alpha (TNFα) is a well-studied cytokine mainly secreted by macrophages in the AT but also by adipocytes (77). It is believed that TNFα acts locally in AT in a paracrine or autocrine manner. It is also believed that the TNFα affects insulin action distal to the insulin receptor, since it reduces maximal insulin responsiveness and not basal glucose transport (78). TNFα levels are elevated in obesity and inversely associated with insulin sensitivity (79). TNFα has previously been shown to stimulate lipolysis in adipocyte cell lines (80) and inhibit insulin-stimulated lipogenesis in the AT from obese women (79). TNFα-knockout mice are resistant to obesity-related IR, probably due to the inhibitory effect of TNFα on gene expression of the insulin receptor, insulin receptor substrate 1 (*IRS1*) and *SLC2A4*, encoding GLUT4 (81, 82).

1.3.4.2 IL-6

Interleukin-6 (IL-6) is a proinflammatory cytokine associated with obesity (83). IL-6 is positively associated with BMI and impairs insulin sensitivity in humans (84). In T2D patients, elevated IL-6 levels were predictive of the development of T2D. In obesity, when the macrophages are infiltrated into the AT, IL-6 is believed to contribute to IR.

1.3.4.3 CCL2

Monocyte chemoattractant protein 1 (MCP-1) encoded by the *CCL2* gene, is another pro-inflammatory cytokine secreted by cells in the AT. It contributes to the chronic inflammation seen in obesity by stimulating macrophage infiltration into the AT. *CCL2* levels are increased in the obese state (85). Both humans and mice with obesity have increased *CCL2* expression in their WAT (86), and at least in mice, the elevated *CCL2* level is sufficient to induce inflammation in the AT, and affect insulin sensitivity (87).

1.4 THYROID STIMULATING HORMONE

Other endocrine factors than catecholamines and insulin that also regulate adipose tissue function are thyroid stimulating hormone (TSH) and thyroid hormone triiodothyronine (T3).

Thyroid hormones are central players in the regulation of our metabolism. They control body functions such as body weight (88), heart rate (89), respiratory capacity (90) the nervous system (91) and more.

The main biological function of TSH and its receptor, thyroid stimulating hormone receptor (TSHR), is to control the release of thyroid hormones T3 and thyroxine (T4)

from follicular cells in the thyroid gland. TSH is involved, both directly (92-94) and indirectly via thyroid hormones (95), in the regulation of basal metabolism (88, 95, 96). In the thyroid gland, the TSHR regulates the production and release of thyroid hormones. Circulating thyroid hormone levels are regulated by negative feed-back on the hypothalamic-pituitary-thyroid axis (Figure 1.5). Release of TSH from the pituitary gland is initiated by thyroid releasing hormone (TRH) from the hypothalamus. TSH binds to the TSHR on the thyroid gland, which leads to the synthesis and release of T3 and T4. T4 is further enzymatically converted by deiodination to active T3 in peripheral tissues. T4 and T3 suppress the release of TRH and TSH respectively, by negative feedback regulation, on the hypothalamus and the pituitary gland (97).

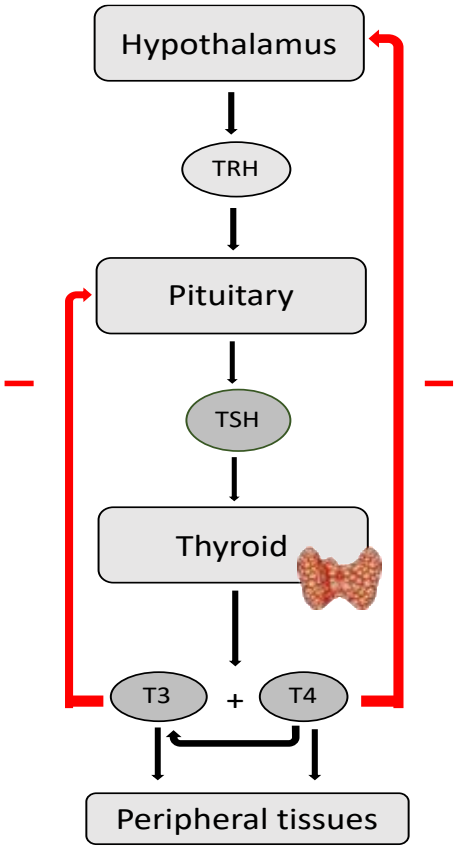


***Figure 1.5**. Feedback regulation of thyroid hormones.*

Sub-clinical hypothyroidism, a condition in which the thyroid gland fails to produce normal amounts of thyroid hormones and TSH levels are increased in order to restore normal levels (98), is associated with weight gain, cardiovascular disease and IR (99, 100). Furthermore, sub-clinical hypothyroidism is a condition associated with a pro-inflammatory state and IR, where an elevation of pro-inflammatory proteins such as IL-6 and elevated FFAs has been reported (101, 102).

1.4.1 Thyroid stimulating hormone and obesity

Body composition and TSH are closely related (103). TSH, via its stimulatory effect on the release of thyroid hormones, is involved in the regulation of energy metabolism and thermogenesis (93, 95) and also in glucose and lipid metabolism (88).

In adults with obesity, TSH has been reported to be elevated, although results indicating unaltered TSH and thyroid hormones have also been presented (104, 105). A positive correlation between weight gain over 5 years and a progressive increase in TSH level have also been reported. Even slightly elevated TSH levels might be involved in the occurrence of obesity (106), but the cause of the higher TSH concentrations observed in the obese state, and the underlying mechanisms are unclear (107, 108).

1.4.2 Thyroid hormones and effect on energy balance.

Thyroid hormones regulate energy expenditure by regulating cellular respiration (109, 110). Thyroid hormones act on the hypothalamus, modulating AMPK activity, increasing the activity of the sympathetic nervous system and thereby regulating whole-body energy homeostasis. Centrally administered T3 also upregulates thermogenic markers in BAT (111).

The relationship between obesity and the thyroid is complex and it is still not known whether changes in thyroid function are primary or secondary to obesity (106, 112). Hyperthyroidism (thyroid hormones fT3 and fT4 above the normal range and/or TSH below the normal range) is known to predispose to weight loss and hypothyroidism to induce weight gain, due to the role of thyroid hormones in thermogenesis and appetite regulation (88, 95). Overfeeding increases, and undernutrition decreases energy expenditure due to the related increased or decreased concentrations and production rate of T3 (113).

Resting energy expenditure is positively associated with T3 levels and it has been suggested that an increase in TSH and secondarily in fT3 in obesity is an attempt to increase resting energy expenditure and inhibit the storage of excess energy into fat (110). In other words, TSH in obesity may be increased as an adaptation to the excessive weight gain (88, 95).

Sub-clinical hypothyroidism is reported to be associated with components of the metabolic syndrome such as glucose tolerance (114, 115). Underlying mechanisms involves effects of T3 on insulin signalling, β-cell function, gluconeogenesis in the liver, lipolysis and lipid oxidation as reviewed (116). These factors raise the question of

whether thyroid hormones in the upper normal range in the obese state, might have an impact on adipocyte function.

1.4.3 Thyroid stimulating hormone receptor in adipocytes

In adipocytes from human neonates, TSHRs regulate lipolysis through the binding of thyroid stimulating hormone (TSH). TSH is the dominating lipolytic hormone when the lipolytic effect of catecholamines is depressed by an increased alpha-2 adrenoceptor activity. (58, 59). As the child grows older the lipolytic effect of TSH is reduced and catecholamines acting on β-adrenergic receptors becomes the main lipolytic regulators (58, 60).

TSHRs were thought to be confined to the thyroid gland; however, it has been proven that they are widely expressed and functional in extra-thyroidal tissues such as human and rat adipocytes (117, 118). Studies have also demonstrated the presence of functional TSHR in adult human adipose tissue (117, 119) but their physiological significance and role in adipocytes is still largely unknown.

Studies in preadipocyte cell lines (120) and animal models (118) suggest that TSHRs are involved in white AT (WAT) proliferation and differentiation of white adipocytes (121). In a small study of adult human AT, the levels of *TSHR* expression decreased during adipogenesis (122). In brown adipose tissue (BAT) TSHRs are suggested to be involved in the regulation of thermogenesis (93), thereby having an effect on energy homeostasis.

More recently in a mouse model with AT-specific knockout of *TSHR*, it was shown that the knockout mice had larger adipocytes compared to controls (123). This finding supports the descriptive results in studies of human AT that TSHR activation may contribute to body composition separately from the effect of circulating thyroid hormone levels (103).

Although the level of TSHRs is reduced in adults compared to children, more recent studies on adult AT report that the expression of TSHRs is altered when the AT undergoes excessive expansion, as in obesity (120, 124).

In brown adipocytes, the expression of *TSHR* has been clearly established, but the physiological role of TSHR in BAT is yet to be established. Mice harbouring a mutation in the *TSHR*, are hypothyroid because of the mutation. Studies have indicated that not only thyroid hormones but also TSH might be directly involved in the regulation of UCP1-expression in mouse BAT (93).

Mouse strains that lack either functional TSH or TSHR display hypothyroidism with thyroid hyperplasia. These strains exhibit dysfunctional TSHR in all body tissues, and it is therefore not possible to study the direct role of TSHR in a specific tissue, such as AT. Thus, in order to study the specific role of the TSHR on adipocyte function, a mouse model with normal thyroid function but with specifically down-regulated expression of *TSHR* in AT was generated by our research group (123).
We used the Cre-lox system to specifically remove a part of the gene coding for the TSHR in adipose tissue (*TSHR* $^{loxP/loxP}$*/Cre* $^{+}$ or *TSHR* KO). This provides an advantage compared to other TSHR knockout mice strains, in which the *TSHR* is non-functional in all cells. The *TSHR* KO mouse has functional TSHRs in all tissues except BAT and WAT. The initial characterisation has shown that the *TSHR* KO mouse is euthyroid and healthy. The *TSHR* expression is reduced in WAT and BAT and the lipolytic effect of TSH in vitro is low (125). In contrast, the expression of TSHR in other organs, such as brain, muscle and kidney, are similar in *TSHR* KO and wild-type (WT) mice. At 8 weeks of age, the white adipocytes were larger in *TSHR* KO than in WT animals which further supports the hypothesis that functional TSHRs in adipocytes are of physiological importance for adipocyte growth and function. Our results indicate that TSHRs are of importance for BAT since *UCP1* expression is lower in *TSHR* KO animals and whole-body temperature is also significantly lower in young animals. These results indicate that TSH-TSHR signalling in brown adipocytes is essential for normal functioning of the UCP1, body temperature regulation and body weight in early life.

1.5 OBESITY: A COMPLEX DISEASE

Obesity is a complex disease. Complex diseases are called “complex” because both genetic and environmental factors contribute to susceptibility risk. It is possible that individuals that are genetically predisposed to develop obesity and obesity-related complications are at greater risk by living in an obesogenic environment by so called gene-environment interactions.

1.5.1 Dysfunctional adipose tissue

In humans, the physiological link between obesity and metabolic disease is currently not fully understood. One proposed factor is the AT-expandability hypothesis (126). When the AT undergoes excessive expansion, as in obesity, it changes its hormonal secretion patterns as well as its sensitivity to signal molecules. Furthermore, the development of hypoxia, oxidative stress, fibrosis and inflammation contribute to the AT becoming dysfunctional (127-129). The capacity of AT to expand is considered to be limited for a

given individual. When this limit is exceeded, lipids begin to accumulate in ectopic tissues causing lipotoxicity leading to IR and metabolic dysfunction (126).
Lipolytic products such as diacylglycerols (DAGs), monoacylglycerols (MAGs) and FFAs not only serve as energy dense substrates for other tissues, they are also signalling molecules involved in multiple biological pathways (130). In obesity, excessive FFAs produced by lipolysis enters the circulation and are transported to peripheral tissues. Therefore, FFAs released from the AT are likely to be key factors in ectopic lipid accumulation in obesity. Studies in humans report that FFAs attenuate insulin-stimulated glucose uptake and oxidation in peripheral tissues, thereby contributing to IR (131, 132). A dysfunctional lipolysis is likely to be a factor contributing to obesity-related metabolic complications and adipose morphology is linked to functions in the adipocyte such as lipolysis.
The reasons and mechanisms behind why some but not all individuals with obesity develop metabolic complications are still unknown. For instance, not all individuals with obesity develop a severe IR (133). There seems to be a strong genetic influence underlying adipose morphology, seen in studies of twins with concordant BMI (134), indicating a genetic variation in the predisposition of developing obesity-related complications. More research is needed to clarify what underlying mechanisms causes the altered adipocyte function in hypertrophic obesity.

1.5.2 Genetic predisposition

Genetic predisposition is one cause for the development of obesity and obesity-related disease. Genome-wide association studies (GWAS) have led to the discovery of numerous genetic variants such as single nucleotide polymorphisms (SNPs) associated with risk markers for various diseases.
In the past few years, GWASs led to the discovery of numerous genetic risk markers for obesity traits. The first locus for BMI, was identified in 2007 and was located in the *FTO* gene (135, 136). Taken together, <3% of variation in BMI in the population is explained by identified genetic loci (137).
Concerning T2D, heritability is estimated to range between 30–70%, based on twin studies (138). According to the DIAGRAM consortium, a research collaboration performing large scale studies to characterise the genetics of T2D, estimated that known genetic variation account for around 50% of the risk for a diabetes-related trait (139).
To further identify genetic loci for complex disease, methods for deep sequencing of all known exons have been developed with the capacity to detect low-frequency and rare

causative variants in the genome (140, 141). These studies are a major tool for identifying genes that contribute to complex diseases in which many different variants contribute to disease susceptibility, with each variant having only a subtle effect. However, large gaps remain in understanding the heritable genetic impact of detected genetic variation on obesity.

For the majority of loci discovered by GWASs, the probable disease-causing genes remain unknown (137). Since lead SNPs often resides in non-coding regions in the genome, expression quantitative trait loci (eQTLs) of genes in the vicinity of SNPs associated with obesity and obesity-related traits could lead to the finding of candidate genes with a functional impact on disease (142).

Adipose tissue eQTL studies, with gene expression levels of target genes in adipose tissue investigating genotype-gene expression level correlations, have implicated genes involved in obesity and metabolic traits. The importance of genetic influence in the susceptibility to T2D is well established (143), but large gaps remain in our understanding of how genetic variations affect molecular processes and thereby contribute to T2D susceptibility. GWASs have identified several genetic loci associated with fasting insulin (FI) and FI adjusted for BMI (144). However, for most of these associations the functional impact of the genetic variation in the loci is unclear. Associations of gene expressions in the AT with human clinical traits could identify candidate genes causing the metabolic trait (78). It is important to understand the molecular changes that comes with obesity in order to further understand how different treatments may potentially contribute mechanistically in obesity-related disorders. Functional studies of candidate genes from GWAS are needed to clarify their physiological impact and what mechanisms are affected in obesity and adipose tissue hypertrophy and the accompanying metabolic risk.

2 HYPOTHESES AND AIMS

2.1 SPECIFIC AIM OF THE STUDIES

2.1.1 Study I

Research has shown that thyroid stimulating hormone levels are affected in obesity. It is unclear what causes elevated TSH-levels in obesity and whether they influence metabolic health. In Study I, we aimed to investigate if TSH status within the normal range is associated with degree of obesity and metabolic risk markers in children with obesity. We hypothesised that TSH levels are increased within the normal range to maintain normal thyroid hormone levels, and that a high-normal TSH is accompanied by a more deranged metabolic profile.

2.1.2 Study II

TSHR has been implicated to have a role in adipogenesis and in the regulation of adipocyte functions. In Study II, we set out to investigate the AT-specific role of TSHRs using an AT-specific knockout mouse model. We aimed to investigate how TSHRs may contribute to adipocyte function in different AT depots in the nutritionally obese state and in the normal-weight state. We hypothesised that reduction in the effect of TSH, a potent lipolytic hormone, should affect fat cell metabolism.

2.1.3 Study III

In Study III, we set out to investigate the impact of candidate genes in genetic loci associated with fasting insulin, on adipogenesis and adipocyte function. The key research question was: can identified candidate genes in these genetic loci be linked to adipose variables, thus linking adipose function to systemic insulin resistance?

2.1.4 Study IV

Given that TSHRs are known to be down-regulated in obesity and are proposed to have a regulating role in adipogenesis. Larger fat cells have been reported when TSHRs are knocked out in the adipose tissue of mice. Therefore, in we aimed to investigate *TSHR* expression in WAT in relation to BMI and WAT phenotypes (WAT morphology, lipolysis and insulin-stimulated lipogenesis). In addition, we investigated if TSHR expression in WAT is connected to expression of adipocyte genes central to adipogenesis, adipocyte lipolysis, insulin signalling and genes encoding adipokines. In individuals with morbid obesity we aimed to investigate if the reduction of TSHRs in obese WAT is a primary effect or if TSHR expression is affected by weight change in

women with obesity. We hypothesised that *TSHR*-expression in human WAT, is affected by weight change and that TSHR signalling in WAT is associated with adipocyte metabolism.

3 RESEARCH APPROACH AND REMARKS ON THE METHODS

The methods and materials used for each study have been described in detail in their corresponding papers. In this chapter, study designs are described in brief and interesting aspects of a few selected methods are addressed.

3.1 STUDY I

3.1.1 The Swedish Childhood Obesity Register (BORIS)

In Study I, data from the BORIS register was used to investigate TSH levels in children with obesity and the association of TSH with metabolic risk markers. BORIS is a national web-based quality register for childhood obesity treatment supervised by the National Board of Health and Welfare. It was started in 2005 with the purpose to quality assure the pediatric obesity care and to be a tool for the health care staff. Since 2005, over 18000 children with different severity of obesity, at different ages and follow-up times, have been included in the register and BORIS. The register is therefore a good source for clinical research.

3.1.2 Study design

The study encompassed a retrospective cohort design consisting of children registered in BORIS. The children and adolescents were enrolled in obesity treatment from pediatric clinics all over Sweden between August 1995 and June 2016. Inclusion criteria were children and adolescents classified as obese according to Cole and Lobstein (2012) (10) with records of TSH levels within the normal range (Age 3-6.9 years TSH 0.7-6.0 mU/L; 7-11.9 years 0.6-4.8 mU/L; 12-17.9 years TSH 0.5-4.3 mU/L (Reference intervals for children and adults, Elecsys Thyroid Test Elecsys 2010 syst. Roche Diagnostics 2009)). Exclusion criteria were: diagnosed thyroid disease (subclinical and overt hypo- and hyperthyroidism, Graves' disease, Hashimoto's thyroiditis, and thyroid hormone treatment in general), the presence of hypothalamic or pituitary disturbances, central nervous system damage, or syndromes (Prader-Willi syndrome, Down syndrome, and Laurence-Moon-Bardet- Biedl syndrome) or other chronic diseases. In Study I, we set the inclusion criteria as described in the flow chart (Figure 3.1).

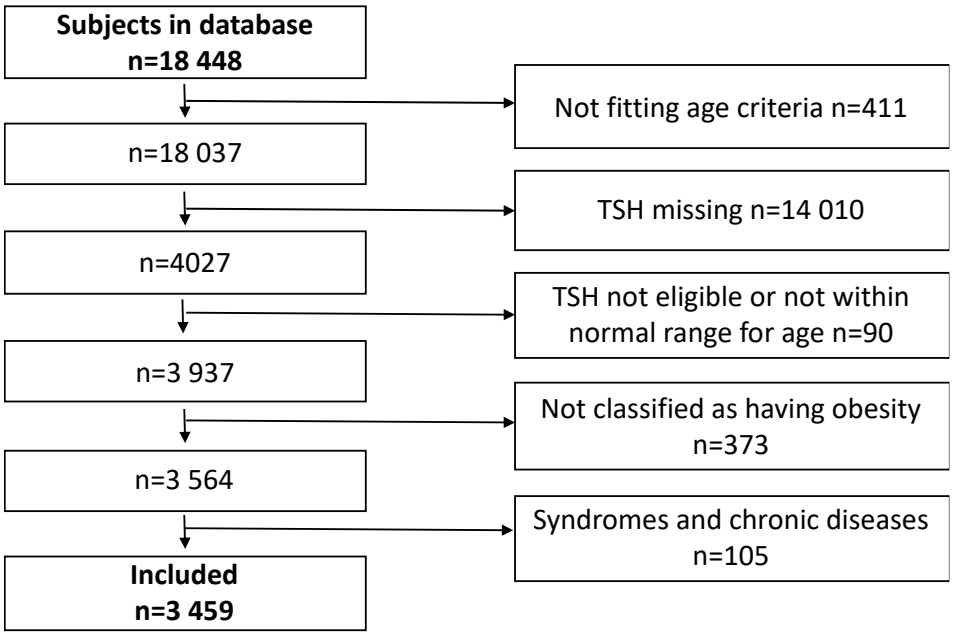


Figure 3.1. *Flow-chart of inclusion.*

After exclusion, a total of 3,459 children, aged 3.0–17.9 years, were identified within each age-specific normal TSH range.

3.1.3 Metabolic risk markers

Fasting blood samples were obtained from all children, and routine biochemical analysis was performed to obtain their levels of TSH, free T3, free T4, fasting insulin, fasting glucose and blood lipid. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated by the formula:

(fasting glucose (mmol/L) x fasting insulin (mIU/L))/22.5

HOMA-IR is a model used to estimate an individual's IR (145). However, there is no unanimous defined cut-off for IR and values above 2.3–2.5 are often defined as IR and values <2.0 are considered normal (146).

3.1.4 Statistical analysis

Linear regression analyses were conducted to investigate associations between TSH and BMI, and between TSH and metabolic risk markers. The models were adjusted as appropriate for BMI, sex and age. One study by Ittermann et al., has shown that smoking may mediate the association between thyroid function and BMI in adolescents (147). They found the relationship to be stronger in smokers than non-smoker. One could therefore suspect smoking to be a cofounder in this study. We did not have information about smoking status of the children and adolescents included and could not take in account this

possible cofounder. However, most individuals in the study are children where smoking is uncommon.

It should be noted that statistical associations do not imply causality, especially in multifactorial conditions and biological processes such as metabolism. They do however, generate research questions for future research.

3.1.5 Ethical permission

Data collection in BORIS was obtained by the regional committee of ethics in Stockholm (2014/381-31/5).

3.1.6 Comments on the design

Study I was a retrospective cross-sectional study. A prospective design would have been preferable since no causal relationship can be determined in a cross-sectional study. Furthermore, the cut-off for "high-normal TSH" is under debate and different cut-offs for elevated TSH have been used previously. This may be a problem due to difficulties in directly comparing our results to previous studies. An elevated TSH level is described in some studies as 4.0–10.0 mU/L (148, 149), whilst others suggest that the limit should be lower (150). Recently, TSH >3.0 mU/L is described as elevated within the normal range (151) and supports the cutoff chosen in the present study.

3.2 STUDY II

In Study II, we used genetically modified mice to study the impact of an AT-specific *TSHR* knockout on AT development and function. Using *in vivo* animal models provides a way to study a factor or condition in the physiological context.

3.2.1 Study design

3.2.1.1 Mice and diet intervention

At 4–5 weeks of age, male animals were divided into 4 groups according to genotype and diet. *TSHR* knockout and corresponding wild-type mice received either a high-fat diet (60% kcal from fat) or a matched control diet (10% kcal from fat) for 20 weeks.

3.2.1.2 Physiological measurements

Physiological phenotypes were determined by measurements of body weight and body temperatures Measurements were performed in the morning at the environmental temperature of 20–22°C.

3.2.1.3 Glucose tolerance test

Glucose tolerance was investigated in a group of female mice both at baseline, at 8 weeks of age, and after 10 weeks (follow-up test) on either a control diet or high-fat diet. They received an oral glucose dose of 1.5 grams of glucose per kilogram of body weight as a 20% aqueous glucose solution (Glucose, APL Pharma Specials, Sweden). Blood was drawn from the tail vein before glucose administration and at 15, 30, 60, 90 and 120 minutes after dose. Plasma glucose was measured in connection with each blood sample using a glucometer (HemoCue system, Hemocue AB Ängelholm, Sweden).

3.2.1.4 Gene expression analysis in white and brown adipocytes

After 20 weeks, interscapular BAT and epididymal WAT fat depots were collected and isolated adipocytes were prepared using the collagenase method (152) to ensure that the following gene expression analysis was conducted on adipocytes and not on AT, which contains several other cell types.

Gene expression of selected genes in adipocytes was determined by real time PCR (CFX thermocycler, BioRad) using Taqman assays (Life Technologies). *TSHR*-expression was investigated in both white and brown adipocytes.

In white adipocytes, the expression of genes central to adipogenesis, lipolysis, insulin sensitivity, and adipokine/cytokine-signals were investigated. In addition, in brown adipocytes, genes central to BAT function, such as thermogenesis, were investigated.

All gene expression assays were normalised against *GAPDH*. Relative gene expressions were calculated using the delta-delta Ct method (153) and expressed as fold-change in the expression of target genes relative to untreated controls (*TSHR* knockout on control- and high-fat diet and wild-type on high-fat diet relative wild-type on control diet).

3.2.2 Statistical analysis

Between-group comparisons were performed using an independent t-test for sample sizes ≥6 (body weights and body temperatures). One-way ANOVA with the Bonferroni posthoc test was used to compare calorie intake and relative gene expressions. $p<0.05$ was considered significant. No adjustment for multiple testing was applied since the gene expression analysis were investigated with specific hypotheses for adipogenesis and adipocyte-specific functions.

3.2.3 Ethical permission

The study protocol has been approved by the Stockholm South Animal Board Ethics committee (D.no S65-13, D7-16).

3.2.4 Mice as a research model

3.2.4.1 Mice versus humans

Rodents are a widely used pre-clinical model for human obesity. There are, however, several differences between rodents and humans when it comes to AT physiology that needs to be considered when deciding whether the research findings can be translated to humans. Beneficial aspects of using an animal model are that non-biological factors, that are considered to be confounders in human studies, can be excluded. Ethnicity, socioeconomics, education and environmental exposure are factors that would need to be considered in human studies. A mouse model is also a good alternative to cell lines since an animal displays greater biological variation than cell cultures.

3.2.4.2 Adipose tissue depots

As in humans, AT in mice is divided between different depots, however, the location of these depots differs between humans and mice (18). The perigonadal fat in mice, also known as the epididymal fat in male mice and periovarian fat in female mice, is often used in AT studies in mice, partly because it is easy to isolate. However, there is no corresponding fat depot in humans and, as different fat depots have been shown to differ in certain functional aspects, this needs to be considered.

In humans, a sexual dimorphism in both total adiposity and depot-specific adiposity exists (154). The sexual dimorphisms are less well-documented in rodents. However, similar to humans, female mice have a greater total fat mass than male mice but remain the insulin sensitivity remains. There are however, inter-depot differences in insulin sensitivity in female mice that do not correspond to humans (155).

Weight-loss studies in mice and humans display a similar fat-depot pattern when it comes to which depots are reduced (156-158). Hypothetically, this may indicate that weight gain also occurs in a similar way between depots in mice and humans.

3.2.4.3 Lipolysis

Lipolysis can be stimulated in mice and humans under similar physiological conditions and by similar activation of the sympathetic nervous system. However, there are important differences for instance in the pathways involved in driving lipolysis.

Catecholamine-induced lipolysis occurs as mentioned in the introduction by activation of specific β-adrenergic receptors. There are four types of adrenergic receptors β1, β2, β3 and α2A. In humans, β1 and β2 adrenergic receptors are most active, whereas the β3-adrenergic

receptor is the most active in mice. The α2A-adrenergic receptor is coupled to inhibitory G-proteins and, when activated, the adenylyl cyclase is inactivated and lipolysis is inhibited. The α2-adrenergic receptor inhibitory pathway does not exist in rodents AT (51); therefore, when studying adrenergic receptor signalling, the lacking of inhibitory α2-adrenergic receptor signalling could account for differences between species.

3.2.4.4 Energy expenditure

The energy expenditure in a small animal such as the mouse is much higher than in humans, and this needs to be taken into consideration when using mice as a model system for human disease (159). The lipid turnover in mice is also different from that in humans. In mice, BAT has a significant impact on thermoregulation and energy expenditure (20). The relationship between surface and volume is different between mice and humans which account for the lower thermal dispersion in humans (13). It is, however, unclear what impact BAT has in human energy homeostasis, although several studies have reported its presence and activity in adult humans (28, 29, 160). It is estimated that, in adults, fully activated BAT can increase energy expenditure by up to 20%, which makes it an interesting target for obesity treatment in humans (13).

Nevertheless, the translation of results from rodent studies of BAT to humans needs to be carefully considered since this particular fat depot clearly has different physiological impact in mice and humans.

3.2.5 Knockout mice

The generation of the *TSHR* adipocyte-specific (*TSHR*$^{loxP/loxP}$ *Cre)* knockout (KO) mouse strain used in the study was bred on a C57Bl6 background as previously described (123). In brief, a Cre-mouse strain expressing the Cre-recombinase under the control of the *Fabp4/ap2*-promoter was purchased from Jackson Laboratories ((B6.Cg-Tg (*Fabp4-Cre*) 1Rev/J stock no. 005069 Jackson Laboratory) and bred with mice in which the LoxP-sites had been inserted in exon 10, encoding the transmembrane β-subunit of the TSHR. As untreated controls, we used corresponding C57Bl6/J mice.

The Cre-Lox system was first used in 1985 when Brian Sauer introduced the system in eukaryotic cells (161, 162). The Cre-Lox system is a method used to generate tissue-specific gene knockout by deleting a part of a gene, thereby inhibiting the synthesis of a functional protein.

More recent research has shown that the *Fabp4/aP2-Cre* used in this mouse strain is not as effective or specific as for example the adiponectin-Cre-lox system (163). This is seen by the effectivity of a 40-45% knockout of *TSHR* expression in these mice, due to the Cre-

recombinase not being expressed in all adipocytes. However, considering the effects seen on body weight development and body temperatures, the smaller and more variable degree of knockout does have a significant effect *in vivo* and thus allows us to study the effect of TSHR removal in AT. One might also argue that a partial knockout phenotype better reflects a pathophysiological condition, since in most conditions such as obesity, TSHRs are reduced but not missing.

3.2.6 Gene expression

In Study II and III, quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify transcripts of interest. Briefly, total mRNA is extracted from a sample of cells or tissue. It is then reverse transcribed into complementary DNA (cDNA). The cDNA is amplified in the qRT-PCR reaction. Detection is enabled by fluorescence labelling of the probe or binding dye of primers depending on what assay is used.

Gene expression is calculated by the delta-delta Ct method (153). The expression of a target gene is normalized to the expression of an endogenous control, also known as housekeeping gene and expressed as fold-change relative to an untreated control sample. The purpose of the endogenous control is to normalize the PCR for the amount of RNA in the reaction.

There are some important aspects to considered when conducting PCR to investigate gene expression. It is a highly sensitive method which allows generation of a product when the target mRNA is present at very low levels. Also, the presence of a transcript product does not necessary correspond to corresponding levels of the functional protein. Furthermore, PCR is often applied in tissue which are heterogenous and the expression of the target cannot be attributed to a specific cell type.

In study II and III qRT-PCR was conducted in adipocytes to ensure adipocyte-specific analysis. A limitation in our studies was that we did not verify the expression by quantification of protein levels. We did however, analyse adipocyte specific functions to assess if the observed gene expression had a functional impact on the phenotype. In study II we measure glucose tolerance and body temperature and in study IV we analyzed glycerol release in cell culture medium for assessment of lipolysis.

3.3 STUDY III

In study III, candidate genes for fasting serum insulin were identified from SNPs identified and listed in a GWAS meta-analysis (144). The identified candidate genes were functionally evaluated in a human mesenchymal stem cell model where hMSCs were

differentiated into adipocytes after knocking down genes with siRNA. The workflow is described in Figure 3.2.

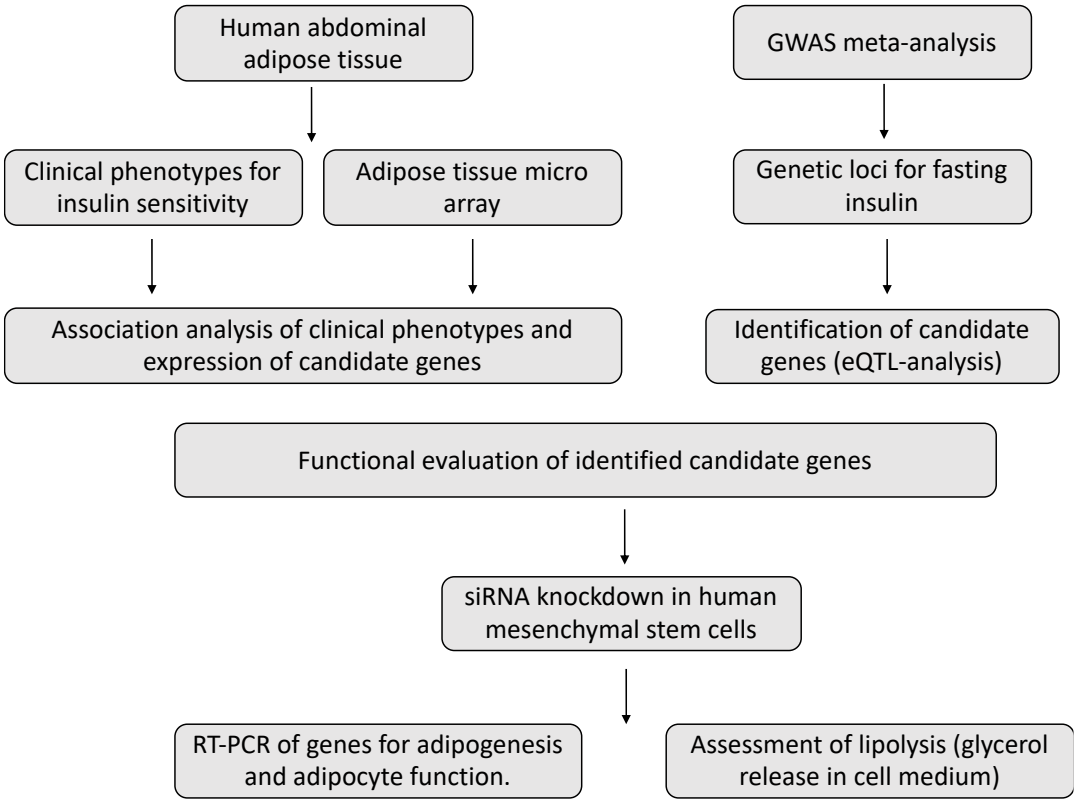


Figure 3.2*. Schematic description of the workflow in Study III.*

3.3.1 Study participants in human WAT studies

Studies III and IV included 114 non-diabetic Swedish women with WAT global transcriptome profile available from a previous study (142). The women were recruited by advertisement from the general adult population in the Stockholm (Sweden) area. They displayed a large inter-individual variation in BMI and were healthy, except that some were obese.

3.3.2 Ethical permission

The studies were performed in accordance with the guidelines set out by The Declaration of Helsinki. All participants received detailed information about the studies and written informed consent was obtained. The studies were approved by the regional ethics board in Stockholm (D. no. 278-03).

3.3.3 Experiments in white adipose tissue

3.3.3.1 Lipolysis experiments

In Studyies III and IV, lipolysis was measured using a bioluminescent method as described by Hellmer et al. (1989) (164) in mature adipocytes prepared from WAT biopsies. The cells were diluted in Krebs-Ringer phosphate buffer with bovine serum albumin (20 mg/ml), glucose (1 mg/ml) and ascorbic acid (0.1 mg/ml) and incubated with different concentrations of noradrenaline (10–12 to 10–4 mmol/l) for 2 hours at 37ºC. Isoprenaline is a synthetic, selective β-adrenoreceptor agonist, and was used as the lipolytic agent. After incubation the samples were put on ice to stop lipolysis. A fat cell free aliquot was removed for the measurement of glycerol release with a luminometer, which measure light emitted in the following reactions:

$$\text{ATP + Glycerol} \xrightarrow{\text{Glycerolkinase}} \text{Glycerol-1-phosphate + ADP}$$

$$\text{ATP + Luciferin + } O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin + AMP + PPi + } CO_2 \text{ + Light}$$

Luciferase and glycerol kinase compete for the use of ATP and a decreased emission of light indicates higher lipolytic activity. During lipolysis, each triglyceride molecule is broken down into three fatty acids and one glycerol molecule. Glycerol is a good marker for lipolysis since fat cells do not contain glycerol kinase and therefore are unable to reuse glycerol.

3.3.3.2 Adipose morphology

In Studies II and IV a measure of adipose tissue morphology was used to define whether the clinical WAT samples displayed hypertrophy or hyperplasia. Spalding KL (2008) and Arner E et al. (2010) (11, 42) have shown that the relationship between adipocyte volume across the range of fat mass is curve-linear. The difference between the actual measured adipocyte volume and the expected adipocyte volume from the mean curve fit is an indication of the adipose morphology independent of total body fat. A positive value indicates hypertrophy, whereas a negative value is indicative of hyperplasia.

These morphology values can be quantitatively assessed and for the individuals included in Studies II and IV were obtained from previous calculations (165). Adipose morphology is a

good marker for adipocyte dysfunction given that hypertrophy is associated with attenuated insulin sensitivity.

3.3.3.3 Lipogenesis experiments

In Studies II and IV, insulin-stimulated lipogenesis was assessed as a measure for insulin sensitivity. Briefly, adipocyte lipogenesis was determined in isolated adipocytes which were incubated in vitro in an albumin-containing buffer with labeled [^{3}H]glucose and unlabeled glucose and varying concentrations (0–70 nmol/l) of insulin. The incubations were conducted for 2 hours at 37°C with air as the gas phase. Incubations were stopped by rapidly chilling the incubation vials to 4°C. The incorporation of labelled glucose into adipocyte lipids was determined, which reflects lipogenesis, and was expressed as the amount of glucose incorporated per adipocyte number, as described previously (166). Values at the maximum effective insulin concentration were used.

The method is used as a risk marker for IR, since a blunted ability of insulin to stimulate fat synthesis through glucose conversion into lipids (lipogenesis) leads to an influx of NEFA into the liver and to systemic IR.

3.3.3.4 Adipose tissue depots

In Studies II and IV, abdominal, subcutaneous AT (SAT) was used. In humans, an increased visceral AT (VAT) mass is associated with insulin resistance and dyslipidemias (167) SAT-derived adipocytes have a greater adipogenic capacity than VAT-derived adipocytes and are associated with improved or at least preserved insulin sensitivity. However, excessive abdominal SAT is also associated with a deranged metabolic profile (168). Understanding the growth mechanisms of SAT may reveal therapeutic targets for obesity-related metabolic disease.

In Study III, visceral AT samples were not obtained for ethical reasons; it is a more invasive method to obtain visceral fat biopsies than subcutaneous fat samples obtained by needle aspiration under local anaesthesia. The results may only apply to subcutaneous fat; however, this depot constitutes >80% of the total fat mass and, through its mass effect, it has a major influence om body weight and energy homeostasis.

3.3.4 Expression quantitative trait locus (eQTL) analysis

In Study III, eQTL-analysis was performed to link identified candidate genes from a GWAS to adipose tissue function.

Today there are hundreds of identified SNPs that are associated with obesity traits such as BMI and fasting insulin, a marker for IR. The eQTL analysis is a method used to find the underlying genes affected by the associated SNP. A schematic overview of the theory behind eQTL analysis is presented in Figure 3.3. When a SNP affects a gene in the vicinity (+/- 500 kilo bases) in the genome, the term "cis" is used. If a SNP affects a gene further away, it is termed trans-eQTL.

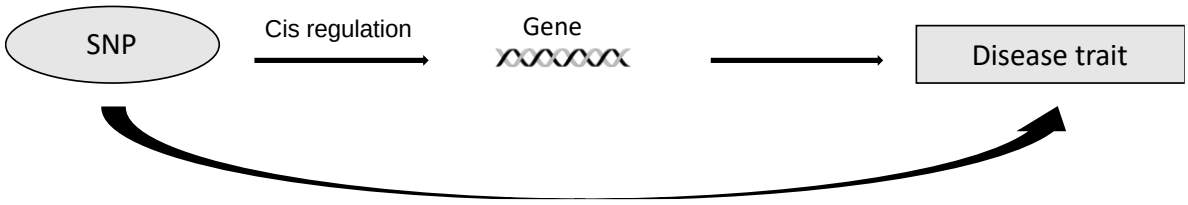


Figure 3.3. *Expression quantitative trait loci (eQTL) analysis. A SNP discovered as associated with a disease trait in a GWAS, and also associated with the expression-level of a nearby gene (cis-eQTL).*

The expression of the nearby gene may or may not be the cause of the measured disease trait and the genes is therefore a candidate gene for disease.

Since lead SNPs often resides in non-coding regions of the genome, eQTLs of genes in the vicinity of SNPs associated with obesity and obesity-related traits could lead to the discovery of candidate genes with a functional impact of disease (77).

Identified genes from eQTL-analysis needs to be followed-up by functional analysis to link the gene with a genetic variant, and with a clinical trait.

3.3.5 Human mesenchymal stem cells

In Study III, hMSCs were differentiated in vitro and used to study the impact of knockdown of candidate genes on adipogenesis and adipocyte function. As well as the differentiation into mature adipocytes, hMSCs can be differentiated into myocytes, chondrocytes and osteocytes. Fibroblast growth factor 2 (FGF2) is added to the culture medium to stimulate proliferation followed by the induction of adipogenesis by adding rosiglitazone, insulin and T3.

hMSCs cell lines are advantageous to use due to their ability to differentiate into an adipocyte phenotype with lipolytic response, insulin sensitivity and the secretion of

adipokines (169, 170). The use of a cell line is also desirable when large number of cells are needed and when minor biological variation is desired.

3.4 STUDY IV

Study IV is based on two different cohorts which were recruited previously for studying the AT (142, 171). For the current study clinical parameters, AT phenotypes determined from adipose tissue biopsies, and gene expression analyzed by AT microarray, were analysed.

3.4.1 Study design

The main study cohort included 114 Swedish women (main study cohort) without diabetes which were recruited from the general adult population in the Stockholm (Sweden) area. Adipose tissue phenotypes (measures of lipolysis, adipose morphology, insulin sensitivity) were determined and associations with *TSHR* expression was analysed adjusted for age and BMI.

Gene expression in WAT was analyzed by Gene 1.0 or 1.1 ST Affymetrix arrays as described (172) and associations of *TSHR*-expression and the expression of genes involved in adipogenesis, lipolysis and insulin signalling were also analysed. The cohort was previously described in detail elsewhere (142).

In a second cohort of women with morbid obesity (n=16), *TSHR* expression was investigated before (obese group) and 2 years after bariatric surgery, when they had reached a non-obese state (post-obese group), and compared to matched never-obese controls (n=16). Abdominal subcutaneous AT biopsies were available before and after intervention for analysis of adipose tissue phenotypes and for microarray analysis.

Women were recruited in association of planned visits to surgical units for gastric bypass surgery because of obesity or through local advertisement for the purpose of studying adipose factors regulating body weight. The post-obese women had reached their lowest post-surgery weight and had been weight stable at this weight for >6 months. Their mean BMI was 16 kg m^{-2} lower as compared with before surgery. The controls had also been weight-stable for >6 months.

The local ethics committee approved the study, and written informed consent was obtained from all participants as described in Study III.

3.4.2 WAT experiments

The adipose tissue specimens (about 1 gram) was subjected to collagenase treatment to obtain isolated adipocytes as described (173). The mean weight and volume of these cells

were determined (172, 174), adipose tissue morphology, insulin-stimulated lipogenesis, spontaneous and hormone-stimulated lipolysis was assessed as described in section **3.3.1**. Spontaneous unstimulated lipolysis in fat pads was also determined in adipose tissue explants as described (175). In brief, pieces of adipose tissue (200 or 300 mg) were incubated for 2 h (100 mg/ml) at 37°C with air as the gas phase in Krebs–Ringer phosphate buffer (pH 7.4) supplemented with glucose (8.6 mmol/l), ascorbic acid (0.1 mg/ml) and bovine serum albumin (20 mg/ml). Glycerol release into the medium was measured using a sensitive bioluminescence method and expressed as amount of glycerol release per 2 hours and 10^7 adipocytes.

3.4.3 Statistical analysis

In the main study cohort, multiple linear regression analyses was performed to investigate associations between *TSHR* expression and clinical parameters (BMI and AT phenotypes) and the expression of adipocyte specific genes. The beta-coefficient, partial regression coefficient represents the predicted change in the dependent variable when the predictor is increased by one unit while other predictors are held constant. Note that beta-coefficients depend on whether the dependent variable was log10-transformed.

False discovery rate (FDR) <5% according to Benjamini and Hochberg (176) was used to adjust for the analysis of multiple parameters.

In the bariatric surgery sub-group, one-way ANOVA with the Bonferroni posthoc test was performed to assess differences in *TSHR* expression between groups. A p-value of <0.05 was considered significant.

4 RESULTS

4.1 STUDY 1

In Study I, we found that the TSH level in children with obesity was positively associated with BMI. We also found TSH level to be inversely associated with markers for insulin sensitivity and lipid metabolism (Table 4.1).

Table 4.1. Associations of TSH with clinical parameters

BMI SDS	0.21 (0.14–0.28)**
Fasting insulin (mU/L)	0.77 (0.27–1.26)*
Fasting glucose (mmol/L)	0.02 (-0.02–0.04)
HOMA-IR	0.19 (0.06–0.31)*
Total cholesterol (mmol/L)	0.05 (0.02–0.08)*
Triglycerides (mmol/L)	0.06 (0.04–0.09)**
LDL cholesterol (mmol/L)	0.01 (-0.02–0.04)
HDL cholesterol (mmol/L)	0.01 (-0.000–0.02)

Multivariable regression analysis of BMI SDS (independent variable) on TSH (dependent variable) adjusted for age and sex, and multivariable regression analysis of TSH (independent variable) on variables of lipid and glucose metabolism (dependent variable) controlling for age, sex and BMI SDS. Data is presented as beta coefficient (95% confidence interval), *p<0.05. **p<0.001.

When children with high-normal and low-normal TSH levels were compared, higher BMI SDS was found in the group with high-normal TSH levels (2.92 ± 0.45 vs 3.00 ± 0.78) with no significant differences in fT3 or fT4-levels.

4.2 STUDY II

In Study II, a transgenic mouse model, with reduced expression of *TSHR* in AT, was used. In the studied animals, *TSHR*-expression was reduced by 37% and 67% in WAT in mice fed a control and a high-fat diet, respectively. In BAT, TSHR-expression was reduced by 36% and 42%, respectively (Paper II, Figure 1).

We demonstrated that reduction of *TSHR* in WAT and BAT resulted in a greater weight gain in knockout mice than WT mice on both control diet and high-fat diet (Figure 4.1).

Figure 4.1. *Data are presented as mean weight per group and per week (grams). Error bars represent standard error of mean. Between-group comparisons using Independent t-test WT on control diet (CD) vs knockout on CD and WT on high-fat diet (HFD) vs knockout on HFD. *p<0.05, **p<0.01, ***p<0.001.*

The body temperature was reduced in knockout mice before start and at the beginning of the study (Figure 4.2).

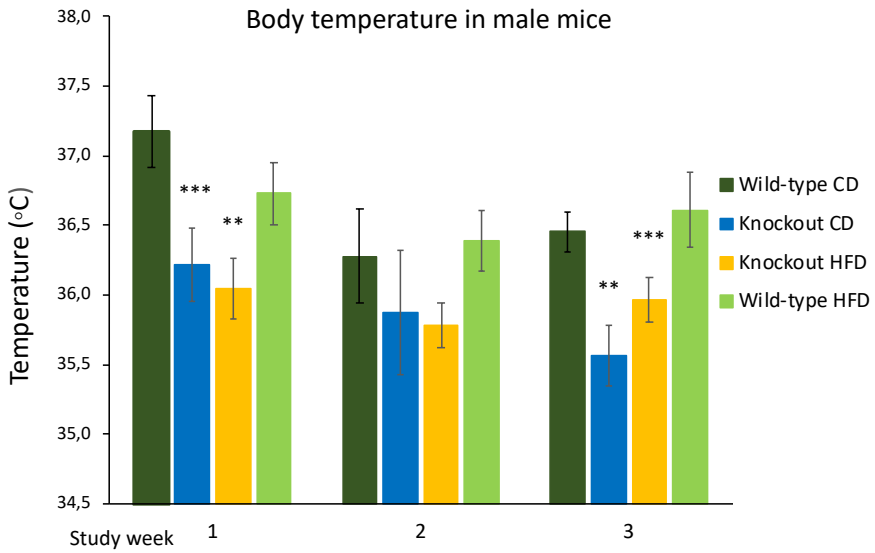


Figure 4.2. *Body temperature measured once weekly during weeks 1-3. Data are presented as mean temperature per group. Error bars represent 95% confidence intervals. Between group comparisons with one-way ANOVA. **p<0.01, ***p<0.001.*

The difference was not as clear at the end of the study period where a statistically significant difference was observed only at week 20 but not at week 18 and 19. We cannot rule out that temperatures could still be lower in KO mice. Due to limited power, statistical significance should be interpreted with care. The reduced expression of *UCP1* at the end of the study indicate that thermoregulation is affected by the *TSHR* knockout throughout the study period.

Glucose tolerance was assessed in female mice by an oral glucose tolerance test. Results showed that knockout mice on control diet had a reduced glucose tolerance compared to wild-type mice at follow-up but not at baseline (Figure 4.2a–b). Furthermore, gene expression of both insulin receptor substrate 1 (*IRS1*) and Solute carrier family 2 member 4 (*SLC2A4*), was down-regulated which are genes involved in the insulin signalling cascade and glucose transport, respectively (Paper II, Figure 6a).

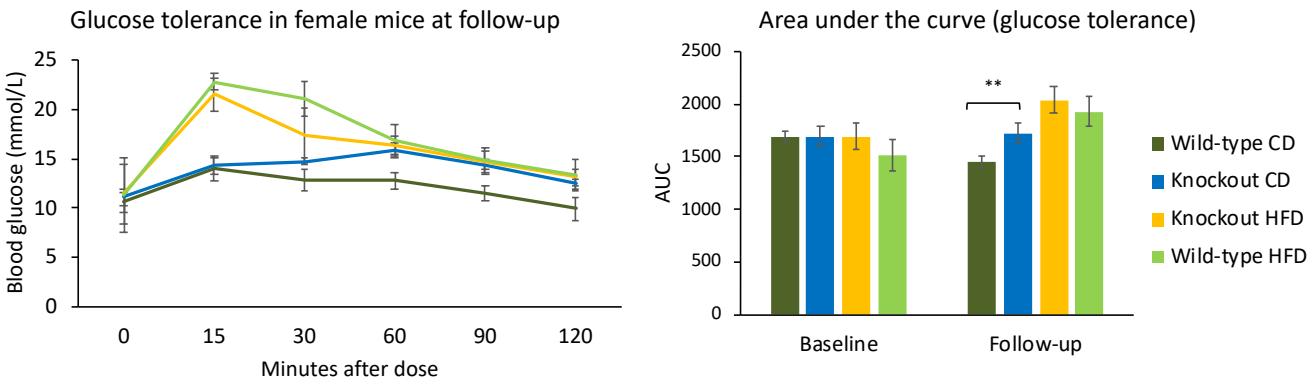


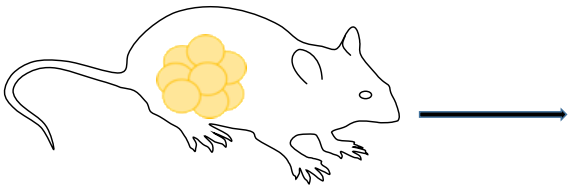
Figure 4.2a. *Glucose tolerance test at follow-up. Data is presented as blood glucose (mmol/L). Error bars represents 95% confidence intervals.* ***Figure 4.2b****. Data is presented as area under the curve at baseline and after 10 weeks on high-fat or control diet. Error bars represents 95% confidence intervals. Between group comparisons using independent sample t-test between WT CD vs KO CD and WT HFD vs KO HFD, respectively. *p<0.05, **p<0.01.*

An overview of the directions of gene expressions affected by the knockout is given in Figure 4.3. In white adipocytes, peroxisome proliferator-receptor gamma (*PPARG*) and CCAAT/enhancer-binding protein alpha (*CEBPA*), which are genes central to adipogenesis, were down-regulated in KO mice and in WT mice on high-fat diet as well, but significantly lower in KO mice on high-fat diet (Paper II, Figure 6a).

Adrenergic signalling, responsible for functions such as lipolysis, was affected in knockout mice. Gene expression of ß1-adrenergic receptor (*ADRB1*) and ß3-adrenergic receptor (*ADRB3*) was lower in knockout mice on the control diet and a similar reduction was observed in WT and KO mice on the high-fat diet (Paper II, Figure 6b).

Expressions levels of the investigated adipokines and cytokines in WAT of the *TSHR* KO mice, were heterogenous. The expression of adiponectin (*ADIPOQ*) was reduced in KO mice on CD and further reduced on HFD whereas leptin expression (*LEP*) was markedly reduced in HFD *TSHR* KO mice (Paper II, Figure 6c).

The pro-inflammatory markers chemokine ligand 2 (*CCL2*) and tumour necrosis factor alpha (*TNFα)* were both up-regulated in KO mice on both diets, but interleukin 6 (*IL-6)* was only affected in high-fat diet mice where it was markedly increased (Paper II, Figure 6c).



Adipose tissue-specific *TSHR* knockout

GENE NAME	KO on CD		KO on HFD		WT on HFD	
	WAT	BAT	WAT	BAT	WAT	BAT
PPARG	↓	↓	↓	n.s.	↓	↓
CEBPA	↓	n.s.	↓	↓	↓	↓
ADRB1	↓	n.s.	↓	n.s.	↓	n.s.
ADRB3	↓	n.s.	↓	↓	↓	↓
LIPE	n.s.		↓		↓	
FABP4	n.s.		↓		n.s	
SLC2A4	↓	n.s.	↓	↓	↓	↓
IRS1	↓		↓		↓	
IL-6	n.s.		↑		↑	
CCL2	↑		↑		↑	
TNF alpha	↑		↑		↑	
IGF1	↑		↑		↑	
UCP1		↓		↓		n.s.
PGC1-alpha		↑		n.s.		n.s.
ADIPOQ	↓	↑	↓	n.s.	↓	n.s.
LEP	n.s.		↓		n.s.	
DIO2		n.s.		n.s.		n.s.

Figure 4.3. *Effect of TSHR knockout in white adipose tissue.*
Effect on gene expression relative to wild-type mice on control diet. Arrows indicate direction of relative expression in intervention groups. Expressions of genes not investigated are left blank.

In brown adipocytes, as well as a reduced expression of *UCP1*, expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1a)* was upregulated when the *TSHR* was removed (Paper II, Figure 7a), as was the expression of *ADIPOQ* (Paper II, Figure 7b). Adipogenic genes, along with *ADRB3* and glucose transporter type 4 (*SLC2A4*) were down-regulated in a similar manner as in white adipocytes (Paper II, Figure 7a and b).

4.3 STUDY III

4.3.1 Fasting serum insulin and adipose phenotypes

The women in the clinical cohort displayed a wide variation in BMI and FSI (Paper III, Table 1), and as expected, BMI was positively correlated with FSI ($r^2 = 0.48$, $p = 2.0 \times 10^{-17}$). BMI was also positively correlated with adipose spontaneous lipolysis **(**$r^2 = 0.15$, $p = 0.0002$) and inversely correlated with insulin-stimulated lipogenesis ($r^2 = 0.08$; $p = 0.0038$). There was no significant association with adipose morphology. FSI was positively correlated with adipose morphology ($r^2 = 0.15$, $p = 2.0 \times 10^{-5}$) and spontaneous lipolysis ($r^2 = 0.23$, $p = 1.5 \times 10^{-6}$) but was negatively correlated with insulin-stimulated lipogenesis ($r^2 = 0.06$, $p = 0.0088$). Associations between FSI and morphology ($p = 1.3 \times 10^{-6}$) or spontaneous lipolysis ($p = 0.0019$) remained significant after adjusting for BMI.

From a GWAS meta-analysis, 17 genomic loci associated with fasting serum insulin (FSI) were identified. Out of 120 coding transcripts of genes within these 17 loci, 48 genes had expression levels associated with FSI, of which 11 remained significant after adjusting for BMI. To link the candidate genes for FSI to WAT function, we used an *in silico* method to determine whether tag-SNPs for each of the 17 loci comprised expression quantitative loci (eQTLs). According to the GTEx database, eight genes in four different loci demonstrated genotype specific expression (Table 4.2).

Table 4.2. SNPs from GWAS meta-analysis that comprise eQTLs

Locus from FSI GWAS			*eQTL from GTEx portal*		*Association with fasting serum insulin and adipose phenotypes (p-values)*			
Chrom	**SNP**	**Effect allele**	**Gene**	**P-value**	**FSI**	**Lipolysis**	**Insulin-stimulated lipogenesis**	**Morphology**
2	rs2972143	G	*IRS1*	$8.0x10^{-08}$	$2.8x10^{-05}$		$3.4x10^{-04}$	$3.3x10^{-02}$
4	rs3822072	A	*FAM13A*	$4.0x10^{-06}$	$5.6x10^{-12}$	$5.6x10^{-07}$	$1.6x10^{-02}$	$8.2x10^{-04}$
6	rs6912327	T	*UHRF1BP1*	$2.0x10^{-25}$				$1.1x10^{-02}$
		T	*SNRPC*	$3.3x10^{-10}$				
7	rs1167800	A	*STAG3L1*	$3.3x10^{-10}$				
		A	*TRIM73*	$5.2x10^{-08}$	$1.5x10^{-02}$			
		A	*POM121C*	$3.6x10^{-07}$	$2.0x10^{-02}$		$3.4x10^{-03}$	$2.6x10^{-02}$
		A	*PMS2P3*	$6.1x10^{-11}$		$8.0x10^{-03}$		$4.8x10^{-02}$

Genes comprising eQTLs according to GTEx portal were analysed by linear regression with clinical phenotypes as dependent variables in respective analysis: fasting serum insulin (FSI), basal lipolysis, hormone-stimulated lipolysis adjusted for study batch, and morphology, adjusted for age.

Four of the genes (*FAM13A, UHRF1BP1, SNRPC* and *POM121C*), were expressed in white adipocytes and was not previously described in connection to WAT function. They were taken forward for functional evaluation by siRNA knockdown in hMSCs. The cells were differentiated into adipocytes and the effect on adipocyte function was evaluated.
For FAM13A, knockdown resulted in increased expression of adipogenic regulators peroxisome proliferator-receptor gamma (*PPARG*) and CCAAT/enhancer-binding protein alpha (*CEBPA*), hormone sensitive lipase encoded by *LIPE* and glucose transporter type 4, encoded by *SLC2A4* (Paper III, Figure 3a). Glycerol levels in cell medium were also increased. *POM121C* knockdown resulted in reduced expression levels of all investigated genes (*PPARG, CEBPA, LIPE, SLC2A4)* (Paper III, Figure 3b) and a reduced glycerol release. Knockdown of *SNRPC* resulted in markedly reduced expression of *ADIPOQ*, encoding the adipokine adiponectin, and *SLC2A4*. Expression of *LIPE and CEBPA* were slightly reduced (Paper III, Figure 3c). *URF1BP1* knockdown resulted in an increase in a modest increase in expression of *PPARG* and *CEBPA*, early during the differentiation (day 7),

with no effect on Day 12 (Paper III, Figure 3d). The release of glycerol in medium was unaffected.

4.4 STUDY IV

In study IV, we report that the expression of *TSHR,* in subcutaneous abdominal AT, was inversely related to basal lipolysis but positively related to hormone-stimulated lipolysis, adjusted for BMI (Table 4.3).

Table 4.3. Association of TSHR gene expression and adipose tissue phenotypes

	STD B	***95% CI***	***P***	***R^2***
	LOG10 BASAL LIPOLYSIS			
TSHR	**-0.28**	**-0.04–(-0.01)**	**0.01A**	0.18
BMI (kg/m^2)	**0.28**	**0.01–0.02**	**0.003A**	
Age	**-0.16**	**-0.01–0.001**	**0.07**	
Study batch	**0.09**	**-0.04–0.15**	**0.34**	
	LOG10 ISOPRENALINE STIMULATED LIPOLYSIS			
TSHR	**0.27**	**0.00–0.03**	**0.01A**	0.12
BMI (kg/m^2)	**-0.18**	**-0.01–(0.00)**	**0.06**	
Age	**0.19**	**0.00–0.01**	**0.04**	
Study batch	**-0.08**	**-0.10–0.04**	**0.42**	

TSHR gene expression as independent variable, AT phenotypes as dependent variables in respective model. Models are adjusted for BMI, age and study batch. Results are presented as standardized beta coefficient (std B), 95% confidence interval (95% CI), p-value (P) and adjusted percentage of variance explained (R^2). [a] indicate a significant result after adjustment for a false discovery rate of 5% (FDR<5%).

In human subcutaneous WAT, *TSHR*-expression was associated with genes central to lipolysis and insulin-signalling (Table 4.4).

Table 4.4. Association analysis of TSHR expression in AT and adipocyte specific genes

	Std B	***95% CI***	***P***	***Adj R^2***
PGC-1A	0.20	0.19–1.32	0.009[a]	0.50
CEBPA	0.03	-1.34–2.22	0.63	0.73
LIPE	0.19	2.67–11.10	0.002[a]	0.69
ADRB1	0.16	-0.12–1.06	0.12	0.10
ADRB2	0.05	-0.23–0.41	0.57	0.24
ADRB3	0.23	0.19–2.45	0.022[a]	0.21
SLC2A4	0.21	0.79–4.08	0.004[a]	0.58
IRS1	0.10	0.75–2.72	0.001[a]	0.76
ADIPOQ	0.10	-1.54–31	0.075	0.21
LEP	-0.03	-9.3–6.2	0.69	0.44

Linear regression analysis of AT TSHR gene expression as independent variable, adipocyte-specific genes as dependent variables in respective model. Models are adjusted for BMI, age and study batch. Results are presented as standardized beta-coefficient (Std B), 95% confidence interval (95% CI), p-value (P) and adjusted percent of variance explained (Adj R^2).

[a] *False discovery rate (FDR) <5%.*

TSHR-expression was associated with the expression of adrenergic receptor *ADRB3* in a positive direction, but not *ADRB1* and *ADRB2.* No association between *TSHR*-expression and adipokines leptin and adiponectin was found.

Furthermore, in the second cohort which underwent bariatric surgery, we found *TSHR* expression to be affected by the weight-loss (Figure 4.4).

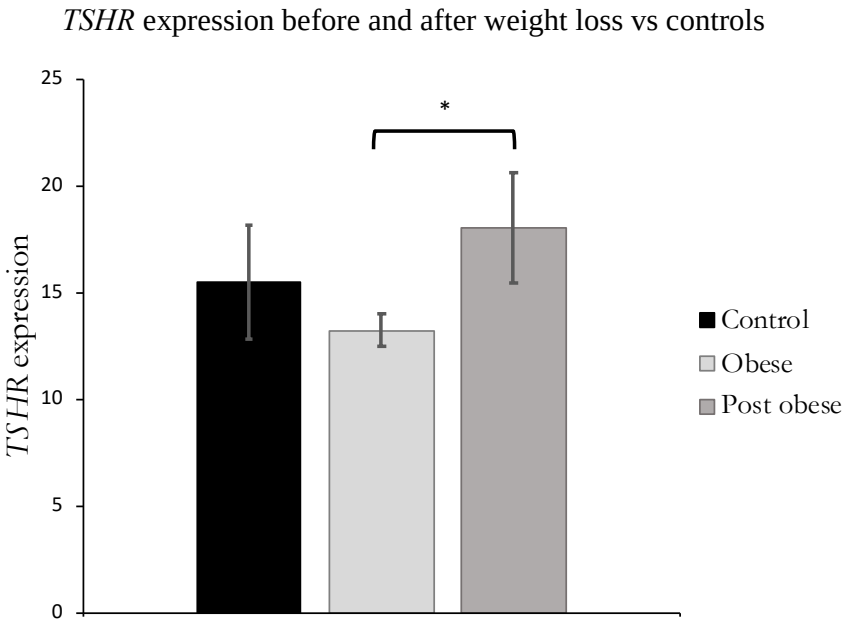


Figure 4.4. *Differences in mean TSHR expression between groups.*
One-way ANOVA with Bonferroni posthoc test between obese and post-obese groups.
Independent t-test between controls and obese and post-obese groups, respectively..
*Error bars represent 95% confidence intervals, *p <0.01.*

TSHR expression in AT was significantly upregulated after weight loss by gastric bypass surgery, (mean difference 4.8, 95% CI (0.89–8.73), p=0.007). No difference was observed in *TSHR* expression comparing the control group with the intervention group either pre- or post-surgery.

5 DISCUSSION

5.1 STUDIES I, II AND IV

In Study I, we found TSH-levels in children with obesity to be elevated and associated with risk markers for glucose metabolism and lipid profile. Further functional investigations in in study II, in our mouse model showed that partial removal of *TSHR* in AT led to modified expressions of genes central for adipocyte development and function. Our functional analysis thus supports that the associations of TSH-levels and metabolic risk markers, found in the cross-sectional cohort study may be of physiological relevance in humans. The causes of an elevated TSH in obesity, and underlying mechanisms are unclear. The elevation of TSH could be compensatory due to increased metabolism, or the disturbed production of thyroid hormones in the obese state, or due to obesity itself directly upregulating the release of TSH. It is possible that the increase in TSH, causes down-regulation of TSHRs in the AT causing a peripheral resistance, contributing to adipocyte dysfunction and predisposes to weight gain.

In Study II, we showed that TSH-TSHR signalling in adipocytes in mice is likely to have a role in body weight regulation mediated by the AT. We observed that animals partially lacking functional TSHRs in the AT were more susceptible to developing obesity than control mice. We also found that a reduced gene expression of *TSHR* in the AT predisposed the studied subjects to develop metabolic complications. Thus, the reduction of TSH-TSHR signalling in AT causes metabolic consequences, both via weight gain and via effects on adipose tissue gene expression, resulting in impaired adipocyte metabolism.
In a previous study, knocking down *TSHR* in mouse 3T3-L1 preadipocytes was shown to delay cell differentiation, implicating the TSHR in the regulation of this process (120). Elgadi et al. (2010) (125) showed that, in the same mice strain as used here, partially knocking out *TSHR* in AT resulted in a greater adipocyte volume also suggesting a potential role for TSHRs in adipogenesis (125).
The *TSHR* knockout mice displayed a decrease in expression of genes central to adipogenesis (*PPARG* and *CEBPA*) (39). The reduction in expression of both *PPARG* and *CEBPA* are in line with previous observations of a reduced adipogenesis when TSHRs are reduced (120, 121), strengthening the evidence of TSHRs having a regulatory role in adipogenesis.

The relationship between TSH and IR is currently unclear. Some studies in adults and children have found TSH-levels to be associated with markers for IR (177-179), whereas other studies reported a lack of association (180, 181).
In our *TSHR* knockout model, glucose tolerance was in fact affected. We report that in older animals with reduced expression of *TSHR*, in conjunction with an oral glucose tolerance test, the expression of genes relevant for insulin signalling and glucose uptake were down-regulated. These findings may indicate a reduced insulin sensitivity in adipocytes with down-regulated *TSHR* expression.
We did not find any difference in glucose tolerance between wild-type and knockout obese mice. Both mice strains on the high-fat diet displayed an IR phenotype which may be due to the fact that both groups were obese and obesity itself is a strong predictor of IR.

In study IV, we set out to investigate how our findings translates to adult humans. We investigated if *TSHR*-expression in WAT was related to BMI, adipose phenotypes and relevant adipocyte-specific genes. Firstly, *TSHR*-expression was inversely correlated with BMI and was down-regulated in AT, in the obese state, which we observed in both human and rodent adipocytes. In humans, *TSHR*-expression was up-regulated after weight loss. Weight-loss was not investigated in our mouse model but it is possible that the same up-regulation could take place in rodent AT, although this would require further studies.
It has previously been shown that the lipolytic effect of TSH in adult humans is minimal (59). The present data indicate that the effects of TSH on adipocyte function and regulation may be of importance in adults.

5.1.1 *TSHR* in human white adipose tissue

In human WAT, the expression of *TSHR* was investigated in relation to adipose phenotypes. A positive association was found between hormone-stimulated lipolysis and *TSHR*-expression, and basal lipolysis was inversely associated with *TSHR*-expression. A high basal and low stimulated lipolysis is reported to predict weight-gain (55).
If *TSHR*-expression is involved in the regulation of lipolysis and a higher *TSHR* expression is involved in the regulation of basal lipolysis, the weight gain in the *TSHR* KO mouse strain could be connected to the effects on lipolysis.
Our findings in the knockout mice together with our findings in human WAT, indicate TSHRs to play a role in the regulation of lipolysis. despite that the direct lipolytic effect is negligible after the neonatal period.

This suggestion is further strengthened by the observation of *TSHR* expression in human WAT is correlated with the expressions *LIPE* encoding the lipolytic enzyme hormone sensitive lipase, independent of BMI.

Partially lacking functional TSHRs may contribute to difficulties in coping with overfeeding due to impaired adipogenesis and altered lipolytic regulation. Down-regulated TSHRs could contribute to lipid retention and AT expansion. An impaired adipogenesis could also explain the reduced insulin sensitivity observed.

Regarding inflammation, the *TSHR* knockout in mice resulted in an increased expression of pro-inflammatory cytokines. In obese mice, the expression of these cytokines was even more increased both in wild-type and knockouts. In the lean knockout mice, the increased expression of pro-inflammatory markers could be due to the increased cell size reported by Elgadi et al. (2010) (125). The pro-inflammatory response may suggest that subjects with reduced TSH-TSHR signalling in AT, could have a reduced ability to cope with excessive expansion of the AT. This in turn leads to an increased inflammatory response, predisposing them to metabolic complications mediated by the chronic low-grade inflammation observed in obesity.

Taken together, our findings support the notion that TSHRs in AT, possibly by their regulatory role in adipogenesis, may have an impact on lipolysis and insulin sensitivity in humans and mice.

5.1.2 *TSHR* in rodent brown adipose tissue

An inverse correlation between the amount of brown adipose tissue, body mass index and percentage body fat has been reported (182). BAT activation is also suggested to increase energy expenditure in humans (31). In our mouse model, *TSHR* knockout resulted in a reduced body temperature compared to control mice, which is most likely is due to the observed reduction in *UCP1*-expression.

PGC-1a is involved in mitochondrial biogenesis and is important for the thermogenic activity in BAT (183). Its expression in knockout mice was up-regulated which may be compensatory for the reduced *UCP1*-expression observed.

Our results show that in our knockout model, a reduction of *TSHR*-expression in BAT, does in fact reduce body temperature and possibly also energy expenditure. This could explain the increased body weight gain observed. The relevance of BAT in humans was not addressed in this thesis and more research is needed to clarify what impact TSHRs have in human BAT.

5.1.3 Study III

Given that obesity is a complex disease and the related metabolic complications are clearly caused by many different factors, we set out to find genes involves in the development of insulin resistance.

In Study III, we found two candidate genes that could be linked to adipocyte dysfunction. We report that the expression of *FAM13A* was associated with a beneficial metabolic profile. *FAM13A*-expression was associated with decreased WAT lipolysis, and knockdown of *FAM13A* resulted in increased lipolysis and expression of *LIPE*, which encodes hormone sensitive lipase, a rate-limiting enzyme in lipolysis (184). Previously, it has been shown that basal lipolytic activity is a strong determination of insulin sensitivity (175). The reduced expression of genes central to adipogenesis and adipocyte function, implicate *FAM13A* as having a regulatory role in adipogenesis. The underlying molecular mechanisms linking *FAM13A* to lipolysis are unknown and beyond the scope of this investigation. However, a recent study reported *FAM13A* to be reduced in adipose tissue of obese mice and to be involved in the insulin signalling pathway by inhibition of IRS1 degradation, thereby promoting the intra-cellular insulin response (185). Insulin has an inhibitory effect on lipolysis in adipocytes. The regulatory role of *FAM13A* on enhancing the insulin signal in the cell could therefore promote the inhibition of lipolysis.

The expression of *POM121C* is positively associated with insulin sensitivity and adipose hyperplasia. Given that adipose hypertrophy previously has been linked to impaired adipogenesis and IR (48), our findings are consistent with an insulin-sensitising function of *POM121C*. Knockdown early in the adipogenesis caused decreased expression of all adipocyte-specific markers suggesting that *POM121C* is necessary for adipogenesis. The reduction of gene expression of markers for adipocyte function, and the reduction of glycerol, is probably secondary to an impaired adipogenesis. *POM121C* encodes a nucleoporin and forms an important component of the nuclear pore complexes (186). It has not, to the best of our knowledge, previously been implicated in human metabolic disease.

Functional analysis of *SNRPC* and *UHRF1BP1* did not suggest that these genes underlie genetic control of FSI. For *SNRPC*, the FSI-associated allele was associated with higher *SNPRPC* expression, suggesting that *SNRPC* contributes to metabolic disease. However, the results from *SNPRPC* knockdown were consistent with an insulin-sensitising function of *SNRPC*. Knockdown of *UHRF1BP1* had marginal effects on markers for adipogenesis and adipocyte function, suggesting that *UHRF1BP* is not a

causative gene for FSI. Of note, expression of neither *UHRF1BP* nor *SNPRPC* was associated with WAT variables with FDR <5%.

5.2 CONCLUDING SUMMARY

Obesity is a complex disease with underlying causes ranging from environmental to genetic factors. Prevention and treatment thus require a multidisciplinary approach and research to elucidate the underlying biological pathways is necessary.

In this thesis, TSH-TSHR-signalling in AT was investigated. Given that TSHRs have been shown to be reduced in AT in the obese state, our results suggest that TSH-TSHR signalling is required for a functional expansion of AT and, when impaired, may contribute to a blunted obesity-related insulin sensitivity. Furthermore, we report that *TSHR*-expression is associated with a decreased basal lipolytic capacity and an increased hormone-stimulated lipolysis. Changes in lipolytic capacity is a result of dysfunctional adipocytes and may contribute to metabolic complications such as IR in peripheral tissues (48, 187).

We also searched for candidate genes for fasting insulin, and we report that *FAM13A* and *POM121C* are candidate genes for fasting insulin and show them to have a potentially protective role in IR by inhibiting lipolysis and promoting adipogenesis, respectively. These candidate genes could thus be potential treatment targets for insulin resistance.

In this thesis, factors that may contribute to adipocyte dysfunction in obesity were investigated. The herein investigated targets, *TSHR*-signalling and the newly identified genes for fasting insulin, may be important contributors in the multifactorial development of obesity and the related metabolic risk.

5.3 FUTURE PERSPECTIVES

The adipose tissue is obviously more than just an energy storing organ. In obesity, the physiological changes that occurs, as well as the underlying genetic control of metabolism, are important factors to consider when trying to elucidate the cause and consequences of obesity and the metabolic complications that follow.

Several factors contribute to metabolic risk in obesity. Two possible contributing factors to adipocyte dysfunction are hormonal signalling and genetic variance. Associations of TSH and obesity as well as obesity-related metabolic disease are well documented although the functional impact of TSH leading to these risks is not clarified. Further research targeting TSHR impact in the adipocyte is necessary in order to fully understand these associations.

The brown adipose tissue is considered a potential target for obesity treatment due to its energy consuming function, but few studies have investigated the functional impact of BAT in obesity treatment. Studies trying to recruit and activate brown adipocytes and triggering the activation of brown adipocyte markers in WAT, so called browning, has been reported but much research is still needed before implementation of such treatment methods in human obesity.

Genetic variants associated with metabolic traits in the obese state seem to act by causing adipocyte dysfunction and IR. IR is an essential factor contributing to obesity-related metabolic disease. Elucidating the functional impact of underlying genes to complex disease is an important part in understanding the mechanisms behind obesity and related comorbidities. These kind of genetic studies increases the potential of finding genetically supported therapeutic targets in obesity and IR.

Current treatment methods for obesity focus on weight-loss and life-style changes, mostly with unsatisfactory results. The most effective treatment today is bariatric surgery. However, considering that bariatric surgery is an invasive method carrying the risk of complications, and also that most individuals with obesity does not meet the criteria for surgery, new treatment strategies are needed (188, 189). Perhaps focusing on the energy metabolism would provide a good complementary strategy.

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9 PAPERS I-IV