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# Novel mechanisms of glucocorticoid-induced insulin resistance in human adipose tissue

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#### **Abstract**

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The global prevalence of obesity and type 2 diabetes (T2D) is increasing. From a public health perspective, it is therefore of interest to identify common underlying mechanisms of these comorbidities. Glucocorticoids are steroid hormones that are important in stress regulation in mammals. Elevated glucocorticoid levels are associated with insulin resistance (IR) and T2D-like phenotypes. Here, glucocorticoids are used to model a state of IR in human adipose tissue to identify potential pharmacological targets.

In *Paper I* the impact of T2D on lipid turnover was examined in a cohort of 20 T2D subjects and 20 healthy controls. Plasma levels of non-esterified fatty acids (NEFA) were shown to be elevated in T2D subjects during oral glucose tolerance test (OGTT) compared to healthy controls. *In vitro* lipolysis and assessments of mRNA and metabolites in subcutaneous adipose tissue (SAT) were performed. Results showed that elevated NEFA levels in T2D subjects could be attributed to impaired lipid storage.

In *Paper II* we explored the role of cannabinoid receptor type 1 (CNR1) in glucocorticoid-induced IR. The *CNR1* gene was upregulated after exposure to glucocorticoids in SAT. Moreover, *CNR1* gene expression in SAT was associated with markers of IR and elevated in T2D subjects compared to healthy controls. Furthermore, using a CNR1-specific antagonist, we found that CNR1 may mediate lipolysis in SAT.

In *Paper III-IV*, we examined the role of FK506 protein 5 (FKBP51) in glucocorticoid-induced IR. Its corresponding gene, *FKBP5*, was found to be upregulated in SAT and omental adipose tissue (OAT) following glucocorticoid-exposure. In addition, *FKBP5* gene expression in SAT was associated with markers of IR and tended to be elevated in T2D subjects compared to healthy controls. Furthermore, co-incubating an FKBP51-specific inhibitor with glucocorticoids in SAT partly prevented glucocorticoid-impaired adipocyte glucose uptake.

We identified CNR1 and FKBP51 as potential pharmacological targets in T2D and glucocorticoid-induced IR. Both were shown to be elevated in human adipose tissue after glucocorticoid-exposure. Their SAT gene expression levels were also associated with markers of IR and tended to be elevated in T2D. Both may be involved in perturbations of adipocyte metabolism, including glucose and lipid metabolism.

**Keywords:** cannabinoid receptor type 1 fkbp51 dexamethasone type 2 diabetes adipocytes

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*To my Family & To my Friends*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I** Pereira MJ, Skrtic S, Katsogiannos P, Abrahamsson N, **Sidibeh CO**, Dahgam S, Månsson M, Risérus U, Kullberg J, Eriksson JW. Impaired adipose tissue lipid storage, but not altered lipolysis, contributes to elevated levels of NEFA in type 2 diabetes. Degree of hyperglycemia and adiposity are important factors. *Metabolism*. 2016 Dec;65(12):1768-1780. doi: 10.1016/j.metabol.2016.09.008.
- II** **Sidibeh CO**, Pereira MJ, Lau Börjesson J, Kamble PG, Skrtic S, Katsogiannos P, Sundbom M, Svensson MK, Eriksson JW. *Role of cannabinoid receptor 1 in human adipose tissue for lipolysis regulation and insulin resistance*. *Endocrine*. 2017 Mar;55(3):839-852. doi: 10.1007/s12020-016-1172-6.
- III** Pereira MJ, Palming J, Svensson MK, Rizell M, Dalenback J, Hammar M, Fall T, **Sidibeh CO**, Svensson PA, Eriksson JW. *FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance*. *Metabolism*. 2014 Sep;63(9):1198-208. doi: 10.1016/j.metabol.2014.05.015.
- IV** **Sidibeh CO**, Pereira MJ, Boersma G, Castillejo-Lopez C, Abalo XM, Skrtic S, Lundkvist P, Katsogiannos P, Hausch F, Eriksson JW. *FKBP5 expression in human adipose tissue: Potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes*. Manuscript.

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# Abbreviations

2-AG	2-Arachidonoylglycerol
11 $\beta$ -HSD	11 $\beta$ -hydroxysteroid dehydrogenase
AC	Adenylyl cyclase
ACEA	Arachidonyl-2'-chloroethylamide
ACTH	Adrenocorticotropic hormone
AEA	Anandamide
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl- <i>N</i> -4-morpholinyl-1 <i>H</i> -pyrazole-3-carboxamide
AS160	Akt substrate of 160 kDa
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CGI-58	Comparative Gene Identification-58
CNR1	Cannabinoid receptor type 1
CNR2	Cannabinoid receptor type 2
CRF	Corticotropin-releasing factor
DAGL	Diacylglycerol lipase
DG	Diglyceride
ECS	Endocannabinoid system
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
FBS	Fetal Bovine Serum
FKBP5	FK506 binding protein 5
FKBP52	FK506 binding protein 4
GEO	Gene Expression Omnibus
GLUT4	Glucose transporter type 4
HPA axis	Hypothalamic-pituitary-adrenal axis
HSL	Hormone-sensitive lipase
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
KRH	Krebs-Ringer media

MAPK	Mitogen-activated protein kinase
MG	Monoglyceride
MGL	Monoacylglycerol lipase
NEFA	Non-esterified fatty acids
OAT	Omental adipose tissue
OGTT	Oral glucose tolerance test
PDE3B	Phosphodiesterase
PEST	Penicillin-streptomycin
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
SAT	Subcutaneous adipose tissue
SVF	Stromal vascular fraction
SNP	Single Nucleotide Polymorphism
T2D	Type 2 diabetes
TG	Triglyceride
ECS	Endocannabinoid System

# Introduction

## Obesity and Type 2 Diabetes

The World Health Organization recently reported that in 2014, the prevalence of obesity was such that, out of 1.9 billion overweight adults, 600 million are considered obese (1). This number has more than doubled since 1980 and obesity is associated with increased morbidity, mortality and several comorbidities. These include hypertension, dyslipidaemia and the onset of type 2 diabetes (T2D). The T2D onset usually stems from a state of obesity and pancreatic beta cell dysfunction that contributes to insulin resistance (2). With the increasing prevalence of obesity and T2D, it is of interest for public health to explore the underlying causes and mechanisms responsible for obesity and insulin resistance due to their shared association with T2D onset.

## Adipose tissue

This thesis encompasses white adipose tissue, unless stated otherwise.

Adipose tissue plays a pivotal role in both obesity and insulin resistance. Obesity goes hand in hand with an increase of adipose tissue mass, where adipose tissue serves as the principal organ for the long-term storage of energy (3). However, the role of adipose tissue extends beyond simply being a passive storage depot of excess energy. Indeed, adipose tissue is crucial in terms of systemic metabolic control where it is a master regulator of energy homeostasis (3,4). Adipose tissue secretes signal proteins known as adipokines. This links adipose tissue to several other peripheral and central organs and tissues such as skeletal muscle, liver, kidneys and brain in complex cross-talks (5). The adipokines consist of both pro- and anti-inflammatory factors and are thus important also in the regulation of the immune system (6).

The discovery of adipokines such as leptin and adiponectin are considered significant milestones in the study of adipose tissue, adipocyte biology and energy homeostasis. This is because of their shown associations with obesity and insulin sensitivity (4,7). Leptin, also known as the satiety hormone, can interact with receptors in the hypothalamus and the periphery. As such, it is capable of mediating processes such as satiety, food intake and energy ex-

penditure (4). Adiponectin has previously been shown to have protective roles pertaining to insulin sensitivity, inflammation, apoptosis and the cardiovascular system (4,8). Early studies have demonstrated that the *in vivo* administration of adiponectin in mice improves hepatic insulin sensitivity (9).

Throughout the body, adipose tissues are localized in specific depots. The most well-studied is the subcutaneous adipose tissue (SAT) depot, which is also the most abundant (10); and the intraabdominal omental adipose tissue (OAT) (11). OAT is part of the visceral adipose tissues, which include the mesenteric and epiploic adipose tissues (10). OAT is more metabolically active than SAT and is in close proximity to vital organs, with direct access to the liver through the hepatic portal vein (11,12).

Adipose tissue is composed of numerous cell types, including adipocytes, non-adipocyte cells including endothelial cells, pre-adipocytes and various types of immune cells including macrophages (5,13). Taken together, the non-adipocyte cells are referred to as the stromal vascular fraction (SVF).

In states of obesity or T2D, the balance of adipokine secretion from adipose tissue becomes dysfunctional. This leads to adverse effects to the adipokine secretion profile in which there is an upregulation of pro-inflammatory cytokines such as tumour necrosis factor alpha, interleukin 6, visfatin, leptin, interleukin 8 and macrophage migration inhibitory factor (13-15). The upregulation of pro-inflammatory cytokines in adipose tissue has been attributed to adipose tissue macrophages and their accumulation and proliferation during obesity (16,17). Anti-inflammatory cytokines such as interleukin 10 and adiponectin, on the other hand, are downregulated (18,19). These are events that, together with a malfunctioning processing of excess energy via adipose tissue expansion through hypertrophy (cell size increase) and hyperplasia (cell number increase) (20), contribute to whole-body insulin resistance.

## Adipocyte metabolism

### Glucose uptake

Glucose serves as the primary source of energy in mammalian cells (21). Its uptake into cells is facilitated by glucose transporter proteins, of which glucose transporter type 4 (GLUT4) features prominently in the most metabolically active and insulin-sensitive tissues such as skeletal muscle and adipose tissue (21,22). GLUT4, stored in intracellular storage vesicles, is translocated to the plasma membrane of cells. The GLUT4 translocation is part of a cascade of reactions initiated by the binding of insulin to the extracellular portion of the plasma membrane-bound insulin receptor (22,23) (Figure 1). The binding of insulin to the insulin receptor leads to the phosphorylation of tyrosine kinase at the intracellular portion of the insulin receptor. The acti-

vated tyrosine kinase phosphorylates insulin receptor substrate-1 which subsequently activates phosphatidylinositol-3-kinase (PI3K). Activated PI3K proceeds to activate protein kinase B (PKB, also known as Akt) and atypical protein kinase C, both of which are involved in GLUT4 vesicle translocation (22,23) (Figure 1). The act of translocation involves the docking and subsequent fusion of the GLUT4 vesicle with the plasma membrane. In this configuration, GLUT4 is exposed to the extracellular environment, allowing for the uptake of circulating glucose into the cell. The GLUT4 storage vesicles are later internalized (22). Absorbed glucose undergoes glycolysis to produce the intermediate molecule acetyl-CoA. Acetyl-CoA is then used to generate energy in the form of adenosine triphosphate via the citric acid cycle. Alternatively, glucose undergoes conversion into fatty acids for energy storage; a process known as *de novo* lipogenesis (24).

## Lipid storage

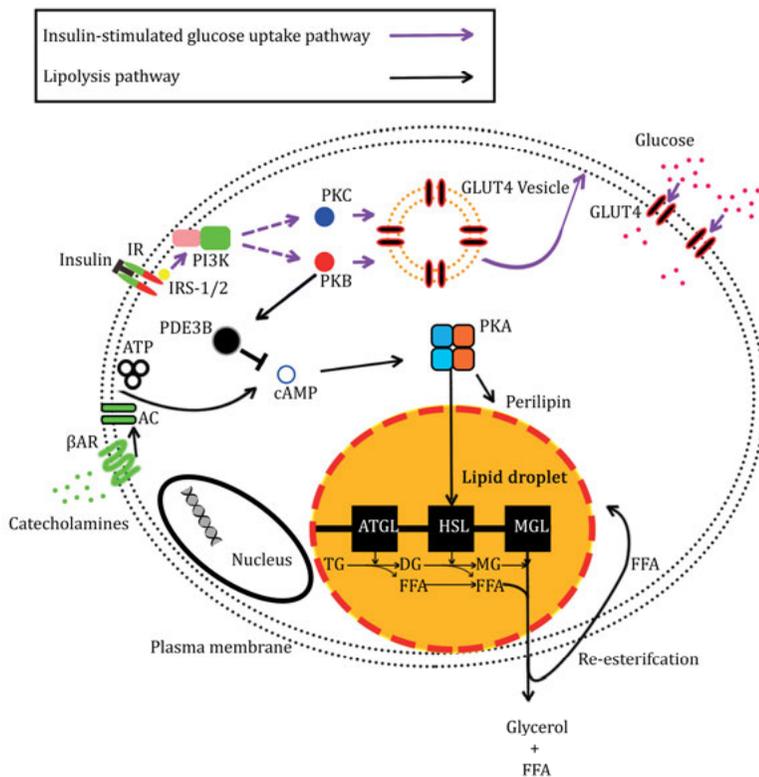
Lipogenesis or *de novo* lipogenesis is the esterification of fatty acids together with glycerol to produce triglycerides (TG) in the form of a lipid droplet. TG is the major form of fat stored by adipocytes (25). Lipogenesis is distinguishable from *de novo* lipogenesis in the principal source of substrates. While lipogenesis uses existing lipids or fatty acids (25,26), *de novo* lipogenesis consumes carbohydrates such as glucose (25,26).

In lipogenesis, fatty acids can be taken up as free fatty acids from the circulation where they are bound to albumin (26). Alternatively, free fatty acids can be obtained from the enzymatic breakdown of TGs that are carried by lipoproteins such as chylomicrons or very low-density lipoproteins (26). The TGs in these carrier proteins need to be broken down by lipoprotein lipase to release free fatty acids (26). There are several transporter proteins, such as fatty acid binding protein and CD36, that facilitate the localization of fatty acids from cell membranes to sites in the cytosol where the enzyme acyl-CoA synthase is active (26). Acyl-CoA synthase in turn esterifies fatty acids with CoA, forming acyl-CoA. Acyl-CoA is then esterified with glycerol-3-phosphate to form TG in a process that, in sequence, is facilitated by glycerol-3-phosphate, acylglycerol-3-phosphate acyltransferases, phosphohydrolase and diacylglycerol acyltransferase (25,26).

Where lipogenesis promotes the storage of excess available energy (derived from foods in the form of glucose or free fatty acids) in the form of TG; lipolysis is the process by which TG is broken down into free fatty acids and glycerol (13) (Figure 1). Naturally, because insulin promotes energy storage, it stimulates glucose uptake and inhibits lipolysis (27,28).

The primary initiators of lipolysis are the beta-adrenergic receptors and the enzyme adenylyl cyclase (AC). AC is coupled to beta-adrenergic receptors that are expressed on the cell surface. Agonism of beta-adrenergic receptors, e.g. with catecholamines, leads to the activation of AC via signal-

transducing G proteins (Figure 2) (29). AC's main function is to facilitate the conversion of adenosine triphosphate to cyclic adenosine monophosphate (cAMP). cAMP subsequently activates protein kinase A (PKA, also known as cAMP-dependent protein kinase). PKA is a kinase with multiple targets of phosphorylation. These include phosphodiesterase (PDE3B), hormone sensitive lipase (HSL), the lipid droplet-coating protein perilipin as well as the adipose triglyceride lipase (ATGL)-recruiter and co-activator comparative gene identification-58 (CGI-58) (30-32). The main role of PDE3B in this setting is negative feedback control by the hydrolysis of cAMP to AMP (33,34). This in turn reduces or terminates the activity of PKA.



*Figure 1.* Simplified illustration of insulin action on the adipocyte insulin-stimulated glucose uptake pathway (purple arrows) and the lipolysis pathway (black arrows). Adenylyl cyclase (AC), adipose triglyceride lipase (ATGL), adenosine triphosphate (ATP), beta adrenergic receptor (βAR), cyclic adenosine monophosphate (cAMP), diglyceride (DG), free fatty acid (FFA), glucose transporter type 4 (GLUT4), hormone sensitive lipase (HSL), insulin receptor (IR), insulin receptor substrate-1/2 (IRS-1/2), monoglyceride (MG), protein kinase A, B, C (PKA, PKB, PKC), phosphatidylinositol-3-kinase (PI3K), phosphodiesterase (PDE3B).

Accessibility of the enzymes to their lipid substrates in the lipid droplet is determined by the lipid droplet-associated protein perilipin. Perilipin coats the lipid droplet and functions as a protective barrier, restricting lipases and preventing basal lipolysis (35). Perilipin also prevents lipid-droplet-bound CGI-58 from interacting with ATGL. ATGL is partly activated by AMP-activated kinase but requires CGI-58 for full activation (32).

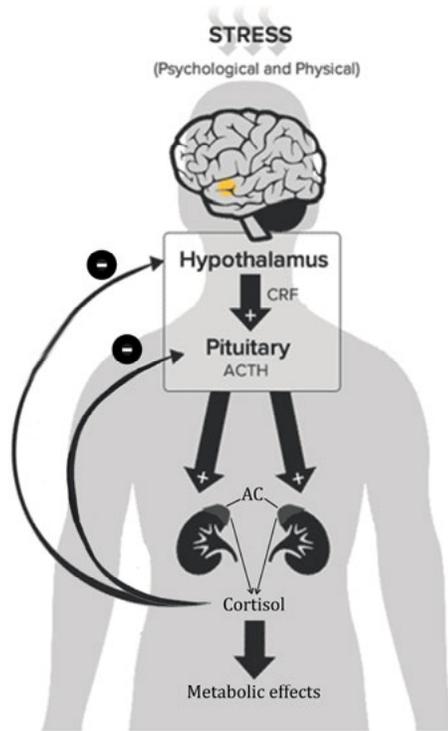
Upon activation of perilipin and CGI-58 by PKA; the TG droplet scaffold is disrupted. ATGL is then recruited and activated by CGI-58 whilst PKA-activated HSL is recruited to the lipid droplet via interaction with activated perilipin (30,32). The lipases ATGL, HSL as well as monoacylglycerol lipase (MGL), then work in a sequence (Figure 1) of catalytic reactions that produce free fatty acids and glycerol. The catalysis of TG is principally performed by ATGL and generates a free fatty acid and a diglyceride (DG). HSL continues to catalyze the hydrolysis of DG into a free fatty acid and monoglyceride (MG). In what is then the final step of lipolysis, MGL hydrolyses MG into a free fatty acid and a glycerol (Figure 1). The free fatty acids released into circulation may then be re-esterified for lipogenesis or be used as a source of energy by other tissues via beta oxidation (36).

## Glucocorticoids and insulin resistance

Glucocorticoids are a family of steroid hormones that have a plethora of different physiological effects related to the immune system, metabolism, homeostasis and stress regulation. In an immediate stress-response, the role of glucocorticoids is to maintain homeostasis by attenuating the response of normal defensive mechanisms - i.e. inflammation, immune system (37). The human endogenous glucocorticoid, cortisol, is the end-point hormone of the hypothalamic-pituitary-adrenal axis (HPA axis) (Figure 2). The HPA axis is a complex feedback system that is activated primarily in response to stress. It regulates homeostatic systems such as the metabolic system, the cardiovascular system, the immune system, the reproductive system as well as the central nervous system (37,38).

Upon exposure to stress, corticotropin-releasing factor (CRF) is synthesized and secreted by neurons in the paraventricular nerve of the hypothalamus. CRF is released into vessels that access the anterior pituitary gland (38). When CRF binds to receptors on corticotropic cells in the anterior pituitary gland, the subsequent release of adrenocorticotrophic hormone (ACTH) into the systemic circulation is triggered. Circulating ACTH stimulates the synthesis of cortisol in the adrenal glands that are situated on top of the kidneys (Figure 2) (38). The production of cortisol in the adrenal cortex is facilitated by 11 $\beta$ -hydroxysteroid dehydrogenase type 1 and type 2 (11 $\beta$ -HSD). These enzymes are responsible for the conversion of inactive cortisone to active cortisol (11 $\beta$ -HSD type 1) or the reverse reaction (11 $\beta$ -HSD type 2)

(39). High levels of 11 $\beta$ -HSD type 1 have been implicated in obesity, T2D and insulin resistance in adipose tissue (40-42); adding further support for the role of cortisol in T2D and related comorbidities.



*Figure 2.* The hypothalamic-pituitary-adrenal axis. Image modified from Integrative Therapeutics (43). Adrenal cortex (AC), adrenocorticotropic hormone (ACTH), corticotropin-releasing factor (CRF).

A normal range of circulating cortisol is considered to be 0.2-0.6  $\mu\text{M}$ , as measured in the morning (44). A maximum physiological level of cortisol is in the range of 1-2  $\mu\text{M}$ , during stress (45). Dysfunctional regulation of the HPA-axis can lead to Cushing's syndrome which is the manifestation of symptoms associated with excess exposure to circulating levels of glucocorticoids (46). The underlying causes could be exogenous pharmacological administration of glucocorticoids or from an endogenous source of cortisol (46,47). The pharmacological use of glucocorticoids is rooted in their anti-inflammatory and immunosuppressive properties. The latter is especially utilised in cases of transplantation surgery as a preventive measure of graft rejection (48,49).

Elevated glucocorticoid levels, such as in Cushing's syndrome or from prolonged pharmacological use, is associated with adverse effects such as dyslipidaemia, increased obesity, increased insulin resistance and the onset

of T2D (48). Therefore, subjects suffering from elevated plasma glucocorticoid levels share several characteristics with T2D subjects.

## Glucocorticoid effects on adipocyte metabolism

It has been suggested that a major net effect of glucocorticoids is to maintain glucose homeostasis by increasing glucose plasma levels. This is in an effort to promote maximal brain function (50). Brain activity and functionality is of particular importance during stress (51). A mechanism of action to raise or maintain systemic glucose levels is then by stunting systemic insulin action.

It is well-established that glucocorticoids can directly interfere with the insulin action in human adipocytes by imposing a state of insulin resistance (52). This is evident when assessing the glucose uptake capacity of isolated adipocytes that have been exposed to glucocorticoids (53). The exact mechanism by which this occurs remains elusive. It has been proposed that in both human and murine adipocytes, glucocorticoids downregulate the phosphorylation of AS160 (Akt substrate of 160 kDa) (54). AS160 is a substrate of PKB/Akt that is a regulator of GLUT4 translocation (54,55). Furthermore, glucocorticoids have previously been shown to reduce the glucose uptake capacity and expression levels of IRS-1 and PKB/Akt in human adipocytes derived from OAT but not from SAT (53).

Glucocorticoids increase adiposity by promoting the differentiation of human adipocytes through interaction with the glucocorticoid receptor (GR) (56). Glucocorticoids also contribute to increasing central adiposity by the redistribution of peripheral adipose tissue depots to central adipose tissue depots (57) – a higher abundance of GRs in OAT than in SAT is a possible contributor (58).

Although variable results have been reported (56), most studies report that glucocorticoids stimulate lipolysis (56). Stimulation of lipolysis and the subsequent release of free fatty acids have been proposed as additional mechanisms by which glucocorticoids impair glucose uptake (50,59). Similar to the effects of glucocorticoids on glucose uptake, their means of regulating lipolysis are poorly understood. However, a direct effect has been proposed by the glucocorticoid treatment of adipocytes from rats (60). The downregulation of both mRNA and protein levels of PDE3B, as well as the upregulation of mRNA and protein levels of HSL and ATGL were demonstrated.

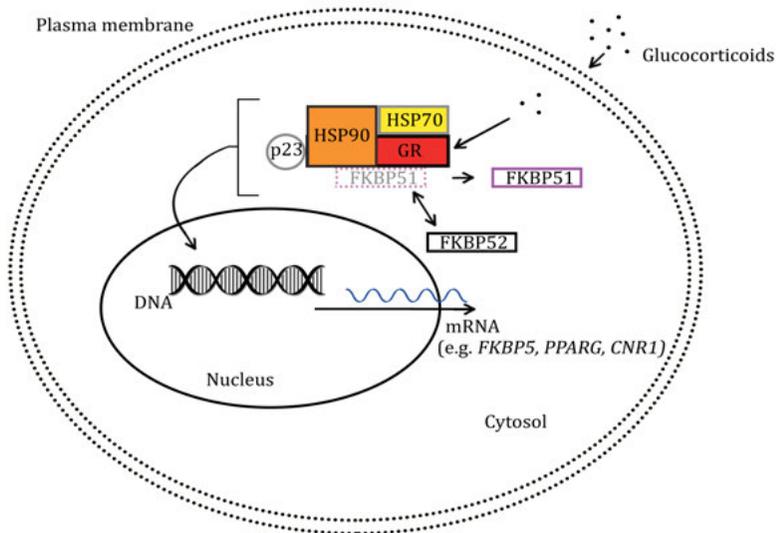
## FK506 binding protein 5

The expression of FK506 binding protein 5 (FKBP51) in several tissues has been shown to be regulated by glucocorticoids (61). Its expression levels are

elevated in metabolically active tissues such as adipose tissue compared to other tissues (61). FKBP51 is an immunophilin and is thus a target of immunosuppressive agents, through which they can exert their effects (62,63). FKBP51 also has a key role in stress response of the HPA-axis, and many studies on FKBP51 have focused on depression, anxiety and stress (64). Due to its relationship with glucocorticoids, FKBP51 is also of interest in metabolic conditions (64,65). In recent years several papers have implicated a metabolic role of FKBP51 (66). Recently, FKBP51 has also been implicated in adipocyte differentiation (67).

FKBP51 plays a critical role in stress regulation by mediating the glucocorticoid response through interaction with the GR and its downstream signalling. The GR resides in the cytosol and forms a complex together with multiple co-factors, chaperones and proteins, including heat shock proteins 70 and 90 (HSP90), p23, FKBP51 and FK506 binding protein 4 (FKBP52) (Figure 3) (68). FKBP51 and FKBP52 compete for the binding to the glucocorticoid receptor-associated HSP90. An FKBP51-associated GR-complex has a low affinity for glucocorticoids, whereas an FKBP52-associated glucocorticoid receptor complex has a high affinity for glucocorticoids (64). When a glucocorticoid such as cortisol diffuses through the cell membrane, it binds to the FKBP52-associated glucocorticoid receptor complex (64) (Figure 3).

This allows the entire complex to translocate into the nucleus where it binds to specific DNA response elements, triggering the activation or suppression of gene transcription (64) (Figure 3). One of the principal genes that are upregulated in this chain of events is the gene corresponding to FKBP51; *FKBP5*. FKBP51 thereby regulates its own expression in what is referred to as an ultra-short negative feedback loop (64,68-70).



*Figure 3.* Illustration of glucocorticoid receptor complex and its action within a cell following glucocorticoid stimulation. FKBP51 and FKBP52 compete (double-headed arrow) for the binding to heat shock protein 90 (HSP 90). An FKBP52-associated GR complex is able to translocate into the nucleus. Heat shock protein 70 (HSP 70), FK506 binding protein 5 (FKBP51), FK506 binding protein 4 (FKBP52).

## Cannabinoid receptor type 1

Cannabinoid receptor type 1 (CNR1) is a membrane-bound receptor that is primarily expressed in the central nervous system. However, peripheral and metabolically active tissues such as skeletal muscle and adipose tissue also express CNR1 (71).

CNR1 is part of the endocannabinoid system (ECS) – a complex network of endogenous ligands (endocannabinoids) that specifically bind to the G protein-coupled receptors, the cannabinoid receptors.

The ECS, composed of CNR1 and CNR2, their lipid ligands (endocannabinoids) 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the endocannabinoid synthesising and degrading enzymes; plays an important

role in the regulation of energy homeostasis (72,73). 2-AG is synthesized by diacylglycerol lipase (DAGL) and degraded by monoacylglycerol lipase. AEA is synthesised by N-acyl phosphatidylethanolamine phospholipase D and degraded by fatty acid amide hydrolase (74). DAGL enzymes are encoded by two separate genes, denoted DAGL-ALPHA and DAGL-BETA, where DAGL-ALPHA has been reported to play a greater role than DAGL-BETA in 2-AG synthesis in adipose tissue (75). It is generally accepted that CNR1 inhibits AC leading to a reduction in cAMP (76) in most tissues and models (77). However, in human pre-adipocytes activation of CNR1 has been shown to increase cytoplasmic cAMP, a key mediator of lipolysis (78,79).

CNR1 has been shown to regulate food intake in the hypothalamus (80) and the ECS is upregulated in both the central nervous system and the periphery in obesity (81,82). Given the role of the ECS in appetite regulation and obesity, there has been much recent pharmacological research for the development of anti-obesity drugs. One such anti-obesity drug, rimonabant, a CNR1-specific antagonist, was released and marketed in the mid-2000s (71). Although successfully reducing the weight of patients, it was coupled with adverse effects such as depression and anxiety and an increased suicide rate was reported. Rimonabant was eventually withdrawn from the market and discontinued as an anti-obesity drug due to its side-effects (71,83-85).

# Aim

The major aim was to evaluate the underlying causes and mechanisms of glucocorticoid-induced insulin resistance. In particular, the glucocorticoid-induced insulin resistance that manifests itself in adipose tissue and affects the metabolic functions of adipocytes and other peripheral tissues.

## Specific aims:

- *Paper I:* We aimed to explore the regulation of adipose tissue lipid turnover in the context of T2D, glycaemic control and obesity. For this purpose, 20 T2D subjects and 20 healthy control subjects were recruited. The subjects were well-matched by gender, age and BMI.
- *Paper II:* CNR1 is a membrane-bound receptor whose corresponding gene experienced a high upregulation in adipose tissue following treatment with the synthetic glucocorticoid dexamethasone. The aim was to extensively validate previous findings and explore the role of CNR1 in T2D and states of insulin resistance in adipose tissue.
- *Paper III:* Here we aimed to assess the genetic profile of adipose tissue treated with glucocorticoids – specifically the synthetic glucocorticoid dexamethasone. Prolonged or elevated glucocorticoid exposure is linked to several adverse metabolic effects. It was therefore of interest to elucidate on the underlying mechanisms, many of which are directly attributed to alterations in genetic makeup.
- *Paper IV:* Based on the findings in *Paper III*, the aim here was to further examine the role of FKBP51 in T2D and glucocorticoid-induced insulin resistance. Furthermore, we wanted to explore the role of FKBP51 in adipocyte biology and metabolism. This included aspects such as glucose and lipid metabolism as well as adipogenesis.

# Methods

See the respective papers for detailed method descriptions.

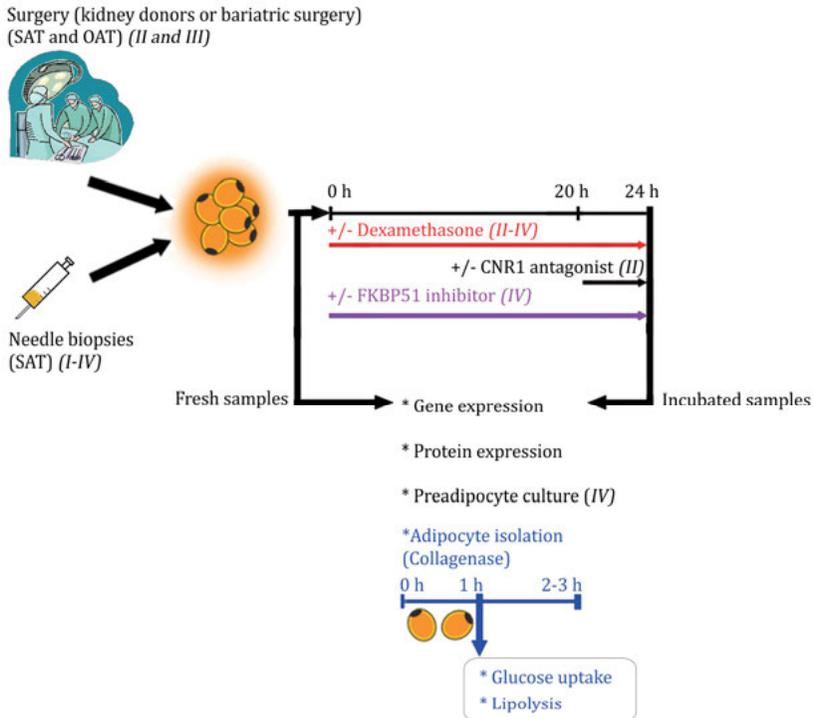
## Adipose tissue donors

Paired samples of SAT and OAT were obtained from non-diabetic subjects undergoing kidney donation or bariatric surgeries (*Paper II*: 13M/31F, 24-66 yrs, BMI 20.7-56.3 kg/m<sup>2</sup>; *Paper III*: 10M/15F, 28-60 yrs; BMI: 20.7-30.6 kg/m<sup>2</sup>). In addition, SAT was obtained from separate groups of non-diabetic volunteers (*Paper II*: 5M/21F, 21-72 yrs, BMI 21.3-32.9 kg/m<sup>2</sup>, *Paper IV*: 20F/9M, 18-72 years, BMI 22.7-47.2 kg/m<sup>2</sup>) by needle aspiration of the lower abdominal region as previously described (86). Anthropometric measurements were performed to obtain body composition with bioimpedance. Fasting blood samples were collected for analysis of plasma glucose, insulin and lipids at the Department of Clinical Chemistry at Sahlgrenska University Hospital or Uppsala University Hospital; Sweden.

Subjects with T2D, endocrine disorders, cancer or other major illnesses, as well as those having ongoing medication with, systemic glucocorticoids, immune modulating therapies or beta-adrenergic blockers were excluded from the studies. Females taking oral contraceptive pills with oestrogen were not included in the study (*Paper I*).

In a separate cohort (*Paper I, II and IV*), 20 T2D subjects were group-wise matched with 20 healthy control subjects by gender (10F/10M), age (58±9 vs 58±11) and BMI (30.7±4.9 vs 30.8±4.6 kg/m<sup>2</sup>). Fasting blood samples were collected, and oral glucose tolerance test (OGTT) and SAT needle biopsies were performed as previously described (86).

The study protocols were approved by the respective Regional Ethics Review Boards in Gothenburg (Dnr 336-07) and Uppsala (Dnr 2013/330 and Dnr 2013-183/494). Written informed consent was obtained from all study participants.



*Figure 4.* Illustration of adipose tissue handling and experiments. Shown methods were used in all papers, unless stated otherwise. Roman numerals indicate the respective papers in which specific methods were used. Subcutaneous adipose tissue (SAT), omental adipose tissue (OAT).

## Adipose tissue incubations and assessments

Adipose tissue samples from surgery were cut into smaller pieces and incubated in Dulbecco's Modified Eagle Medium (DMEM) containing 6 mM glucose (Invitrogen Corporation, Paisley, UK), 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (PEST, Invitrogen) with or without the synthetic glucocorticoid dexamethasone (0.003-3  $\mu$ M, Sigma Chemical Co., St. Louise, MO, USA) for 24 h in 37  $^{\circ}$ C, 5% CO<sub>2</sub>. Following incubation, adipose tissue samples were snap-frozen in liquid nitrogen and used for the assessment of FKBP51 and CNR1 gene (microarray analysis, 3  $\mu$ M dexamethasone, *Paper III*; real-time PCR, 0.003-3  $\mu$ M dexamethasone, *Paper II and III*) and protein (0.003-3  $\mu$ M dexamethasone *Paper II and III*) expression levels. In addition, some of the incubated tissue samples were used to isolate adipocytes with collagenase (Sigma) and perform glucose uptake measurements as previously described (*Paper I-IV*) (87). SAT samples were also incubated with or without cortisol or dexamethasone (1  $\mu$ M and 24 h for both) for real-time PCR analysis of *CNR1* (*Paper II*).

SAT was also incubated with or without dexamethasone (0.3  $\mu\text{M}$ ) for 24 h in 37°C, 5%  $\text{CO}_2$  and with or without the CNR1 antagonist/inverse agonist AM281, (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide, Sigma, 3  $\mu\text{M}$ ), for the final 4 h of incubation (*Paper II*) (Figure 4). SAT was also incubated with or without the CNR1 agonist, ACEA (Arachidonyl-2'-chloroethylamide, Cayman Chemical Company, Ann Arbor, MI, USA, 1  $\mu\text{M}$ ) (*Paper II*), or the FKBP51-specific inhibitor, SAFit1 (45) (*Paper IV*), for 24 h (Figure 4). Adipose tissue was used to test the effects of dexamethasone and the CNR1 antagonists or FKBP51-specific inhibitor on adipocyte lipolysis (CNR1 antagonist, *Paper II*) and glucose uptake (CNR1 antagonist and FKBP51-specific inhibitor; *Paper II and IV*, respectively) as previously described (86,87). Glucose uptake and lipolysis assays were normalized to the number of cells. Adipocyte cell size and number was determined as previously described (88-90). Moreover, preadipocytes from SAT were used to explore the role of FKBP51 in adipocyte differentiation (*Paper IV*).

A concentration of 0.3  $\mu\text{M}$  dexamethasone corresponds to a maximum physiological level of cortisol, 1-2  $\mu\text{M}$  under stress (45). Therefore, a 0.3  $\mu\text{M}$  dexamethasone concentration was selected to ensure a maximum effect on CNR1 or FKBP51 expression levels.

## Glucose uptake in isolated primary adipocytes (*Papers I-IV*)

Adipocytes were isolated from fresh or incubated samples of adipose tissue with collagenase in Hank's medium 199 (Gibco, Paisley, UK) supplemented with 6 mM glucose (Sigma), 4% bovine serum albumin (BSA, Sigma), 150 nM adenosine (Sigma) and pH 7.4, for 60 min at 37°C in a shaking water bath as previously described (91,92). Adipocytes were then washed in glucose-free Krebs-Ringer-Hepes (KRH), supplemented with 4% BSA (Sigma), 150 nM adenosine (Sigma), pH 7.4. Adipocytes were diluted ten times in KRH media and were stimulated or not with insulin (1000  $\mu\text{U}/\text{ml}$ , Actrapid, Novo Nordisk, Bagsvaerd, Denmark) for 15 min in a shaking water bath at 37 °C. Thereafter, D-[U-14C] glucose (0.26 mCi/L, 0.86  $\mu\text{M}$ ; NEC042V250UC, PerkinElmer, Waltham, Massachusetts, USA) was added and the accumulation of glucose followed for 45 min. The reaction was stopped by the transfer of the cell suspension into ice-cold tubes with silicon oil (SERVA Electrophoresis GmbH, Heidelberg, Germany) and the adipocyte-associated radioactivity was measured in a beta-counter. Glucose uptake was determined by the rate of transmembrane glucose transport and calculated as previously described (93).

## Glucose uptake in adipocytes differentiated *ex vivo* (Paper IV)

Glucose uptake was performed in *ex-vivo* differentiated human adipocytes. Effects of dexamethasone on glucose uptake were also assessed. After 14 days of differentiation, adipocytes were deprived of glucocorticoids for 48 hours. Adipocytes were then incubated with 0.3  $\mu$ M of dexamethasone (Sigma) for 24 hours followed by glucose uptake analysis performed as previously described (54,94,95).

## Lipolysis (Papers I-IV)

Adipocytes were isolated from fresh or incubated samples of adipose tissue with collagenase (Sigma) in Hank's medium 199 (Gibco), as previously described (91,92).

Adipocytes were diluted to a lipocrit of 2-3% and the cell suspension was incubated with or without isoproterenol (0.5  $\mu$ M, Sigma) and with or without insulin (100  $\mu$ U/mL, Actrapid) in a gently shaking water-bath at 37 °C for 2 h. Glycerol released into the media was measured by colorimetric absorbance at 540 nm with Free Glycerol Reagent (Sigma). Cellular lipids were extracted (96) and adipocyte size and cell number was measured as previously described (97). The glycerol released into the media was normalized per cell number and calculated relative to basal control in each experiment.

## Isolation of human adipocytes and cells of the stromal vascular fraction (SVF) for immunoblotting analysis of FKBP5 (Paper III)

Adipocytes and the SVF were isolated from SAT and OAT, as previously described (91,92), for analysis of FKBP5 protein expression. 500 mg of adipose tissue was digested with collagenase in Hank's medium 199 as previously described (91,92). Isolated adipocytes were filtered through a 250  $\mu$ m nylon mesh. SVF in the media below the mature adipocytes was collected for isolation. The floating mature adipocytes were washed and separated from media by filtration through dinonyl phthalate (Merck, Darmstadt, Germany). The SVF was isolated from medium by centrifugation for 3 min at 1200 rpm and washing with phosphate buffered saline (PBS, Medicago, Uppsala Sweden). The adipocytes and the SVF were then used for immunoblotting analysis of FKBP5.

## Culture and *ex vivo* differentiation of human preadipocytes (*Paper IV*)

In order to investigate the role of FKBP51 in adipogenesis, human preadipocytes from SVF were isolated and cultured for differentiation. For the separation of the SVF from mature primary adipocytes, fresh SAT was digested with collagenase in Hank's medium 199, as previously described (91,92). The SVF was then cultured in preadipocyte medium, DMEM: Nutrient Mixture F-12 (DMEM/F12; 10% FBS, Invitrogen; 1% PEST, Invitrogen; 0.4% Gentamicin, Thermo Fisher Scientific, Waltham, Massachusetts, USA; 4.25 ng/ml human basic fibroblast growth factor, Sigma) until a confluency of 70-80% was reached. Cells were expanded until ~1 000 000 cells had been accumulated. Cells were then transferred to 24-well plates at a 15 000 cells/cm<sup>2</sup> density. Upon reaching 100% confluence, cells were differentiated as previously described (94,98). Preadipocytes were differentiated for 14 days with samples for gene and protein expression levels being collected on days 0, 7 and 14. In addition, quantification of differentiation was performed by Oil Red O staining and subsequent imaging on days 7 and 14 of differentiation.

## Gene expression analyses

Assessment of lipogenic/lipolytic mRNA levels, endocannabinoid levels and *FKBP5* mRNA expression in freshly harvested SAT (*Paper I, II and IV*)

Immediately after the biopsies, the SAT from T2D and healthy control subjects was snap frozen in liquid nitrogen. The mRNA expression levels of *CNR1* and the major enzymes responsible for the synthesis and degradation of the two principal endocannabinoids, 2-AG and anandamide AEA, was measured (*Paper II*). 2-AG levels in SAT were also assessed but AEA levels were not detectable. mRNA expression levels of *FKBP5*, other glucocorticoid-regulated genes as well genes involved in adipogenesis and glucose and lipid and energy metabolism were also measured. mRNA expression levels were obtained with Rnaseq at Exiqon A/S, Vedback, Denmark and 2-AG quantification was done by Metabolon Inc's (Durham, North Carolina, USA) TrueVision<sup>TM</sup> as previously described (86).

Microarray (*Paper III*) and real-time PCR (*Paper II, III and IV*)

Gene expression was assessed by mRNA measurement using microarray analysis (Affymetrix, Human Exon 1.0 ST Array, Santa Clara, CA, USA, *Paper III*) in accordance with the guidelines detailed by the manufacturer.

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE42902. To determine the tissue distribution of *FKBP5* gene expression, microarray expression profiles (Human U133 plus 2.0 DNA microarray, Affymetrix) from metabolically active tissues were downloaded from the GEO database (data set GSE3526). *FKBP5* gene expression was determined using the 224840\_at probeset.

For real-time PCR, total RNA was isolated from adipose tissue using the Rneasy Lipid Tissue mini kit (Qiagen, Hilden, Germany, *Paper II, III and IV*). The RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was converted to cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and relative quantification of mRNA was performed. All reagents for real-time PCR were purchased from Applied Biosystems and used according to the manufacturer's protocol. The relative quantification of gene expression was analyzed using the ABI Prism 7700HT Sequence Detection System (Applied Biosystems) or the CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A standard curve of pooled cDNA was used to determine the concentration of the target gene. The gene expression levels were normalized to the housekeeping genes 18S rRNA (*Paper II and III*) or *GUSB* (*Paper IV*) (Applied Biosystems); both well-established reference genes for gene expression studies of human adipose tissue (99,100).

### Single nucleotide polymorphism analysis (*Paper III*)

By extracting results from previously published human genome-wide studies, single nucleotide polymorphisms (SNPs) in the *FKBP5* region and their associations with diabetes and diabetes-related traits were assessed. Publicly available results from the following genetic consortia were used: DIAGRAM, MAGIC, Global Lipids Genetics Consortium and GIANT. All of these studies are based on individuals of European descent. Additive models were used for genetic analysis. The *FKBP5* gene region was defined as the region ranging from 110kb upstream to 40kb downstream of the gene boundaries. We extracted all genotypes from this region corresponding to chr6:35609kb-35914kb from HapMap 3 (build 36) (101). This yielded a high number of SNPs in high linkage disequilibrium. We therefore determined the number of independent SNPs by using the 'indep' command in PLINK (102,103), yielding a total of 22 SNPs. All p-values were Bonferroni-corrected with a factor of 22 to compensate for multiple testing.

## Protein expression analyses

### Immunoblotting (*Paper II, III and IV*)

Adipose tissue (*Paper II-IV*), adipocytes (*Paper III*), SVF (*Paper III*) or preadipocytes (*Paper IV*) were homogenized in ice-cold lysate buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine and 0.1 μM okadaic acid; all reagents from Sigma) and lysed at 4°C for 2 h. The supernatant, a fat free extract, was obtained by centrifugation at 12 000 g, 15 min, 4°C, and was collected and saved at -80°C. Protein content was measured with the BCA protein assay kit (Thermo Fisher Scientific).

Total lysate (equal amounts of 10-20 μg/lane) were subjected to SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with primary antibodies corresponding to FKBP51 (1:1000, Cell Signaling Technology (CST), Danvers, MA, USA (*Paper III*) or 1:250, Abcam, Cambridge, United Kingdom (*Paper IV*)), extracellular regulated kinase (ERK) (1:1000, CST, *Paper II*), phospho-ERK (Thr202/Tyr204) (1:1000, CST, *Paper II*), HSL (1:1000, CST, *Paper II*) or phospho-HSL (Ser563) (1:1000, CST, *Paper II*). This was followed by immunoblotting with secondary antibody linked to horseradish peroxidase (1:2000, CST, *Paper II, III and IV*). Beta-actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA, *Paper III*) or GAPDH (1:1000, Millipore, Temecula, CA, USA; or CST, *Paper II and IV*) were used as loading controls.

### Immunohistochemistry (*Paper II*)

Adipose tissue that was incubated for 24 h in the absence or presence of dexamethasone was snap frozen in liquid nitrogen. Frozen sections (12 μm thick) were mounted on glass slides (Superfrost<sup>®</sup> Plus, Thermo Fisher Scientific). The sections were fixated in ice-cold acetone:methanol (1:1) (acetone from Sigma, methanol from Merck Millipore, Billerica, MA, USA) for 10 min and washed in PBS-T (Medicago). Sections were then blocked in a blocking solution containing PBS 1X (Medicago), 1% BSA (Sigma), 0.3 % Triton X-100 (Sigma), 0.1% Sodium Azide (Sigma) for 1 h. This was followed by overnight incubation of the sections at 4 °C with the CNR1 primary antibody (Ab23703, Abcam, diluted 1:300). After incubation, sections were washed in PBS-T (Medicago) and then incubated for 1 h at room temperature with secondary goat anti-rabbit Alexa fluor 594 (Life Technologies, Eugene, OR, USA; diluted 1:1000). Sections were then mounted with coverslips by using ProLong Gold Antifade Mountant with DAPI (4',6-diamidino-2-phenylindole, Life Technologies) and allowed to dry overnight

in darkness. Sections were then visualized under a confocal microscope and images were captured with a Laser Scanning Microscope ZEISS LSM780 (Carl Zeiss AG, Oberkochen, Germany). Quantification was performed by using Image Processing and Analysis in Java (ImageJ, National Institutes of Health, Bethesda, MD, USA).

## Statistical Analysis

All data are presented as mean $\pm$ SEM, unless stated otherwise. Pairwise comparisons were performed between treated and untreated adipose tissue within the same individual to minimize confounding variables. The normality of datasets was determined by using the Shapiro-Wilk test of normality. Wilcoxon signed-rank test and Kruskal-Wallis test were used to analyse differences in gene expression and protein levels between treated and untreated adipose tissue. Differences in glucose uptake and lipolysis were performed with paired t test. Spearman's correlation test was used to measure the statistical dependence between *FKBP5* or *CNRI* gene expression, dexamethasone effects and metabolic variables. Significant variables were subsequently included in a step-wise manner into multivariate linear regression analyses. A P-value <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL) or specific software for the microarray and SNP analysis.

# Results

See the respective papers for detailed results.

## Paper I – Impaired adipose tissue lipid storage, but not altered lipolysis, contributes to elevated levels of NEFA in type 2 diabetes. Degree of hyperglycemia and adiposity are important factors

AUC (area under the curve) NEFA (non-esterified fatty acids) levels during OGTT were shown to be elevated in T2D subjects in comparison to healthy control subjects. However, both plasma glycerol AUC during OGTT and subcutaneous adipocyte lipolysis were similar between T2D and healthy control subjects. This would suggest normal adipose tissue regulation of lipolysis in both groups. At the same time, several genes associated with lipogenesis were downregulated in T2D subjects versus healthy controls. There were no differences in expression levels of lipolytic genes. NEFA AUC during OGTT in T2D subjects negatively correlated with gene expression levels of genes associated with lipogenic genes, but did not correlate with any lipolytic genes or *in vitro* adipocyte lipolysis.

Furthermore, when subjects were subdivided according to obesity status, there were higher levels of plasma glycerol and NEFA in T2D obese subjects compared to T2D non-obese subjects. Moreover, markers of beta oxidation were elevated in T2D subjects.

In addition, NEFA AUC during OGTT correlated positively with HbA<sub>1c</sub> and negatively with the Matsuda index of insulin sensitivity. According to multivariate analysis, both HbA<sub>1c</sub> and the Matsuda index were shown to be strong predictors of NEFA AUC during OGTT in all subjects.

## Paper II – Role of cannabinoid receptor 1 in human adipose tissue for lipolysis regulation and insulin resistance

*CNR1* gene expression levels in freshly harvested SAT were 2-fold elevated in T2D subjects compared to healthy control subjects. In addition, bivariate correlation analyses showed positive correlations between *CNR1* gene expression and markers of insulin resistance such as AUC glucose during OGTT, fasting glucose and HbA<sub>1c</sub>. AUC glucose during OGTT remained the only predictor of *CNR1* gene expression following multivariate data analysis. Overall, the endocannabinoid-synthesizing enzymes were upregulated in T2D subjects versus healthy control subjects, whereas the endocannabinoid-degrading enzymes showed a tendency towards downregulation in T2D subjects.

The synthetic glucocorticoid dexamethasone upregulated *CNR1* gene expression dose-dependently in both SAT and OAT by up to 25-fold following 24 h incubations. Moreover, *CNR1* was also upregulated by dexamethasone at the protein level in SAT by about 2-fold versus untreated control. The *CNR1* gene expression levels of incubated non-treated SAT and OAT were found to correlate positively with HOMA-IR. *CNR1* gene expression levels only in incubated dexamethasone-treated SAT correlated positively with HOMA-IR.

Incubation of SAT with dexamethasone for 24 h increased isoproterenol-stimulated lipolysis in adipocytes when compared with control and reduced the anti-lipolytic effect of insulin. Co-incubation with AM281 for the last 4 h of incubation prevented both effects. Freshly harvested subcutaneous adipocytes that were pre-treated with AM281 for 30 minutes, with the compound present throughout the lipolysis experiment, showed a reduced isoproterenol-stimulated lipolysis by 11% compared to non-treated adipocytes.

SAT incubation with dexamethasone for 24 h was shown to significantly reduce both basal and insulin-stimulated glucose uptake in subcutaneous adipocytes. However, the addition of AM281 for the final 4 h of incubation did not prevent the impairment of glucose uptake by dexamethasone.

## Paper III – FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance

In both SAT and OAT, the genes *FKBP5* and *CNR1* were the most responsive, in terms of upregulation, after incubation with the synthetic glucocorticoid dexamethasone. This was shown with ~7-fold higher expression levels versus non-treated control in microarray analysis

Dexamethasone dose-dependently increased FKBP51 gene and protein expression levels in both SAT and OAT depots; with a ~20-fold upregulation in gene expression levels according to real-time PCR. The relative depot-specific FKBP51 protein expression levels showed ~10-fold higher levels of FKBP51 in OAT than SAT. Furthermore, we showed that 80-90% of FKBP51 in adipose tissue could be attributed to adipocytes, whilst the SVF contributed 10-20% of adipose tissue FKBP51.

*FKBP5* gene expression was found to positively correlate with markers of insulin resistance such as HOMA-IR and insulin in SAT, but not in OAT. In addition, the dexamethasone-induced change of *FKBP5* gene expression in SAT negatively correlated with fasting insulin, HbA<sub>1c</sub> and HOMA-IR.

Dexamethasone decreased both the basal and insulin-stimulated glucose uptake in both adipose tissue depots. Adipocytes from individuals with higher (50<sup>th</sup> percentile and above) endogenous *FKBP5* gene expression levels in OAT had reduced insulin effects on glucose uptake in both subcutaneous and omental adipocytes when compared to individuals with lower OAT *FKBP5* gene expression levels. In contrast, higher *FKBP5* gene expression levels in SAT did not predict the insulin-stimulated effects on adipocytes from either depot.

In an exploration of *FKBP5* polymorphisms to investigate if the gene may contribute to the susceptibility of developing T2D or related phenotypes; 11 SNPs were found to be associated to T2D, 2h OGTT glucose, HDL-cholesterol and triglyceride levels.

## Paper IV – FKBP5 expression in human adipose tissue: Potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes

*FKBP5* gene expression levels in SAT were 16% higher in males than females. Moreover, there was a tendency towards 10% higher *FKBP5* gene expression levels in SAT of T2D subjects compared to healthy controls. In addition, *FKBP5* gene expression levels in SAT were found to positively correlate with several markers of insulin resistance, including AUC glucose during OGTT, fasting glucose and HOMA-IR. AUC glucose during OGTT remained the sole predictor of *FKBP5* gene expression in SAT following multivariate data analysis.

Associations in SAT between *FKBP5* gene expression levels and genes concerning several aspects of adipocyte biology and metabolism were also found. This included positive correlations with genes regulated by the glucocorticoid receptor complex, including *GR* and *CNR1*. Moreover, *FKBP5* gene expression levels negatively correlated with pro-lipolytic genes such as *ATGL* and *MGLL*. However, *FKBP5* gene expression levels were also found to positively correlate with gene expression levels of the anti-lipolytic *PDE3B*. In addition, *FKBP5* gene expression levels also negatively correlated with lipogenic (*DGATI*, *DGAT2*) and adipogenic (*PPARG*, *CEBPA*) genes. Moreover, both gene and protein expression levels of FKBP51 were shown to increase during differentiation.

As expected, incubation of SAT with the synthetic glucocorticoid dexamethasone reduced basal and insulin-stimulated glucose uptake in isolated adipocytes. Co-incubation of dexamethasone with the FKBP51-specific inhibitor, SAFit1; partly prevented the impairment of glucose uptake by dexamethasone. However, this prevention was more pronounced in adipocytes that responded strongly to dexamethasone treatment compared to low-responders.

Table 1. Correlations between gene expression in fresh SAT samples and markers of insulin resistance. AUC = Area under the curve, HbA<sub>1c</sub> = Glycosylated haemoglobin, HOMA-IR = Homeostatic model assessment of insulin resistance, Matsuda = Matsuda insulin sensitivity index, ↑ = Positive correlation, ↓ = Negative correlation, ↔ = No correlation, \* = Tendency

Gene	Parameters	AUC Glucose	HbA <sub>1c</sub>	HOMA-IR	Matsuda
<i>CNR1</i>		↑	↑	↑*	↔
<i>FKBP5</i>		↑	↔	↑	↓

# Discussion

## *Paper I*

In studies of insulin action on the lipolytic machinery, study results have been discordant. Where some studies have shown that the anti-lipolytic effect of insulin is impaired in T2D subjects, others have shown no differences in lipolysis between T2D and matched control subjects (104). Our results are in agreement with the latter, showing no differences of *in vivo* or *in vitro* adipocyte lipolysis between T2D and well-matched healthy control subjects. Rather, it is differences in circulating NEFA levels that separate T2D subjects from healthy controls in the context of lipid metabolism. We show that NEFA levels are significantly higher in T2D subjects during OGTT in comparison to healthy control subjects. In accordance with these observations, we found that genes corresponding to lipogenesis are down-regulated in T2D subjects versus healthy controls. The expression levels of lipolytic genes did not differ between the two groups. Taken together, these results imply that an impaired machinery of lipid storage rather than lipolysis is responsible for elevated levels of NEFA in T2D. In further support of these findings, we found that plasma levels of  $\alpha$ -hydroxybutyrate and  $\beta$ -hydroxybutyrate were also elevated in T2D subjects compared to controls.  $\alpha/\beta$ -hydroxybutyrate are two metabolites that are markers of beta oxidation. Elevated levels of  $\alpha/\beta$ -hydroxybutyrate suggest an increased availability of substrates for beta oxidation, e.g. free fatty acids. This is in agreement with the elevated levels of NEFA in circulation.

We also showed that obesity status, rather than T2D may play a role in *in vivo* lipolysis in humans. Both plasma glycerol and NEFA AUC during OGTT were significantly higher in T2D obese subjects compared to T2D non-obese subjects. *In vitro* adipocyte lipolysis from obese subjects also showed a tendency toward higher isoproterenol-stimulated lipolysis compared to adipocytes from non-obese subjects.

The re-esterification of NEFA in adipocytes depends on the availability of glycerol-3-phosphate. Glycerol-3-phosphate is an intermediate of glycolysis (105), and its supply is therefore dependent on glucose uptake. A reduced glucose uptake in adipocytes from T2D subjects compared to healthy control subjects could therefore lead to reduced glycerol-3-phosphate levels in T2D. This could partly explain elevated NEFA levels in T2D, as less NEFA would be re-esterified to form TG.

## *Paper II*

The overactivity of the endocannabinoid system in T2D was shown by assessing SAT levels of 2-AG and gene expression levels of *CNR1* as well as endocannabinoid synthesizing and degrading enzymes. Associations between the measured metabolites and markers of insulin resistance were also demonstrated. This agreed with several studies showing an increased activity of the endocannabinoid system in T2D and/or obese subjects (106-108). Taken together these results suggest a potential role of the endocannabinoid system in promoting insulin resistance.

In agreement with the findings in *Paper III*, we showed that the synthetic glucocorticoid dexamethasone dose-dependently increases *CNR1* gene expression in both adipose tissue depots. This dexamethasone-induced upregulation was further validated at the protein level in SAT.

The *in vitro* role of *CNR1* in the glucocorticoid regulation of glucose and lipid metabolism in human subcutaneous adipocytes was explored. Here we showed that a *CNR1*-specific antagonist, AM281, partly prevents the stimulatory effects of dexamethasone on lipolysis in adipocytes. Lipolysis-stimulation following incubation with a *CNR1*-specific agonist ACEA further showcased a role of *CNR1* in lipid metabolism.

We examined the possible mechanisms involved in the mediation of lipolysis in *CNR1* by assessing the effects from incubation with dexamethasone and AM281 on phosphorylation and total protein levels of ERK1/2 and HSL in adipose tissue. ERK1/2 is a protein involved in the MAPK-pathway known to be regulated by the endocannabinoid system. ERK1/2 has also been implicated as a kinase capable of phosphorylating HSL (109,110), thus furthering the activity of lipolysis. However, in our study, we did not find ERK and HSL activation and protein levels to be affected by dexamethasone or AM281 incubation. Another possible pathway of mediating lipolysis would be via cAMP. *CNR1* activation has been shown to increase cAMP in human preadipocytes (79). This could explain why we see a reduction of lipolysis when incubating *CNR1* with a specific antagonist.

*CNR1* antagonism has been shown to improve tissue-specific glucose uptake in rat-derived skeletal muscle (111) and nucleus accumbens (112). In our study with human adipose tissue, we found that AM281 did not affect dexamethasone's inhibitory effect on glucose uptake in subcutaneous adipocytes. However, AM281 appears to prevent dexamethasone-stimulated lipolysis. It is possible that *CNR1* has different mechanisms with respect to glucose metabolism in different tissues. Different species (i.e. human adipocytes versus rat skeletal muscle) and different *CNR1*-specific antagonists could also explain the differences between the present study and the others. This should be investigated further.

## *Paper III-IV*

Because of the key role of FKBP51 in regulating the sensitivity of the GR-complex (64), one may have predicted that FKBP51 would be strongly upregulated by glucocorticoids. Furthermore, the broader role of FKBP51 in metabolism is, to the best of our knowledge, poorly understood. Our findings imply that FKBP51 is multifaceted in its functions. Apart from regulating glucocorticoid receptor sensitivity, FKBP51 seems to be involved in several aspects of adipocyte biology. Its gene expression is associated with the expression of genes linked to many metabolic functions in SAT. This includes genes related to glucose and lipid metabolism, but also lipid storage and adipocyte differentiation (adipogenesis). In agreement with previous findings (67), we found that FKBP51 gene and protein expression levels increase during differentiation. This highlights FKBP51 as a possible marker of adipogenesis. In addition, significant associations were found between SNPs of the *FKBP5* gene with T2D and diabetes-related phenotypes. This implies that there are variants of the *FKBP5* gene associated with systemic metabolic disorders.

*FKBP5* gene expression levels in SAT correlated with *in vivo* markers of insulin resistance such as insulin, HOMA-IR, subcutaneous adipocyte diameter and AUC glucose during OGTT (97). There was also a tendency of *FKBP5* expression levels to be higher in T2D subjects. While we cannot explain why some of these associations are present in SAT but not OAT, we demonstrate evidence for cross-talk between SAT and OAT. *FKBP5* gene expression levels in OAT are associated with reduced potency of insulin in glucose uptake stimulation, not only in omental but also in subcutaneous adipocytes. This implicates cross-talk between the two adipose tissue depots. Perhaps in a manner where perturbations in OAT carry a greater influence on SAT than vice versa. Indeed, insulin resistance in OAT has been attributed to a higher degree of macrophage infiltration into OAT compared to SAT in obesity (113). Alterations of the adipokine secretion profile of OAT are therefore likely to be greater than in SAT.

FKBP51 has been suggested as a negative regulator of PKB/Akt phosphorylation via its Akt-interacting domains (114). The FKBP51-specific inhibitor SAFit1 was used is likely to bind to these domains, thus interfering with the suppression of PKB/Akt by FKBP51 (115,116). In a similar vein, a recent study proposed that FKBP51 regulates glucose uptake in mice via regulation of AKT2-AS160 (117). The study looked at extensor digitorum longus (EDL) myotubes from wild type or FKBP51-knockout mice. Results showed increased phosphorylation of AKT2 and AS160 in FKBP51-knockout mice following insulin stimulation. In contrast to our findings in human adipocytes, improved insulin activation of glucose uptake remained unchanged in white adipose tissue of FKBP51-knockout mice compared to wild type mice. With these findings, it would be of interest to extend our

study approach to include human skeletal muscle where insulin-mediated glucose metabolism is more prominent compared to adipose tissue.

## Concluding remarks

We showed that CNR1 and FKBP51 are two proteins associated with insulin resistance in human white adipose tissue. We demonstrate that CNR1 and the endocannabinoid system in SAT is upregulated in states of insulin resistance, such as in T2D as well as insulin resistance induced by glucocorticoid exposure. Our results also suggest an involvement of CNR1 in glucocorticoid-regulated lipolysis in subcutaneous adipocytes. Our findings give further support to the concept of a role of the peripheral endocannabinoid system in insulin resistance. This advances CNR1 in peripheral tissues as a potential attractive drug target for the treatment of dyslipidemia and insulin resistance associated with T2D.

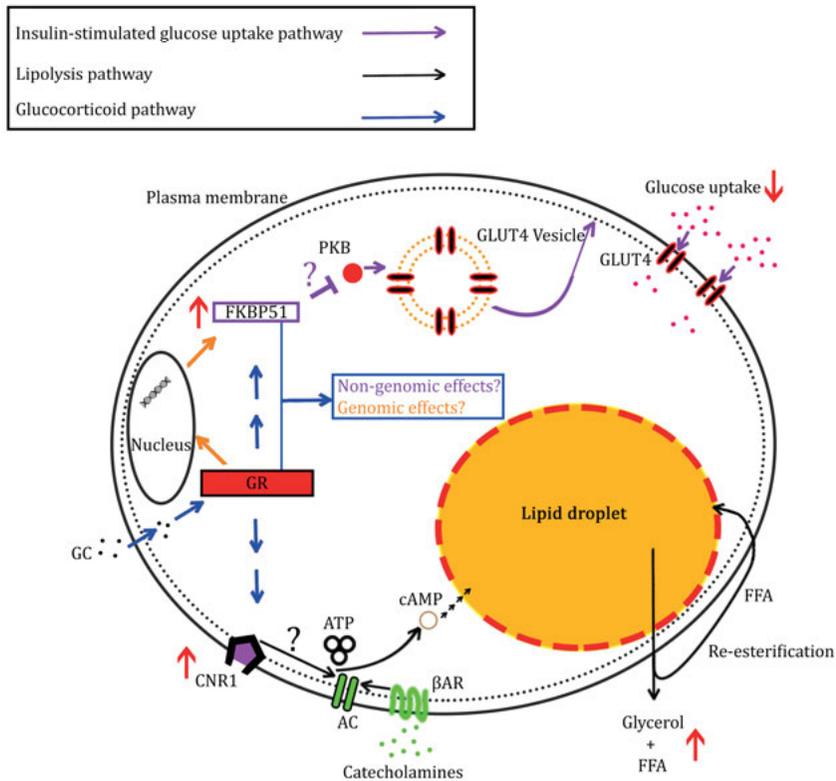
Moreover, our data indicate that the *FKBP5* gene is upregulated by glucocorticoids in both SAT and visceral adipose tissue and that its expression in adipose tissue is associated with markers of insulin resistance. Several SNPs in the *FKBP5* region were shown to be associated with T2D and diabetes-related traits. Moreover, *FKBP5* gene expression tends to be elevated in T2D subjects. Furthermore, glucocorticoid-impaired glucose uptake can be partly prevented by the specific inhibition of FKBP51. We hypothesize that FKBP51 partly mediates metabolic alterations that are brought on by glucocorticoid excess.

Future studies should address the link between CNR1, FKBP51 and insulin resistance and further elucidate underlying mechanisms. Based on our findings and previous results, we hypothesize that CNR1 regulates lipolysis by increasing the activity of AC. Elevated CNR1 levels by glucocorticoids and subsequent antagonism could therefore explain our observations.

We hypothesize that FKBP51 interferes with the glucose uptake machinery by directly binding to PKB/Akt with its FKBP-type domains (118). FKBP51 therefore regulates PKB/Akt activity via a non-genomic pathway. Furthermore, we believe that FKBP51 could also be considered a marker of adipocyte differentiation.

Future studies should explore these proposed mechanisms of action of CNR1 and FKBP5 (Figure 5). This could lead to the emergence of these proteins as potential novel pharmacological targets for the treatment of insulin resistance.

Overall, the study model is limited by its *in vitro* nature that does not take into account complex crosstalks between adipose tissue and other peripheral tissues and the brain. A strength is the inclusion of T2D subjects versus



*Figure 5.* Illustration of proposed mechanisms of action in adipocytes of CNR1/FKBP51 in high exposure to glucocorticoids. Red arrows indicate up- or downregulation. E.g. increased expression of CNR1/FKBP51. E.g. increased lipolysis and reduced glucose uptake. Purple arrows indicate the insulin-stimulated glucose uptake pathway. Black arrows indicate the lipolysis pathway. Blue arrows indicate the glucocorticoid pathway. Adenylyl cyclase (AC), adipose triglyceride lipase (ATGL), adenosine triphosphate (ATP), beta adrenergic receptor ( $\beta$ AR), cannabinoid receptor type 1 (CNR1), cyclic adenosine monophosphate (cAMP), FK506 binding protein 5 (FKBP51), free fatty acid (FFA), glucocorticoids (GC), glucocorticoid receptor (GR), glucose transporter type 4 (GLUT4), lipid droplet (LD), protein kinase B (PKB).

healthy controls, adding further support to our observations in the glucocorticoid model. Altogether, the presented studies demonstrate the strength of our glucocorticoid model of insulin resistance in human adipose tissue. The present studies showcase possible mechanisms of CNR1 and FKBP51 that contribute to perturbations in adipocyte glucose and lipid metabolism. These perturbations are associated with the comorbidities of T2D and obesity, including insulin resistance and dyslipidaemia. The proposed CNR1 and FKBP51 mechanisms could therefore be of interest as pharmacological targets.

## Svensk sammanfattning (Summary in Swedish)

Övervikt och typ 2 diabetes (T2D) ökar i global omfattning. Ur ett folkhälsooperspektiv är det av intresse att identifiera gemensamma mekanismer som ligger till grund för dessa sjukdomstillstånd. Glukokortikoider är steroidhormoner som spelar en viktig roll i stress, immunförsvaret och metabolism i däggdjur. Förhöjda nivåer av glukokortikoider har associerats till insulinresistens (IR) och T2D-liknande fenotyper. Här utnyttjas glukokortikoider för att modellera ett tillstånd av IR i human fettvävnad för att identifiera potentiella måltavlor för läkemedel.

I *Artikel I* undersöktes vilken inverkan som T2D har på omsättning av lipider. Detta gjordes i en kohort bestående av 20 individer med T2D och 20 friska kontroller. Individer med T2D visade sig ha högre plasmanivåer av icke-esterifierade fettsyror under oralt glukostoleransetest (OGTT) i jämförelse med friska kontroller. *In vitro* lipolys och utvärdering av mRNA och metabola faktorer i underhudsfett (UF) genomfördes. Resultaten visade att förhöjda nivåer av icke-esterifierade fettsyror i individer med T2D, kunde tillskrivas försämrade förmåga att lagra fetter.

I *Artikel II* utforskade vi vilken roll som cannaboid-receptor typ 1 (CNR1) hade i glukokortikoid-inducerad IR. Vi fann att *CNR1* genen hade ett förhöjt uttryck i UF efter glukokortikoidbehandling. Vidare fann vi genuttrycket av *CNR1* i UF var associerat med flera markörer av IR och förhöjd i patienter med T2D jämfört med friska kontroller. Med en CNR1-specifik antagonist fann vi att receptorn kan ha förmåga att reglera lipolys i UF.

I *Artikel III-IV*, undersökte vi vilken roll FK506 protein 5 (FKBP51) hade i glukokortikoid-inducerad IR. Den gen som motsvarar FKBP51, *FKBP5*, hade ett förhöjt uttryck i UF och omentalt fett (OF) efter glukokortikoidbehandling. Vidare fann vi att genuttrycket av *FKBP5* i SF var associerat med markörer för IR. Det fanns dessutom en tendens för *FKBP5* genen att vara förhöjd hos patienter med T2D. Genom saminkubation av en FKBP51-specifik inhibitor med glukokortikoider i SF kunde vi påvisa att inhibering av FKBP51 delvis kan förhindra den hämning av glukosupptag i adipocyter som glukokortikoidbehandling medför.

Avslutningsvis har vi här identifierat CNR1 och FKBP51 som potentiella måltavlor för läkemedel i T2D och glukokortikoid-inducerad IR. Båda har visat sig ha ett förhöjt uttryck i human fettvävnad efter glukokortikoidbe-

handling. Dessutom är deras genuttryck i UF associerade med markörer för IR och tenderar att ha ett förhöjt genuttryck i T2D. Båda kan dessutom vara involverade i aspekter av metaboliska störningar i fettceller; bland annat inom glukos- och lipidmetabolism.

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