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THE HPA AXIS IN TYPE 2 DIABETES

SOME ASPECTS IN RELATION TO INSULIN SENSITIVITY, BETA-CELL FUNCTION AND IGF-I/IGFBP-1

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To mom and dad

ABSTRACT

Type 2 diabetes (T2D) is characterized by insulin resistance and β-cell failure, abdominal obesity, hypertension and dyslipidemia. These symptoms are also characteristic of states of hypercortisolism. The purpose of this thesis was to investigate how cortisol and regulation of the hypothalamus-pituitary-adrenal (HPA) axis is affected in subjects with T2D compared with healthy subjects (**study I**). We investigated the effects of improved insulin sensitivity and β-cell function during treatment with pioglitazone (**study II and III**) or sitagliptin (**study IV**), as well as effect of gender (**study I and III**), on serum cortisol and the HPA axis. We further examined the relationship between cortisol and insulin-like growth factor-I (IGF-I) and hepatic insulin sensitivity assessed using IGF-binding protein-1 (IGFBP-1).

Is adrenal sensitivity to ACTH affected by T2D or gender? (Study I)
Serum cortisol was measured basally, after stimulation of the HPA axis with 1 μg adrenocorticotropic hormone (ACTH) and feedback inhibition with 0.25 mg dexamethasone (DEX) in patients with T2D (n = 21, HbA_{1c} = 49 ± 2 mmol/mol) and healthy controls (n = 39). Adrenal sensitivity to ACTH was higher in healthy women compared to men. This gender difference was lost in T2D, due to increased cortisol response in men. Neither basal serum cortisol nor sensitivity to DEX differed between subjects with T2D and controls.

Effect of pioglitazone on cortisol and IGF-I in T2D and IGT (Study II)
Overweight men (BMI ≥28 kg/m^2) with T2D (n = 10, HbA_{1c} = 70 ± 7) and impaired glucose tolerance (IGT; n = 10) were treated with pioglitazone 30-45 mg daily for 12 weeks in addition to pre-existing therapy. Basal and stimulated cortisol did not differ at baseline. Improved insulin sensitivity and β-cell function after treatment was associated with decreased basal and peak cortisol after ACTH in T2D, while IGF-I increased. Paradoxically, basal and peak cortisol increased in IGT.

Are there gender differences in the effects of pioglitazone in T2D? (Study III)
Men (n = 28) and women (n = 20) with T2D and HbA_{1c} >57 mmol/mol despite treatment with metformin and sulphonylurea were treated with pioglitazone 30-45 mg daily for 26 weeks. Basal cortisol increased in women despite improved insulin sensitivity. IGF-I and IGFBP-1 increased regardless of gender.

Is sitagliptin effect related to cortisol or hepatic insulin sensitivity? (Study IV)
Patients admitted to hospital for ACS and in whom an oral glucose tolerance test revealed previously unknown T2D (n = 24) or IGT (n = 47) were randomized to sitagliptin 100 mg once daily for 12 weeks, or placebo. Cortisol decreased regardless of treatment, but was unaffected by sitagliptin as was IGF-I and IGFBP-1.

Conclusions
Study I showed that adrenal sensitivity to ACTH is elevated in men with T2D, abolishing the gender difference seen in healthy subjects. This underscores the importance of accounting for gender in future studies on the HPA axis and T2D. In **study II and III**, improved insulin sensitivity and β-cell function by pioglitazone was associated with changes in basal and stimulated cortisol, but the effect differed between groups. IGF-I increased during pioglitazone therapy in patients with T2D. This may be an effect of improved lipid metabolism and contribute to improved insulin sensitivity. Cortisol levels decreased over the coming weeks after ACS, along with improved insulin sensitivity (**study IV**). The effect of sitagliptin did not appear to be exerted via lowering cortisol, or increasing hepatic insulin sensitivity as measured by IGFBP-1. Differences in findings between our studies may depend on heterogeneity of the groups, as e.g. metabolic control and obesity affect the HPA axis.

SAMMANFATTNING

Typ 2-diabetes (T2D) kännetecknas av insulinresistens och β-cellssvikt, bukfetma, högt blodtryck och höga blodfetter. Dessa symtom är även typiska vid tillstånd då stresshormonet kortisol är förhöjt. Syftet med denna avhandling var att undersöka hur kortisol, och regleringen av hypothalamus-hypofys-binjurebarksaxeln (HPA-axeln), påverkas hos patienter med T2D jämfört med friska (**studie I**). Vi studerade hur kortisol och HPA-axeln påverkades när insulinkänslighet och β-cellsfunktion förbättrades med pioglitazon (**studie II och III**) eller sitagliptin (**studie IV**), samt effekten av kön på detta system och behandlingseffekt (**studie I och III**). I samtliga studier analyserades relationen mellan kortisol och insulin-liknande tillväxtfaktor-I (IGF-I) och IGF-bindarprotein-1 (IGFBP-1).

Påverkas binjurebarkens känslighet för ACTH av T2D eller kön? (studie I)
Patienter med T2D (n = 21, HbA_{1c} = 49 ± 2 mmol/mol) jämfördes med friska kontroller (n = 39). Serumkortisol mättes basalt, efter stimulering av HPA-axeln med 1 µg adrenokortikotropt hormon (ACTH) och efter intag av 0.25 mg av kortisolanalogen dexametason (DEX) för att testa känslighet för feedbackhämning. Känsligheten för ACTH var högre hos friska kvinnor jämfört med män. Denna könsskillnad saknades vid T2D p.g.a. ökat kortisolsvar hos män. Varken basalt kortisol eller hämning efter dexa skiljde sig åt mellan patienter med T2D och kontroller.

Effekten av pioglitazon på kortisol och IGF-I vid T2D och IGT (studie II)
Överviktiga män (BMI ≥28 kg/m^2) med T2D (n = 10, HbA_{1c} = 70±7) och nedsatt glukostolerans (IGT; n = 10) behandlades med pioglitazon 30-45 mg dagligen i 12 veckor utöver deras tidigare diabetesmedicinering. Basalt och stimulerat kortisol skiljde sig inte mellan grupperna före behandling. I samband med att insulinkänslighet och β-cellsfunktion förbättrades under behandling, så sjönk basalkortisol samt peak-kortisol efter ACTH hos T2D-patienterna, medan IGF-I ökade. Däremot steg basal- och peak-kortisol hos patienterna med IGT.

Finns könsskillnader i pioglitazons effekter vid T2D? (studie III)
Män (n = 28) och kvinnor (n = 20) med T2D och HbA_{1c} >57 mmol/mol trots behandling med metformin och sulfonylurea fick tilläggsbehandling med pioglitazon 30-45 mg i 26 veckor. Basalkortisol ökade hos kvinnor trots ökad insulinkänslighet. IGF-I och IGFBP-1 ökade oberoende av kön.

Är sitagliptins effekt beroende av kortisol eller hepatisk insulinkänslighet? (studie IV)
Patienter som lades in på sjukhus p.g.a. ACS och där oralt glukostoleranstest visade tidigare okänd T2D (n = 24) eller IGT (n = 47) randomiserades till sitagliptin 100 mg dagligen i 12 veckor, eller placebo. Basalkortisol minskade oavsett behandling. Sitagliptin hade ingen effekt på kortisol, IGF-I eller IGFBP-1.

Slutsatser
Studie I visade att binjurebarkens känslighet för ACTH är förhöjd hos män med T2D. Således saknades vid T2D könsskillnaden som sågs hos friska. Detta understryker vikten av att beakta skillnader mellan kvinnor och män i framtida studier på HPA-axeln och T2D. Kortisolnivån påverkades efter förbättring av insulinkänslighet och β-cellsfunktion med pioglitazon (**studie II och III**), men typen av förändring skiljde sig mellan grupper med olika glukostolerans. IGF-I ökade hos patienter med T2D vid pioglitazonbehandling. Detta återspeglar sannolikt förbättrad lipidomsättning, och kan bidra till ökningen i insulinkänslighet. Kortisolnivån sjönk under de kommande veckorna efter ACS samtidigt som insulinkänsligheten förbättrades. Sitagliptins effekter tycks inte vara beroende av kortisol eller förbättrad insulinkänslighet i levern (mätt via IGFBP-1). Skillnader i resultat mellan delstudierna kan bero på heterogena grupper, med varierande metabol kontroll, vikt mm vilket påverkar HPA-axeln.

LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to by their Roman numerals.

I. **Arnetz L**, Rajamand Ekberg N, Brismar K, Alvarsson M. Gender difference in the adrenal response to ACTH is abolished in type 2 diabetes. *Submitted for publication.*
II. **Arnetz L**, Rajamand Ekberg N, Höybye C, Brismar K, Alvarsson M. Improved insulin sensitivity during pioglitazone treatment is associated with changes in IGF-I and cortisol secretion in type 2 diabetes and impaired glucose tolerance. *ISRN Endocrinology. 2013;148497. Epub 2013 Jan 15.*
III. **Arnetz L**, Dorkhan M, Alvarsson M, Brismar K, Rajamand Ekberg N. Gender differences in non-glycemic responses to improved insulin sensitivity by pioglitazone treatment in patients with type 2 diabetes. *Acta Diabetologica. 2013 Jan 7. [Epub ahead of print]*.
IV. **Arnetz L**, Hage C, Rajamand Ekberg N, Alvarsson M, Brismar K, Mellbin L. Improved glycemic control with sitagliptin treatment is not related to cortisol or hepatic insulin sensitivity. *Manuscript.*

LIST OF PUBLICATIONS NOT INCLUDED IN THESIS

I. **Arnetz L**, Lantz M, Brismar K, Rajamand Ekberg N, Alvarsson M, Dorkhan M. Effect of pioglitazone of thyroid hormones and IGF-I in patients with type 2 diabetes. *Thyroid Disorders Ther. 2013;3:139. In press.*

II. Spectre G, **Arnetz L**, Östensson C-G, Brismar K, Li N, Hjemdahl P. Twice daily dosing of aspirin improves platelet inhibition in whole blood in patients with type 2 diabetes and micro- or macrovascular complications. *Thrombosis and Haemostasis. 2011 Sep;106(3):491-9.*

LIST OF ABBREVIATIONS

11βHSD	11β-hydroxysteroid dehydrogenase
ACS	Acute coronary syndrome
ACTH	Adrenocorticotropic hormone
AIR_g	Acute insulin response to glucose
ALAT	Alanine amino transferase
AUC	Area under the curve
BMI	Body mass index
CBG	Cortisol binding globulin
CRH	Corticotropin-releasing hormone
CV	Coefficient of variation
CVD	Cardiovascular disease
Δ	Delta, i.e. "change in"
D-AUC	Delta area under the curve
DEX	Dexamethasone
DPP-4	Dipeptidyl peptidase-4
FFA	Free fatty acid
γ-GT	Gamma-glutamyl transferase
GH	Growth hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GLP-1	Glucagon-like peptide-1
HDL	High-density lipoprotein
HGP	Hepatic glucose production
HOMA-β	Homeostatic model of β-cell function
HOMA-IR	Homeostatic model of insulin resistance
HPA	Hypothalamus-pituitary-adrenal
IGF-I	Insulin-like growth factor-I
IGFBP-1	Insulin-like growth factor binding protein-1
IGI	Insulinogenic index
IGT	Impaired glucose tolerance
LDL	Low-density lipoprotein
NGT	Normal glucose tolerance
OAD	Oral antidiabetic drug
OGTT	Oral glucose tolerance test
PI/I	Proinsulin/insulin ratio
PPARγ	Peroxisome-proliferator activated receptor-γ
Repa	Repaglinide
RIA	Radioimmunoassay
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SEM	Standard error of the mean
SU	Sulphonylurea
T2D	Type 2 diabetes
TG	Triglycerides
TZD	Thiazolidinedione
VAT	Visceral adipose tissue

“For every fact, there is an infinity of hypotheses”

-Robert Pirsig, *Zen and the Art of Motorcycle Maintenance (1974)*

TABLE OF CONTENTS

1 INTRODUCTION

1.1 TYPE 2 DIABETES (T2D)

T2D affects 350 million people worldwide today, an increase of 150% compared with 30 years ago. Major contributing factors are obesity, physical inactivity and ageing superimposed on a genetic predisposition (1). Prevalence increases with age in both men and women, and T2D may be more common in older women compared with men (2).

Impaired glucose tolerance (IGT; see *1.1.1.2*) and impaired fasting glucose (IFG) are together at least twice as common as T2D (3). These glucose abnormalities are sometimes known as pre-diabetes, as they often progress to T2D. They often go undiagnosed, but just like T2D they entail an increased risk of cardiovascular disease (CVD) (4).

1.1.1 Pathophysiology – β-cell dysfunction and insulin resistance

Maintenance of normoglycemia is dependent on the β-cells, liver and peripheral organs, particularly the skeletal muscles and adipose tissue (see fig 1 and 1.1.3) (5). Insulin stimulates glucose uptake and lipogenesis, and inhibits hepatic glucose production (HGP) and lipolysis. Therefore, insulin resistance and T2D are associated with dyslipidemia, especially elevated triglycerides (TG), as well as hyperglycemia (6). Both insulin resistance and impaired β-cell function contribute to the development of T2D (7).

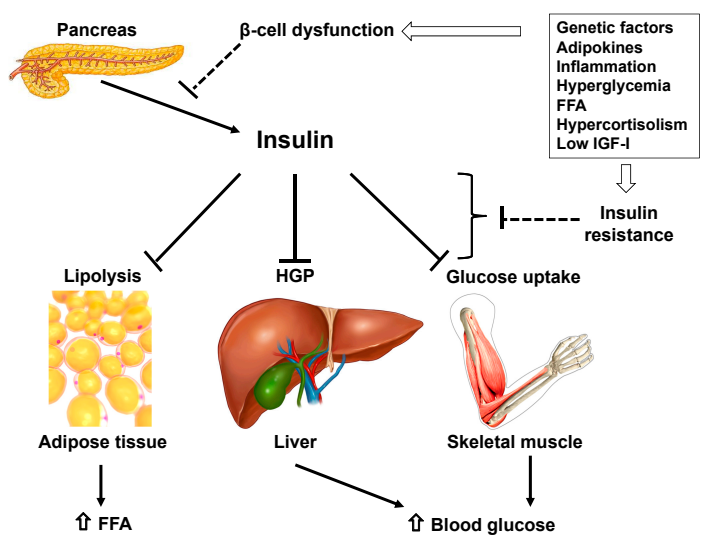

Fig. 1. Functions of insulin, and pathology in T2D. FFA = free fatty acids, HGP = hepatic glucose production, IGF-I = insulin-like growth factor-I. Arrows signify stimulation; lines with flat ends signify inhibition.

1.1.1.1 β-cell dysfunction

Serum insulin levels are determined by insulin secretion, degradation and sensitivity (8). β-cell dysfunction is manifest many years before clinical T2D develops (9). Remaining β-cell function may be from 50 to less than 15% at the time of T2D diagnosis (10).

A reduced acute insulin response to glucose (first-phase insulin secretion) is a strong predictor of T2D (10), and in manifest T2D both the first and second phase of insulin secretion are decreased (11, 12). The loss in β-cell function is strongly influenced by genetic factors, but insulin resistance is an important acquired trigger (10).

Multiple factors contribute to β-cell dysfunction in T2D. Chronically elevated levels of glucose and free fatty acids (FFA) impede insulin signaling, phenomenona known as *glucotoxicity* (13) and *lipotoxicity* (14). β-cells may be subjected to deposition of islet amyloid polypeptide and apoptosis triggered by FFA (10, 15). Dysfunction of the incretin system is another important factor (see 1.7).

1.1.1.2 Insulin resistance

Insulin resistance is a condition in which cells fail to respond adequately to the normal actions of insulin. This results in elevated HGP and lipolysis, and reduced peripheral glucose uptake despite hyperinsulinemia (16). Cortisol, glucagon, catecholamines, growth hormone (GH) and hyperinsulinemia all downregulate insulin's effects at the receptor and post-receptor level (6).

Insulin resistance is common in obese subjects, but most maintain normal fasting glucose through compensatory hyperinsulinemia (17). However in a subset of patients, β-cell function progressively fails to the point that hyperglycemia develops (18). Therefore, β-cell dysfunction is the deciding factor in whether or not insulin resistance progresses to T2D (7).

Impaired glucose tolerance (IGT)

Subjects with IGT are often overweight and have normal fasting plasma glucose but are insulin resistant, with elevated glucose and insulin after an oral glucose tolerance test (OGTT) (19). Insulin levels are lower after OGTT in subjects with IGT and a family history of diabetes compared to those without (20), underscoring the impact of genetic factors on β-cell dysfunction. The annual progression from IGT to T2D ranges from 3 to over 8% (21).

Insulin signaling

Glucose uptake is insulin dependent in most cell types. When insulin binds to insulin receptors on the cell surface, intracellular second messengers such as insulin-like receptor substrate 1 (IRS-1) and phosphoinositol 3-kinase (PI-3K) are activated and recruit glucose transporters (GLUT) to the cell surface. Insulin resistance is associated with disturbed signaling in a multitude of steps in this pathway (22).

Skeletal muscle

Skeletal muscle is the dominant site of post-prandial glucose disposal (23). While the exact cause of insulin resistance in skeletal muscle in unknown, contributing factors

may be mitochondrial dysfunction and detrimental effects of unmetabolized FFA on insulin signaling (24). Skeletal muscle biopsies from patients with T2D contain fewer and smaller mitochondria than samples from healthy controls, with reduced mitochondrial respiration and FFA oxidation (25, 26).

Liver
In the fasting state, HGP from glycogenolysis and gluconeogenesis ensures stable blood glucose levels. After a meal glucose and insulin levels rise, and the liver switches from glucose production to storage. The increase in insulin, delivered to the liver via the portal vein, and decline in glucagon (see 1.7) inhibit HGP (27). In T2D, HGP is increased in fasting and resistant to suppression after meals due to hepatic insulin resistance and elevated glucagon levels (5, 22, 28). Elevated FFA are important in the pathogenesis of hepatic insulin resistance (see 1.1.2)

1.1.2 Lipolysis and insulin resistance in adipose tissue

The adipose tissue consists of preadipocytes, adipocytes, and a vascular stromal fraction containing blood vessels, macrophages and endothelial cells (29). It is not just a storage site, but also an important endocrine organ. Although it accounts for only a fraction of total insulin-mediated glucose disposal, insulin resistance in the adipose tissue is associated with increased risk of IGT and T2D (30). Subcutaneous adipose tissue (SAT) comprises on average 80% of the body's fat depot, but is less metabolically active than visceral adipose tissue (VAT).

Insulin inhibits lipolysis and thereby release of FFA from the adipose tissue (31). When insulin levels are low, such as in fasting, the cell shifts to utilization of FFA for energy rather than glucose. Fat is stored in intracellular droplets as TG, which are hydrolyzed to FFA and glycerol through lipolysis (see *1.1.4.2*) mediated mainly by hormone sensitive lipase (HSL) and adipose triglyceride lipase (32). Compared with SAT, VAT is more sensitive to lipolytic signals and less sensitive to insulin's antilipolytic effect, resulting in increased lipolysis and FFA release (33). FFA secreted into the portal vein from VAT flood the liver where they contribute to hepatic insulin resistance and increased gluconeogenesis (34). Visceral obesity is therefore associated with more severe insulin resistance than peripheral obesity (35). Mitochondrial function may also be impeded, as in skeletal muscle (36).

1.1.2.1 Adiponectin
Adiponectin is a hormone synthesized exclusively in adipose tissue (37). It increases insulin sensitivity of the liver (reducing HGP) and skeletal muscle (38). Adiponectin synthesis is stimulated by insulin, insulin-like growth factor I (IGF-I; see 1.3) and peroxisome-proliferator activated receptor-γ (PPARγ; see 1.6), and inhibited by glucocorticoids, β-adrenergic stimulation, cytokines and androgens (39-41). Serum adiponectin is higher in women than in men (42). Levels decrease in insulin resistance and obesity (43), and are lower in patients with T2D compared with controls (44).

1.1.3 Indices of insulin sensitivity and secretion

1.1.3.1 Whole-body insulin sensitivity

The gold standard for assessing whole body insulin sensitivity is the *hyperinsulinemic euglycemic clamp* (45). However, as this method is time and labor consuming, alternative methods have been developed. Both T2D and IGT can be diagnosed using an *OGTT* (see 3.2.2), which reflects the body's ability to dispose of glucose (46). Insulin sensitivity can be estimated from the OGTT using the *area under the curve (AUC)* for glucose and insulin (47). As the AUC is affected by the basal levels, which vary between individuals, the AUC can be standardized to *delta-area under the curve (D-AUC), which* calculates the AUC corrected for basal levels.

1.1.3.2 Index of hepatic insulin sensitivity

The *homeostatic model of insulin resistance (HOMA-IR)* is based on fasting levels of glucose and insulin (see 3.4.2) (48). HOMA-IR correlates well with more complex techniques, although its results must be interpreted with its limitations in mind (49).

1.1.3.3 Indices of β-cell function

HOMA-β estimates β-cell function based on fasting glucose and insulin levels (see 3.4.3) (48). It is important to keep in mind that it provides only an approximation, less accurate than other methods that test β-cell function dynamically (46).

The *insulinogenic index (IGI)* is an index of β-cell function derived from measurements during the OGTT (see 3.4.4). Although it is closely correlated with actual insulin secretion (50), it is less accurate than the frequently sampled intravenous (iv) glucose tolerance test or clamp studies (51).

Proinsulin is spliced to insulin and C-peptide in the β-cells. Around 2% is released along with insulin into the circulation in healthy individuals (52), more so in T2D (53). Both proinsulin and the *proinsulin-insulin ratio (PI/I)* are markers of reduced β-cell function and β-cell stress (54, 55).

1.1.4 Microdialysis and its use in studying insulin sensitivity of the adipose tissue

1.1.4.1 Basic principles of microdialysis

Microdialysis technique allows for continuous monitoring of biochemical events in various tissues in vivo. The system consists of a microdialysis pump, a double-lumen catheter with a semipermeable tubular membrane that is inserted into the tissue of interest and perfused with sterile Ringer solution, and a collection vial (microvial; fig. 2) (56). As the fluid passes the membrane, low molecular weight substances in the interstitial fluid surrounding the catheter diffuse into (*recovery*) or out of (*delivery*) the perfusion fluid depending on the concentration gradient (fig 3). Usually the perfusion fluid mimics the composition of the medium surrounding the catheter, but lacks the substances of interest to study e.g. glucose. The fluid is termed *perfusate* while in the microdialysis system, and *dialysate* once collected in the microvial.

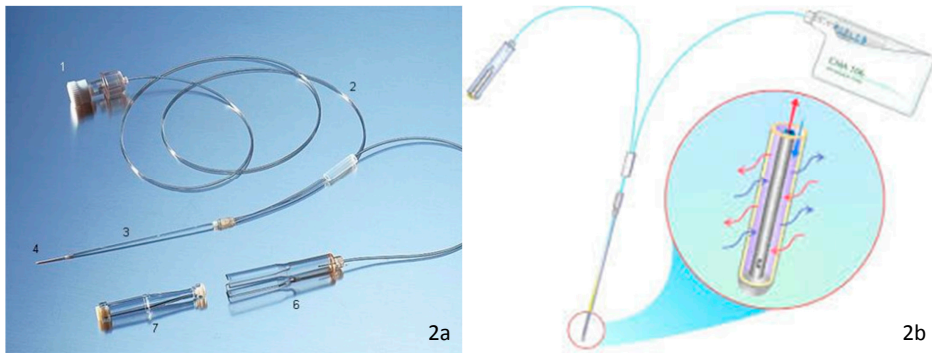

Figure 2. Microdialysis system (2a). 1= pump connector, 2 = inlet tube, 3 = microdialysis catheter, 4 = microdialysis membrane, 5 = outlet tube, 6 = microvial holder, 7 = microvial. Microdialysis system with pump connected (2b), showing analyte flux. CMA microdialysis; www.microdialysis.com

The type and amount of substances collected during microdialysis are determined by:

- *Molecular weight cut-off*, i.e. the upper size limit for particles that can diffuse across the membrane
- The composition of the perfusate, and hence the *concentration gradient.*
- The length and area of the membrane
- The *flow rate* of the dialysate; the lower the flow rate, the higher the recovery
- Temperature and tortuosity of the fluids

In SAT, a flow rate of 0.3 µL/minute and membrane length of 30 mm provides nearly 100% recovery both in healthy subjects and those with T2D (57).

1.1.4.2 Studying metabolism in SAT using microdialysis

Glucose enters the cell via GLUT4 and is converted to pyruvate through glycolysis (fig. 3). If the oxygen supply is adequate, pyruvate enters the mitochondria and the Creb's cycle, generating energy in the form of adenosine tri-phosphate (ATP). Alternately, it is converted to lactate in anaerobic metabolism, also generating ATP although to a lesser extent (24). Glycerol is produced from lipolysis of TG, which yields glycerol alongside FFA. Pyruvate, lactate and glycerol are building blocks for hepatic gluconeogenesis and can diffuse out of the cells and back to the liver for this purpose (58). Glucose, pyruvate and lactate can be measured in the interstitium during microdialysis of the SAT, and reflect glucose utilization. Measurement of glycerol reflects lipolysis (59).

After glucose ingestion, interstitial glucose, pyruvate and lactate in SAT rise, returning to baseline levels within three hours (60). Both glucose utilization and lipolysis in SAT are influenced by insulin; hence, measurement of glucose and products of glycolysis and lipolysis can be used to estimate insulin sensitivity in the SAT (61). Interstitial glycerol concentrations are increased in subjects with T2D, despite hyperinsulinemia (61). Lactate generation from adipose tissue is higher in obese subjects after glucose intake compared with healthy controls (62).

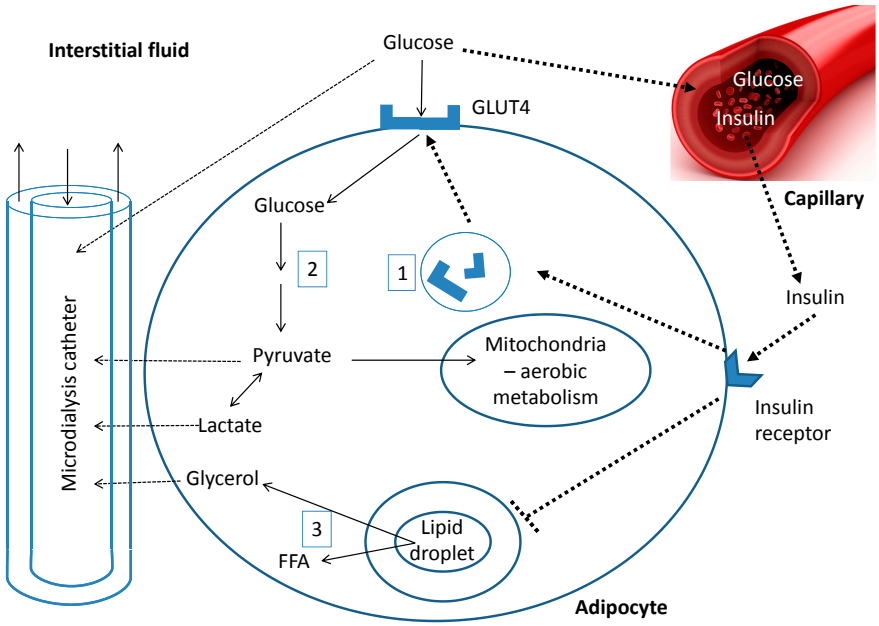

Figure 3. Microdialysis in SAT. 1 = Vesicle containing glucose transporter 4 (GLUT4). 2 = glycolysis. 3 = lipolysis.

1.2 THE HYPOTHALAMUS-PITUITARY-ADRENAL (HPA) AXIS

1.2.1 Cortisol – molecular mechanisms and effects

1.2.1.1 Effects of cortisol

Cortisol is a glucocorticoid that is essential to life. It enables adaptation to fasting and states of stress by mobilizing glucose and FFA for energy, increasing cardiovascular tone, and inhibiting energy demanding processes such as digestion and, in the long term, reproduction (63).

The net effect of cortisol is to increase blood glucose. Even increases of serum cortisol within the physiological range increase insulin resistance (64). Cortisol may reduce insulin receptor number or affinity (65) and lowers peripheral glucose disposal (66). It increases HGP by upregulating genes involved in gluconeogenesis, counteracting insulin which instead downregulates them (67).

Glucocorticoids increase lipolysis via HSL (68) and adrenergic mechanisms (69). Insulin and cortisol both increase expression of lipoprotein lipase (LPL) in adipose tissue, contributing to adipogenesis (70). Under normal circumstances adipose tissue mass remains constant, due to a balance between cortisol and insulin promoting lipid accumulation, and sex steroids and GH (see *1.3.1.3*) promoting lipolysis (71). The increased tendency toward lipolysis in VAT is in part attributed to high density of GRs (72), and β_3 adrenergic receptors which are regulated by cortisol (69).

1.2.1.2 Glucocorticoid receptors (GR)

GR are subdivided into GR type I (GR-I, the mineralocorticoid receptor) and II (GR-II, the classic glucocorticoid receptor). GR-I has high affinity for aldosterone and cortisol and is activated at lower (basal) cortisol levels. GR-II has lower affinity but high capacity for cortisol and responds to higher concentrations. The degree of GR expression determines cellular glucocorticoid sensitivity (73).

GRs belong to the steroid hormone receptor family and exert their effect by non-genomic as well as classic genomic mechanisms. The non-genomic effects occur within seconds to minutes from hormone exposure when cortisol affects ion channels or binds to receptors on the cell membrane, activating second messengers (74). For the genomic effects, glucocorticoids must diffuse into cells where they bind to GR in the cytosol. These complexes translocate into the nucleus and interact with glucocorticoid response elements (GREs) in the DNA, activating or inactivating gene transcription (63).

1.2.2 Regulation of the HPA axis

The neurons that regulate the HPA axis are located in the hypothalamic paraventricular nucleus (PVN) (73). Stimulating impulses are initiated from the cortex and amygdala in response to stress, whereas variation in inhibitory input from the suprachiasmatic nucleus regulates the circadian sleep-wake cycle (see *1.1.2.1*) (73). Vasopressin (anti-diuretic hormone) is co-released with corticotropin releasing hormone (CRH) and acts synergistically with it to potentiate release of adrenocorticotropic hormone (ACTH) (73). CRH reaches the pituitary gland via the portal circulation between the hypothalamus and pituitary, and stimulates ACTH synthesis and release (63). ACTH stimulates cortisol synthesis and release from the zona fasciculata of the adrenal cortex, and contributes to release of dihydroepiandrostenedione and aldosterone from the zona reticularis and glomerulosa respectively (73).

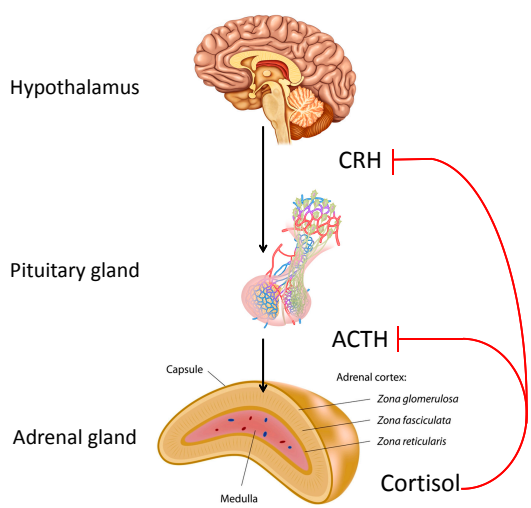

Figure 4. The hypothalamus-pituitary-adrenal (HPA) axis.

Rising cortisol levels initiate negative feedback on the basal and stress-induced activity of the HPA axis. Cortisol binds to GR in the hypothalamus and hippocampus, reducing

CRH and ACTH secretion. ACTH also inhibits CRH release from the hypothalamus. If the cortisol exposure is brief (minutes), rapid feedback occurs via non-genomic mechanisms (73). Longer cortisol exposure inhibits both basal and stimulated ACTH release through genomic effects, reducing mRNA levels of the ACTH precursor pro-opiomelanocortin (POMC) (75). GR-I is most important for feedback inhibition under basal conditions, whereas GR-II dominates after stress.

1.2.2.1 Circadian rhythm

CRH and vasopressin are secreted in a pulsatile manner, with increased pulse amplitudes in the early morning. This results in increased amplitude and frequency of ACTH and cortisol secretion (63). Cortisol levels peak at 6-9 a.m. and then fall during the day, reaching a nadir in the evening and early night (76).

1.2.2.2 Activation of the HPA axis during stress

In acute stress, the amplitude of CRH pulses increases (63). ACTH and cortisol levels peak within 5-30 minutes, and are normalized within hours if the stimulus is brief (77). The PVN is densely innervated with adrenergic and noradrenergic fibers arising from the locus ceruleus and the nucleus tractus solitarius (NTS), among other sites. Upon stressful stimuli these neurons are activated, releasing catecholamines in the PVN that stimulate CRH release. Signaling from the NTS also increases cardiovascular tone (73). During prolonged stress, the expression of CRH mRNA is increased and ACTH and cortisol remain elevated (77). However in chronic stress, CRH receptors in the pituitary and GR in the hippocampus may ultimately be downregulated, resulting in lower stress-induced cortisol secretion and impaired feedback inhibition (77, 78).

1.2.3 Hypercortisolism

While short-term elevations of cortisol are vital to survival, chronic hypercortisolism is associated with hypertension, hyperglycemia, dyslipidemia, and insulin resistance (79). Glucose-stimulated insulin secretion is reduced (80), partially because of elevated FFA, which contribute to insulin resistance (81). Muscle mass is lost due to proteolysis (82).

Hypercortisolism also triggers abdominal obesity. This is obvious in Cushing's syndrome, in which body composition is normalized after successful treatment (79). VAT expands while peripheral fat depots decrease (83) due to increased expression of LPL without concomitant increase in lipid mobilization. Both of these effects are due to cortisol in the presence of insulin (71) and occur primarily in visceral depots due to higher GR density (72).

1.2.4 Other determinants of cortisol levels and action

1.2.4.1 Cortisol-binding globulin (CBG)

Only 5-10% of the circulating pool of cortisol is free, and thereby able to diffuse across cell membranes and interact with GR. 70-75% is bound to CBG and 15-20% to albumin (65). CBG levels are negatively correlated with glucocorticoid activity (84). When serum cortisol exceeds 400-500 nmol/L the binding capacity of CBG becomes saturated and free cortisol increases exponentially (85). At the same time, the lower the % protein-bound hormone, the higher the metabolic clearance rate (86).

1.2.4.2 Peripheral cortisol metabolism

Cortisone is a glucocorticoid closely related to cortisol but metabolically inactive (87). The two are interconverted peripherally by 11β-hydroxysteroid dehydrogenase (11βHSD). 11βHSD type 1 (11βHSD) reduces cortisone to cortisol and 11βHSD type 2 (11βHSD2) does the opposite (88). 11βHSD1 is expressed in insulin's target tissues (liver, adipose tissue and to some extent skeletal muscle) (89), whereas 11βHSD2 is expressed in e.g. the kidney, allowing aldosterone more access to GR-I (90). The degree of local expression of 11βHSD1 regulates the cortisol exposure of each individual tissue and is e.g. higher in VAT than SAT (91, 92). 11βHSD1 is downregulated in visceral obesity (93). Cortisol is excreted via the urine in free form or metabolized form, primarily via 5α-reductase in the liver (94).

1.2.5 Evaluation of the HPA axis

The insulin tolerance test (ITT) has been considered the gold standard for evaluation of the HPA axis. However, as it is time-consuming and potentially dangerous, alternative methods have been developed (95). Basal morning cortisol correlates well with peak cortisol during the ITT (96). On the other hand, it does not provide any information on the dynamics of the HPA axis. This can be achieved by administering CRH or ACTH to stimulate the axis (97), or dexamethasone (DEX; a cortisol analogue that binds to GR) to examine the sensitivity to feedback inhibition (98).

The standard 250-μg ACTH stimulation test induces supraphysiological ACTH levels, and may therefore not be sensitive enough to reveal more discrete disturbances in the HPA axis (99). The low-dose, 1-μg test provides a more "physiological" stimulation of the adrenal cortex compared with the standard dose (see *3.2.1.1*) (99). Results also correlate more strongly with the ITT (99). The same applies to the DEX test. In most subjects, serum cortisol is completely suppressed by 1 mg of DEX, while 0.25 mg gives a smaller reduction (100). Hence, the low-dose DEX test may be better suited for detecting discrete disturbances in the sensitivity of the HPA axis to feedback inhibition (see *3.2.1.3*) (101). For example, subjects with abdominal obesity display normal reduction of serum cortisol to 1 mg of DEX, but have reduced sensitivity to lower doses (98).

The range for normal morning levels of basal serum cortisol is wide (200 - 800 nmol/L) and previous studies from our group on patients with IGT and T2D have found such patients to have levels within this range when measured at 8 a.m. (102, 103). Studies in normal populations have shown the range in serum cortisol to be smaller at 10 a.m. compared to 8 a.m. (104). Measurement of cortisol at 10 a.m. may provide better opportunity to discover differences between groups.

1.3 INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP-1)

1.3.1 The somatotropic axis – GH and IGF-I

1.3.1.1 Growth hormone (GH)

GH is secreted in pulses from the pituitary gland in response to hypothalamic growth hormone releasing hormone (GHRH) and amino acids (105). It promotes anabolism when nutrional supply is adequate, mainly via IGF-I (106). IGF-I feeds back to the hypothalamus and pituitary gland to inhibit GH secretion (105).

In states of fasting or starvation, GH exerts effects independently of IGF-I to mobilize substrates for energy (fig. 5) (105). It raises FFA by stimulating lipolysis, increases HGP and reduces glucose uptake. Elevation of FFA reduces insulin sensitivity (105). When blood glucose and FFA levels are high, i.e. when nutrients are plentiful, GH secretion is inhibited (107).

1.3.1.2 IGF-I

IGF-I is a peptide hormone, mainly produced in the liver (108, 109). It shares structural and functional properties with insulin, and also increases insulin sensitivity both through its own effects and by inhibiting GH (110). IGF-I stimulates glucose uptake, although less acutely than insulin, and inhibits HGP (111). Synthesis also occurs locally in tissues such as the ovaries (112), kidney (113) and adipose tissue, where IGF-I has autocrine and paracrine effects. IGF-I levels decrease with age (114).

Although GH stimulates synthesis of IGF-I, they have partially opposite metabolic effects (fig. 5). While GH increases FFA (lipolysis) and glucose levels, IGF-I reduces them and increases insulin sensitivity (105, 110).

IGF-I receptors (IGF-IR) are similar in structure to insulin receptors and act via the same intracellular signaling cascade (115). IGF-I can bind to IGF-IR, the insulin receptor and hybrid receptors composed of subunits of IGF-IR and the insulin receptor. IGF-IR is expressed in most cells in the human body with the exception of the liver and possibly the adipose tissue (110). However IGF-I exerts effects in these tissues via insulin and hybrid receptors (110, 116).

1.3.1.3 Regulation of IGF-I production – growth hormone, insulin and nutrients

GH, sufficient insulin levels and nutritional status are required for IGF-I synthesis (fig. 5) (105). Insulin stimulates IGF-I synthesis by increasing transcription of the hepatic *IGF-I* gene (108, 109). This applies particularly to insulin delivered to the liver via the portal vein, which also upregulates hepatic GH receptors (117). When insulin levels are low during fasting, IGF-I synthesis decreases despite GH being elevated, which reduces the risk of hypoglycemia (118). FFA stimulate IGF-I synthesis, contrary to their inhibiting effect on GH (110).

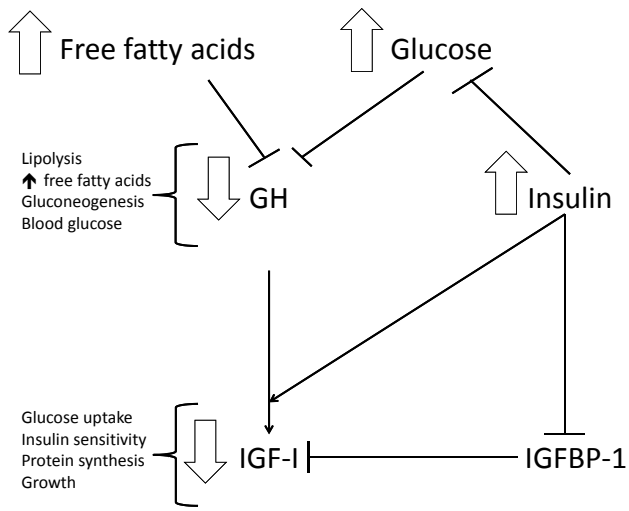

Figure 5. Regulation and effects of GH and IGF-I. Arrows signify stimulation, lines with flat ends signify inhibition, and bold arrows signify changes in T2D.

1.3.2 IGF-binding protein-1 (IGFBP-1)

Free IGF-I has a half-life of around 10 minutes (119) and has a high potential of causing hypoglycemia (120). However ≤1% of all IGF-I circulates freely. The rest is bound by a family of six IGF-binding proteins (IGFBP 1-6) (110). Only free IGF-I is bioactive (121). The dominant IGFBP, IGFBP-3, forms a ternary complex with IGF-I and a third protein, acid-labile subunit (122). This complex acts as a circulating reservoir for IGF-I and increases its half-life to 15 hours (122).

1.3.2.1 Effects of IGFBP-1

IGFBP-1 binds only a small fraction of IGF-I, but is important to its bioavailability as IGF-I can rapidly dissociate from IGFBP-1 into free/active form (123). Free IGF-I and IGFBP-1 are inversely correlated (124). Beyond binding IGF-I in the circulation, it enables its transport across the endothelium into target tissues (125).

1.3.2.2 Regulation of IGFBP-1 production and action

The primary source of IGFBP-1 is the liver (126). The main regulator of serum IGFBP-1 is insulin in the portal vein, which rapidly suppresses transcription of the hepatic *IGFBP-1* gene by binding insulin response elements (IRE; fig. 5) (121). IGFBP-1 is suppressed after meals or during insulin infusion (127, 128), and then rises gradually in fasting when insulin levels decline (127, 129, 130). Fasting serum IGFBP-1 and insulin are inversely correlated (131-134).

Cortisol enhances *IGFBP-1* gene transcription by binding corticoid responsive elements (CRE) (135). However, insulin is a more potent regulator and counteracts glucocorticoid-stimulated IGFBP-1 expression (121). In vivo, the stimulatory effect of glucocorticoids can be seen when insulin levels are low or normal (136, 137) and

IGFBP-1 levels are not elevated in patients with Cushing's syndrome perhaps due to hyperinsulinemia (138). Other factors that stimulate IGFBP-1 synthesis include fasting (130), glucagon (139), catecholamines (140), cytokines (141) and estrogen (142).

1.4 DYSREGULATION OF THE HPA AXIS AND IGF-I/IGFBP-1 IN OBESITY AND DIABETES

1.4.1 The HPA axis in obesity and T2D

The clinical characteristics of hypercortisolism and the metabolic syndrome clearly overlap, with abdominal obesity, hypertension, dyslipidemia, and insulin resistance/T2D (see 1.2.3.). Given these similarities and the profound effects of glucocorticoids on insulin sensitivity and β-cell function, could increased cortisol levels or sensitivity play a role in the development of the metabolic syndrome and T2D?

A healthy HPA axis is characterized by high variability and sensitivity to feedback inhibition. Björntorp et al have found this pattern to be associated with a favorable metabolic profile in men, with low cholesterol, body mass index (BMI), waist-hip ratio (WHR) and blood pressure and high IGF-I. In viscerally obese subjects, the HPA axis showed signs of hyper-reactivity. A final group showed low variability with little response both to stimulation and DEX. This profile was associated with elevated BMI, WHR, blood pressure and lipids, low testosterone levels (in men) and IGF-I (summarized in (98, 143); see fig. 6 and 1.4.6). Most subjects fell in between the two extremes of the highly variable and flat cortisol profiles. The authors hypothesized that dysregulation of the HPA axis gradually develops in the state of visceral obesity –from initially normal function, to hyper-reactivity, to a final state with inefficient stimulation and feedback, and suppression of the sex steroid and somatotropic axes. The sympathetic nervous system is instead upregulated, raising pulse and blood pressure (143).

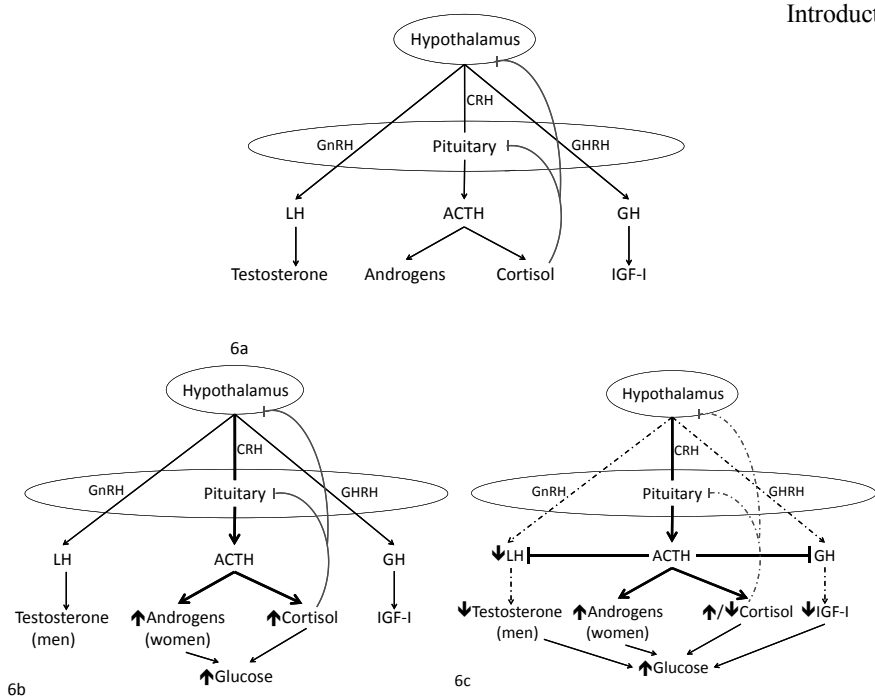

Figure 6. The central hormone axes regulating IGF-I, cortisol and sex steroids in healthy subjects (a), in subjects with visceral obesity with a hyperactive HPA axis (b) and impaired feedback inhibition and inhibition of the other axes.

ACTH = adrenocorticotropic hormone, CRH = corticotropin releasing hormone, GHRH = growth hormone releasing hormone; GnRH = gonadotropin releasing hormone; FSH = follicle stimulating hormone; LH = luteinizing hormone.

1.4.1.1 Basal and stimulated ACTH and cortisol

Most studies have found basal cortisol to be normal in obese subjects, regardless of whether abdominal obesity is factored into the study or not (93, 144-148). Yet, plasma cortisol levels are positively correlated with insulin resistance (149, 150). Basal ACTH has been reported to be either normal ((93, 146, 148, 151, 152) or elevated (144, 145, 153), but these studies only selected obese subjects based on BMI, not body fat distribution.

Studies on CRH responses in obese subjects selected only by BMI also show divergent results. Some reported no differences compared to normal-weight controls (147, 154, 155), while others found higher response in ACTH but not in cortisol (148, 152). The latter was also seen in one study on women with abdominal obesity compared to those with peripheral obesity (156), while another reported higher cortisol response both to CRH and ACTH in such subjects (see 1.5) (157).

In one study that has compared men and women with T2D to weight-matched controls, basal cortisol as well as cortisol and ACTH after CRH were elevated (158). Unstimulated ACTH and cortisol were also higher in patients with diabetic neuropathy compared to those without neuropathy (159).

Genetic factors are probably important in determining which patients with visceral obesity develop disturbances of the HPA axis. A polymorphism of the *GR* gene has been associated with insulin resistance and abdominal obesity, more so in homozygotes (13.7% of men) than in heterozygotes (143).

1.4.1.2 Sensitivity to feedback inhibition

In animal models, chronic activation of the HPA axis eventually results in down-regulation of central GR and impaired feedback inhibition (160). The same occurs in patients with Cushing's syndrome (161). Blunted feedback inhibition of cortisol after DEX of various doses has been shown in men and women with abdominal obesity (98, 162, 163) as well as in patients with T2D and type 1 diabetes (T1D) with acceptable or poor metabolic control (158, 164-166).

1.4.1.3 Role of FFA in glucocorticoid-induced insulin resistance

Visceral obesity is associated with elevated serum FFA, primarily coming from VAT (167). The density of glucocorticoid receptors (GR) is higher in VAT, allowing a greater glucocorticoid effect (72, 98). Glucocorticoids contribute to accumulation of VAT, and raise FFA levels by increasing lipolysis as outlined above. Treatment with supraphysiological glucocorticoid doses causes hyperinsulinemia, hyperglycemia and elevated FFA both in animals and humans (81, 168).

There is also considerable evidence that FFA modulate the activity of the HPA axis. Lipid infusion increases ACTH and cortisol secretion in rodents (169, 170). Pharmacological lowering of FFA improves glucose metabolism (81) and decreases ACTH secretion (144).

1.4.2 GH, IGF-I and IGFBP-1 in type 1 diabetes (T1D)

Even with subcutaneous insulin therapy, patients with T1D have low insulin levels in the portal vein. This results in low IGF-I levels, especially in patients with poor metabolic control (171). Decreased negative feedback by IGF-I leads to elevated GH secretion, potentially contributing to insulin resistance (172). Portal insulinopenia also removes the "brake" from IGFBP-1 synthesis, resulting in several-fold elevation of IGFBP-1 levels (173).

1.4.3 GH, IGF-I and IGFBP-1 in obesity and T2D

Obese subjects often have low spontaneous and stimulated GH-levels (fig. 6) (174). GH deficiency is associated with insulin resistance and visceral fat accumulation (71). Increased activity of the HPA axis may inhibit the somatotropic axis (see 5.9). Elevated FFA and free IGF-I may also inhibit GH secretion (174).

Total IGF-I has been found to be normal (124, 175) or low (176) in obese subjects. Again visceral obesity is detrimental, associated with lower IGF-I (177). Hyperinsulinemia reduces GH receptor expression and signaling in the liver (178). Bioactive IGF-I is instead elevated, most likely secondary to low IGFBP-1 (124, 174,

179). However, as hepatic insulin resistance increases and T2D develops, both total and free IGF-I is suppressed (180, 181). Expression of hybrid insulin/IGF-I receptors increases in skeletal muscle and adipose tissue (182), compensating for reduced insulin sensitivity.

Overweight subjects with retained insulin sensitivity have normal IGFBP-1 levels (183), and adequate elevation of IGFBP-1 during fasting when insulin levels decline (181). However, obese subjects are often insulin resistant resulting in hyperinsulinemia, which suppresses hepatic production of IGFBP-1 (183).

During development of T2D, IGFBP-1 levels rise despite persisting hyperinsulinemia, either because of hepatic insulin resistance, relative portal insulinopenia due to β-cell failure, or stimulatory factors such as counterregulatory hormones and cytokines (184). IGFBP-1 rises less than expected during fasting (181, 185) and is not suppressed after meals (186).

1.4.4 IGF-I and IGFBP-1 as markers and predictors of disease

1.4.4.1 IGFBP-1 - marker of IGF-I, and insulin production and sensitivity

Fasting serum IGFBP-1 is a marker of free IGF-I, and the production of both insulin (187) and IGFBP-1 (133) over the past 24 hours. The fact that insulin acutely inhibits hepatic production of IGFBP-1 allows fasting IGFBP-1 to be used as a marker of hepatic insulin sensitivity (188). The decrease in serum IGFBP-1 during an OGTT, in which insulin levels rise, is correlated with insulin sensitivity during an iv glucose tolerance test (189) and hepatic insulin sensitivity during a euglycemic hyperinsulinemic clamp with glucose tracer, as well as negatively with liver fat content (188).

In T2D the inverse relationship between fasting insulin and IGFBP-1 remains (see *1.3.2.2.*), but the regression line shifts upward and to the right reflecting hepatic insulin resistance or perhaps reduced hepatic insulin extraction (131, 132). The correlation is also weakened, perhaps due to counterregulatory hormones or inflammation stimulating IGFBP-1 (133).

1.4.4.2 Low IGF-I and IGBP-1 – risk factors for glucose abnormalities and CVD

IGF-I is positively correlated with insulin sensitivity in healthy subjects, IGT and T2D (190). High IGF-I levels are prospectively associated with reduced risk of IGT and T2D (191).

Low levels of IGF-I and IGFBP-1 are associated with the metabolic syndrome (192, 193), disturbed glucose metabolism (184, 194) and risk factors for/manifest CVD (184, 195, 196) in cross-sectional studies. They also predict development of abnormal glucose regulation (132) and cardiovascular morbidity and mortality (195, 197). Low basal IGFBP-1 and reduced inhibition of IGFBP-1 during OGTT predict development of glucose abnormalities (132). This is likely due to the relationship between low IGFBP-1 and hyperinsulinemia/insulin resistance (197).

1.4.4.3 High IGFBP-1 – a marker of absolute or relative insulin deficiency

Serum IGFBP-1 is elevated in portal vein insulinopenia, or in insulin resistance with insufficient compensation through increased insulin production (109). In some studies, high IGFBP-1 correlated with increased cardiovascular mortality both in patients with and without previous heart disease as well as T2D patients with acute myocardial infarction (198, 199).

1.4.5 Does increased activity of the HPA axis suppress activity of other axes?

Low GH and sex steroid levels can cause or exacerbate abdominal obesity (71), and abdominal obesity is associated with relative hypogonadism in men (200, 201) and hyperandrogenism in women (202, 203). Disturbed activity in the HPA axis may precede inhibition of the somatotropic and sex steroid axes (204).

In states of prolonged stress, CRH inhibits gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, both directly (205, 206) and via noradrenergic pathways (207). CRH also reduces secretion of follicle stimulating hormone (FSH), luteinizing hormone (LH) (208, 209) and GH from the pituitary gland, the latter partly by raising somatostatin (207, 210).

Prolonged hypercortisolemia reduces secretion of gonadotropins and perhaps also GnRH (211, 212). Cortisol is similar to progesterone in structure, and the two can bind to each other's receptors (213), which may explain why cortisol, like progesterone, can inhibit gonadotropin secretion (214). Cortisol also inhibits GnRH-stimulated LH secretion (215-218) and interferes with the stimulatory effect of FSH and LH on gonadal steroidogenesis (210).

1.5 GENDER DIFFERENCES

1.5.1 Obesity, glucose abnormalities and CVD

Before menopause, women are relatively protected from metabolic disturbances with less VAT than men and lower frequency of CVD (219). After menopause, women also develop a tendency toward centralized obesity and the risk of CVD increases (220, 221). Among patients with T2D, 40% of men but up to 70% of women display abdominal obesity (222).

Women with abdominal obesity are more likely to have metabolic syndrome, hyperinsulinemia, hyperandrogenemia and elevated stimulated cortisol levels (223, 224). Hyperandrogenemia in obese women is partially due to suppression of sex hormone binding globulin by high levels of insulin, and contributes to accumulation of VAT and insulin resistance. This is in direct contrast to men, in whom testosterone is protective against T2D (222).

1.5.2 The HPA axis

Some of the studies described above have only included patients of one gender, or have not factored in gender in the analyses (144, 152, 153, 156, 157). This may be an important cause of discrepant results between different reports, as gender affects regulation of the HPA axis.

Female rats have higher CRH and ACTH mRNA levels in the PVN and amygdala (225) as well as higher basal and stimulated serum levels (73, 222) compared to males. The findings differ in humans, with higher ACTH (145, 226) and higher (145) or similar cortisol levels (226, 227) in men compared with women. However, as in the animal studies, normal-weight as well as obese (non-diabetic) women have increased pituitary and adrenal response to physiological stimulation of the HPA axis or adrenal cortex compared to men, and in some cases increased sensitivity to feedback inhibition (76, 145, 226).

Gonadal steroids may modulate the HPA axis both centrally and peripherally (73). Androgens inhibit activation of the HPA axis in male rats, while estrogen has the opposite effect in females (228, 229). It appears that estrogen inhibits CRH, but it is unclear if this occurs directly via estrogen receptors, or via GR (73). In healthy women, HPA axis activity increases during the luteal phase of the menstrual cycle, due to elevated progesterone (230).

1.5.3 GH, IGF-I and IGFBP-1

Women have higher mean and peak serum GH compared with men (231), even though IGF-I levels do not differ (133, 232). This gender difference may be related to differences in body composition (233, 234) or to estradiol, which potentiates GH secretion (235). Fasting serum IGFBP-1 is higher in women than in men with normal glucose tolerance, even when serum insulin levels are equal (132, 197). This may be due to estrogen, which increases IGFBP-1 in both women (236) and men (237).

1.6 PPARγ AND THE THIAZOLIDINEDIONES

Thiazolidinediones (TZD) are peroxisome proliferator-activated receptor-γ (PPARγ) agonists, a class of drugs that increase insulin sensitivity and are used in the treatment of T2D. The only PPARγ agonist on the market in Sweden today is pioglitazone. Pilot studies on rats have shown that pioglitazone increases IGF-I production and reduces activity of the HPA-axis (238). In a small clinical study, patients with hypercortisolism due to Cushing's disease responded well to a TZD (239). This indicated that TZDs might constitute a useful tool for examining the effect of reduced insulin resistance on the HPA axis and IGF-I.

1.6.1 Peroxisome proliferator activated receptor γ (PPARγ)

PPARs exist in three isoforms, PPARα, -β and -γ (240). All three are activated by fatty acids (241). PPARγ belongs to the nuclear hormone receptor superfamily, which includes retinoid, thyroid and steroid hormone receptors such as GR. It exerts its effects

by binding PPAR-response elements (PPRE), thereby affecting gene transcription (242, 243). To do so it must first form a heterodimer with the retinoid X receptor (RXR) (244). PPARγ is mainly expressed in the adipose tissue, but also in β-cells, endothelium and macrophages (240).

PPARγ upregulates genes necessary for lipogenesis and TG storage, such as LPL and HSL, and reduces lipolysis (245, 246). Despite stimulating expansion of the adipose tissue, PPARγ has positive effects on insulin sensitivity by increasing uptake, storage and oxidation of fatty acids in adipose tissue (240, 247). PPARγ stimulates differentiation of preadipocytes into new, smaller, mature adipocytes with higher storage capacity (245, 248). Animal models with knock-out of PPARγ are insulin resistant and have elevated FFA, despite having fewer adipocytes than controls (245).

PPARγ also plays an important role in glucose metabolism by inducing GLUTs (249). They increase expression of peroxisome proliferator-activated γ coactivator 1 (PGC-1), which regulates mitochondrial biogenesis (22, 250). Finally, PPARγ agonists exert anti-inflammatory effects by inhibiting activation of macrophages and monocytes (238). This reduces expression and secretion of adipokines and cytokines (245, 251).

1.6.2 THIAZOLIDINEDIONES (TZD) - PPARγ AGONISTS

1.6.2.1 Mechanisms of action

TZDs improve insulin sensitivity in the liver and adipose tissue, to great extent through re-distribution of fatty acids from insulin resistant tissues such as the VAT, skeletal muscles and liver to the SAT (240). They increase insulin-stimulated glucose uptake, glycolysis and hepatic glycogenesis (252-254), and decrease gluconeogenesis from pyruvate and lactate (254, 255). In the adipose tissue, TZDs enhance insulin's antilipolytic effect (253). By upregulating glycerol kinase, they divert glycerol toward TG synthesis rather than gluconeogenesis (240). Glucose levels are lowered both in fasting (251) and post-prandially (240). While pioglitazone's sister drug rosiglitazone raises LDL, pioglitazone has no effect on LDL or lowers it (256), while it raises high-density lipoprotein (HDL) (251).

1.6.2.2 Effects in T2D and IGT

TZDs lower insulin levels and improve glycemic control by increasing hepatic and peripheral insulin sensitivity (257). They improve both basal and stimulated β-cell function (10). Mechanisms include lowering FFA levels (and thereby lipotoxicity) and improving insulin signaling (258). They also counteract β-cell apoptosis (10). Reduction of insulin resistance with TZD treatment may also contribute to protecting the β-cells, by reducing β-cell stress (10). Several large studies have shown that intervention with TZDs decreases the risk of developing T2D in high-risk groups, such as subjects with IGT and women with previous gestational diabetes. This is attributed to the β-cell sparing effect (258).

The positive metabolic effects of pioglitazone may be partially attributed to increased mitochondrial number (259) and function (25). Upregulation of genes important for

glucose and lipid oxidation in the mitochondria, such as PGC-1α, are likely important for this effect (259).

TZD treatment may also improve insulin sensitivity by increasing adiponectin levels and adiponectin receptor expression (44, 258). This increase is negatively correlated with hepatic fat content, and positively with hepatic and peripheral insulin sensitivity (245).

Obesity and T2D are characterized by a chronic, low-grade state of inflammation, related to elevated FFA, cytokines and counter-regulatory hormones (258). Inflammation is one cause of lower adiponectin levels in T2D, which is counteracted by TZDs as they reduce cytokine levels and effects (260, 261).

The major side effect of TZD treatment is weight gain. This is accounted for by increased SAT as well as edema, whereas VAT mass and metabolism are reduced (240). However, TZDs have metabolic effects despite this weight gain, and in fact the increase in weight is correlated with improvement of HbA_{1c} (262).

1.6.3 Interaction between TZDs and the HPA axis/cortisol

Largely, TZDs have effects opposite to those of cortisol (263), and several studies have suggested that TZD may reduce cortisol levels and/or effect. A pilot study in mice showed that rosiglitazone decreased ACTH and corticosterone (238). Two small studies subsequently showed the same effects on ACTH and cortisol in patients with Cushing's syndrome (239, 264), although others did not see this effect long-term (265, 266). Treatment of healthy individuals with troglitazone (an older TZD) may also counteract the negative effects of concurrent DEX treatment on insulin sensitivity (267).

It is theoretically possible that PPARγ agonists could inhibit ACTH synthesis. PPRE have been located in accessory proteins to the ACTH receptor found in adipocytes (268), and rosiglitazone counteracts basal and CRH-induced transcription of the *POMC* gene in ACTH-secreting pituitary tumors (238).

1.7 THE INCRETIN SYSTEM

When glucose is ingested orally, the stimulated insulin secretion is significantly larger than if the same dose is given iv. This is known as the incretin effect, accounted for by hormones known as incretins. The most important ones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), account for two-thirds of the insulin response to oral glucose (269). This thesis will focus on GLP-1.

GLP-1 is synthesized in the L-cells in the distal ileum and the colon (258). GLP-1 receptors are expressed in the α- and β-cells of the pancreas, in the central and peripheral nervous system, and in the intestine, heart, kidney and lungs (269). GLP-1 stimulates glucose-dependent insulin secretion, inhibits glucagon secretion, and induces satiety by inhibiting gastric motility and through central effects (fig. 7) (10). The inhibition of glucagon is equally important as the stimulation of insulin in order to inhibit post-prandial hepatic glucose production (HGP) (258). GLP-1 and GIP have

short half-lives in vivo since they are rapidly degraded by the serine protease dipeptidyl peptidase-4 (DPP-4) (10).

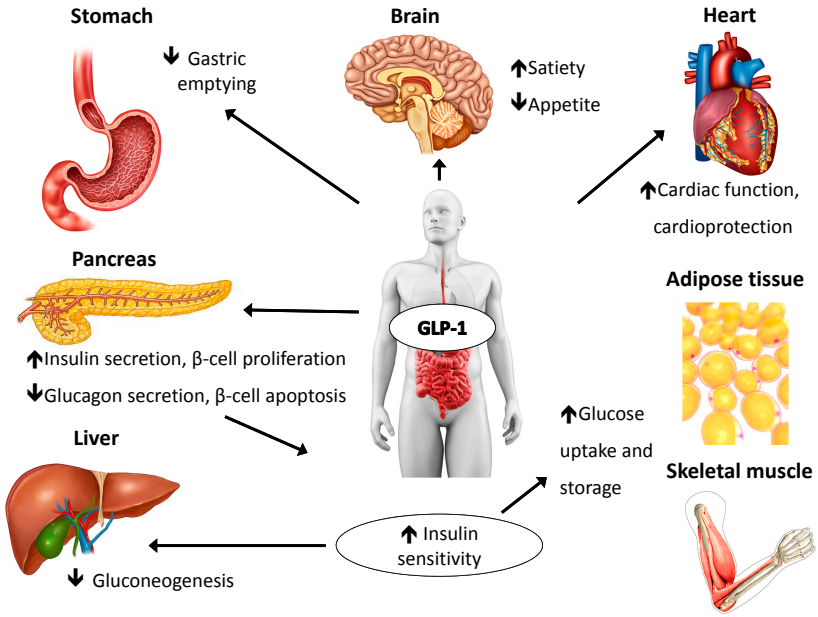

Figure 7. Effects of GLP-1.

1.7.1 Dysfunction of the incretin system in T2D and IGT

Glucose abnormalities are associated with reduced endogenous levels or effect of glucagon-like peptide-1 (GLP-1) (270). This leads to elevated glucagon levels, which along with hepatic insulin resistance contributes to increased HGP (10).

1.7.2 Incretin-based medications for T2D

GLP-1 analogues are administered as subcutaneous injections. They improve glycemic control by the same mechanisms as endogenous GLP-1, which results in lower post-prandial glucose and HGP as well as weight loss. The risk of hypoglycemia is low, as the stimulation of insulin secretion abates at normoglycemia (258). In animal models, GLP-1 analogues stimulate β-cell proliferation and counteract β-cell apoptosis (269).

DPP-4 inhibitors prolong the half-life of GLP-1, and thereby the circulating levels. They primarily affect HGP and glucose levels post-prandially, but also have a modest effect in fasting. While they do lower glucagon, increase insulin and have protective effects on the β-cells (70), DPP-4 inhibitors do not affect gastric emptying. They therefore do not induce weight loss, but are weight neutral and do not cause the nausea often associated with GLP-1 agonists (10).

Sitagliptin is an oral DPP-4 inhibitor taken once daily. A single dose inhibits DPP-4 ≥80% for 24 hours, and doubles GLP-1 secretion after meals without causing hypoglycemia (10).

1.7.3 Sitagliptin after acute coronary syndrome (ACS)

Patients with disturbed glucose metabolism are at increased risk of ACS, a state in which cortisol levels may be elevated (271). Disturbed glucose metabolism is a negative prognostic factor in ACS (272), most likely due to hyperglycemia, inflammation, endothelial dysfunction, dyslipidemia as well as factors thus far unknown (22). Sitagliptin has been shown to improve β-cell function and insulin sensitivity in drug-naïve patients with newly discovered T2D or IGT and recent ACS, and was well tolerated (273). DPP-4 inhibitors have cardioprotective effects, e.g. improving myocardial glucose uptake and endothelial function, and reducing inflammation, atherogenesis and infarct size (273). The mechanisms behind these effects are not completely known.

1.7.4 Interaction between incretins and the HPA axis

GLP-1 is found in the hypothalamus, and GLP-1 receptors are expressed in all the structures of the HPA axis. GLP-1 exerts short-term inhibition of stress-induced ACTH-release from the pituitary gland and ACTH-stimulated cortisol release from the adrenal cortex (274). Stress-induced corticosterone secretion in rodents lead to GLP-1 release in the PVN (275). GLP-1 levels are correlated both with the level of glycemia and with cortisol levels in intensive care patients, suggesting that failure of the incretin system may contribute to stress-induced hyperglycemia (276).

1.8 UNRESOLVED ISSUES

Disturbances in the HPA axis, IGF-I and IGFBP-1 have been seen in patients with visceral obesity and T2D. Results have varied between studies, perhaps due to insensitive test methods or failure to consider differences between men and women. PPARγ agonists (e.g. pioglitazone) improve insulin sensitivity and may lower cortisol levels in patients with Cushing's syndrome. If pioglitazone lowers cortisol levels also in patients with T2D, treatment represents a model for studying the effect of decreased cortisol on insulin sensitivity. The incretin system may also have interactions with the HPA axis. It is possible that the cardioprotective effect of DPP-4 inhibitors (such as sitagliptin) is partly due to lowering cortisol levels after ACS. Studies on gender differences in the HPA axis and the effects of drug therapies will provide information on the role of cortisol in the pathogenesis of T2D, which may contribute to development of better treatments in the future.

2 AIMS

2.1 GENERAL HYPOTHESIS AND AIMS

The general hypothesis was that the HPA axis is affected in T2D. The general aim of the thesis was to gain further knowledge of the role of the HPA axis in T2D and IGT, and the relationship of HPA axis activity with insulin sensitivity, β- cell function and IGF-I/IGFBP-1.

2.2 SPECIFIC AIMS

1. To test the hypothesis that cortisol levels under basal conditions, and after ACTH as well as DEX, are altered in T2D and IGT, and to examine potential relationships between these factors and IGF-I/IGFBP-1 **(Study I - II)**

2. To test the hypothesis that increased insulin sensitivity with pioglitazone treatment reduces cortisol levels in patients with T2D and IGT, and to relate potential changes to β-cell function, and IGF-I/IGFBP-1 (**Study II – III**) and metabolism in adipose tissue **(Study II)**

3. To examine potential gender differences in the basal and ACTH-stimulated cortisol levels, and in the responses to pioglitazone **(Study I, III)**

4. To test the hypothesis that improved glycemic control with sitagliptin treatment in patients with glucose abnormalities diagnosed after acute stress in the form of acute coronary syndrome is related to decreased cortisol and/or increased hepatic insulin sensitivity (**Study IV**)

3 SUBJECTS AND METHODS

3.1 SUBJECTS AND STUDY PROTOCOLS

3.1.1 Study I – Is adrenal sensitivity to ACTH affected by T2D or gender?

Aims

a) To test the hypothesis that cortisol levels basally, after stimulation with ACTH and/or feedback inhibition with DEX, are altered in T2D compared with controls
b) To examine if cortisol levels correlate with insulin sensitivity and IGF-I/IGFBP-1
c) To examine potential gender differences in these aspects of regulation of the HPA axis

Subjects

60 subjects were enrolled in the study: 21 with T2D (11 men, 10 women) and 38 healthy controls (20 men, 18 women). Participants were recruited primarily from a database of subjects who had previously been enrolled in or screened for other studies at the Dept of Endocrinology at Karolinska University Hospital (Stockholm, Sweden). Patients with T2D were permitted oral antidiabetic drugs (OADs), but were excluded if treated with insulin. One male patient with T2D was included in the ACTH and NaCl tests but excluded from the analyses before and after the DEX test (see below), due to initiation of basal insulin therapy in the interim.

Protocol

For all visits, subjects were instructed to fast after ten p.m. the prior evening and refrain from tobacco on the morning of the test. Medical history, medications and family history of T2D were recorded. Weight (without shoes and in light clothing), height, waist and hip circumference were recorded and BMI calculated.

On the first visit, a low-dose ACTH test was performed (see *3.2.1.1*). The first 32 patients (8 with T2D, 24 healthy controls) made an additional visit for a placebo test with NaCl (see *3.2.1.2*). The tests were administered in random order and were single-blinded. These 32 subjects were called back at a later date for a low-dose DEX test (see *3.2.1.3*). Further subjects, who were included at this time, underwent the ACTH and DEX tests, but no NaCl control. Diabetes medications were postponed on all test days until after testing was completed.

3.1.2 Study II – Effect of pioglitazone on cortisol and IGF-I in T2D and IGT

Aims

To test the hypothesis that increased insulin sensitivity with pioglitazone treatment reduces cortisol levels basally and after ACTH-stimulation in patients with T2D and IGT, and to relate potential changes to β-cell function, metabolism in adipose tissue and IGF-I/IGFBP-1

Subjects

Participants were recruited from a database of subjects who had previously been enrolled in or screened for other studies at the Dept of Endocrinology at Karolinska University Hospital. They were contacted via telephone and invited to take part in the study. Inclusion criteria were BMI ≥28 kg/m^2 (confirmed at the screening visit) and male gender. Exclusion criteria were on-going treatment with insulin or a TZD, and clinical history of heart failure. The number of patients screened, included and causes for dropout are summarized in fig. 9. The 1999 WHO criteria were used to define T2D (fasting venous whole-blood glucose ≥6.1 or 2-hour post-load glucose ≥10.0 mmol/L) and IGT (fasting glucose <6.1 and 2-hour post-load glucose ≥6.7 mmol/L) (277). Three patients dropped out due to side effects, which were dizziness, nausea/palpitations and deteriorated vision. The latter was examined by an ophthalmologist and found to be due to cataracts not related to pioglitazone.

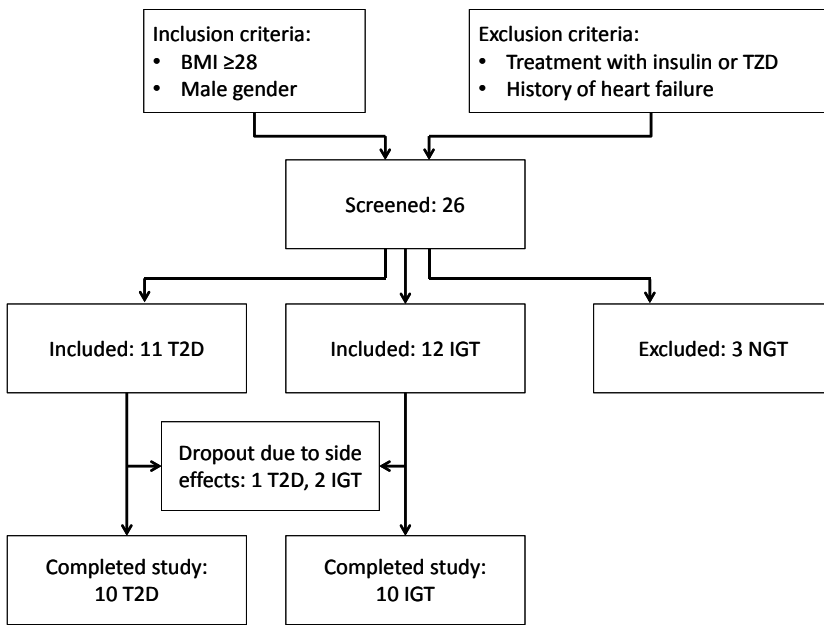

Figure 8. Flow chart of subject recruitment in **Study II**. BMI = body mass index, TZD = thiazolidinedione, T2D = type 2 diabetes, IGT = impaired glucose tolerance, NGT = normal glucose tolerance.

Protocol

The protocol for **Study II** is outlined in fig. 9. All assessments were initiated at 8 a.m. after an overnight fast, and performed with the subject resting in a supine position. No tobacco or heavy exercise was permitted during the morning of either test. Subjects were examined by an MD at the screening visit, including for symptoms of heart failure. Blood pressure, weight and waist circumference (biometric measurements) were recorded. An OGTT was performed, in parallel with microdialysis in SAT (see 3.2.3). Subjects were included if they fulfilled the inclusion criteria and none of the exclusion criteria, and the OGTT showed T2D or IGT (277). They then returned within 1 week for a low-dose ACTH test (see *3.2.1.1*) after which they were started on pioglitazone 30 mg once daily in addition to their pre-existing medications. Diabetes medications normally taken in the morning were postponed on all of the test days, until

after the testing was completed.

Fasting blood samples were drawn prior to the OGTTs at screening, at the interim visit and at the final visit for analysis of complete blood count, electrolytes including creatinine, alanine amino transferase (ALAT), gamma-glutamyl transferase (γ-GT), HbA_{1c} and a lipid profile including total cholesterol, HDL, LDL and TG. A morning urine sample was collected for analysis of microalbuminuria.

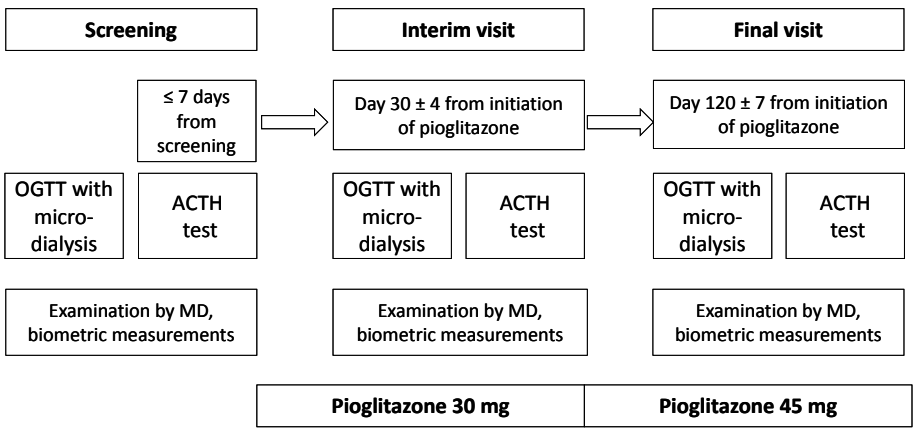

Figure 9. Flow chart of protocol in **Study II**. ACTH test = low dose (1 μg) adrenocorticotropin stimulation test, MD = medical doctor, OGTT = oral glucose tolerance test.

3.1.3 Study III – Are there gender differences in the non-glycemic effects of pioglitazone in T2D?

Aims

a) To test the hypothesis that increased insulin sensitivity with pioglitazone treatment reduces cortisol levels in patients with T2D, and to relate potential changes to IGF-I/IGFBP-1
b) To examine if gender differences exist in these responses

Subjects

Sixty-six patients with T2D and secondary drug failure were screened for the study. Other entry criteria were age 30–75 years and BMI >20 kg/m^2. All subjects were on metformin and a sulphonylurea or repaglinide (SU/Repa) prior to inclusion. *Secondary drug failure* was defined as HbA_{1c} >6.5 % (Mono-S method) in the latest two measurements, separated by at least eight weeks, during on-going treatment with metformin >1,500 mg/day and glibenclamide >7 mg/day, glipizide >10 mg/day, glimepiride >3 mg/day or repaglinide >6 mg/day for at least 3 months.

Subject recruitment and causes for dropout are summarized in fig. 10.
Fifty-four patients completed the study; results on metabolic parameters in these patients have been reported previously (278). Sufficient serum only remained from 48 (28 men and 20 women) for the measurements of IGF-I, IGFBP-1, and cortisol. Thus, only analyses from these patients are included in **Study III**.

Lisa Arnetz

Protocol

This was an interventional, open-label study that spanned 26 weeks. Blood sampling for HbA_{1c}, lipid profile, serum insulin, proinsulin, IGF-I, IGFBP-1 and cortisol as well as recording of biometric parameters, cardiopulmonary symptoms, and other side effects were performed pre-interventionally and at the end of the study. Intermediate visits to screen for side effects were also performed at eight and 16 weeks. Blood tests were taken after an overnight fast, between 7 and 8:30 a.m. after 20 minutes rest in a supine position. Diabetes medications were postponed until after blood samples were drawn. Included subjects received a prescription of 30 mg pioglitazone daily in addition to their preexisting therapy. After 16 weeks, the dose of pioglitazone was increased to 45 mg daily if HbA_{1c} was still >6.5 % and the therapy well tolerated (n = 16 men, 6 women).

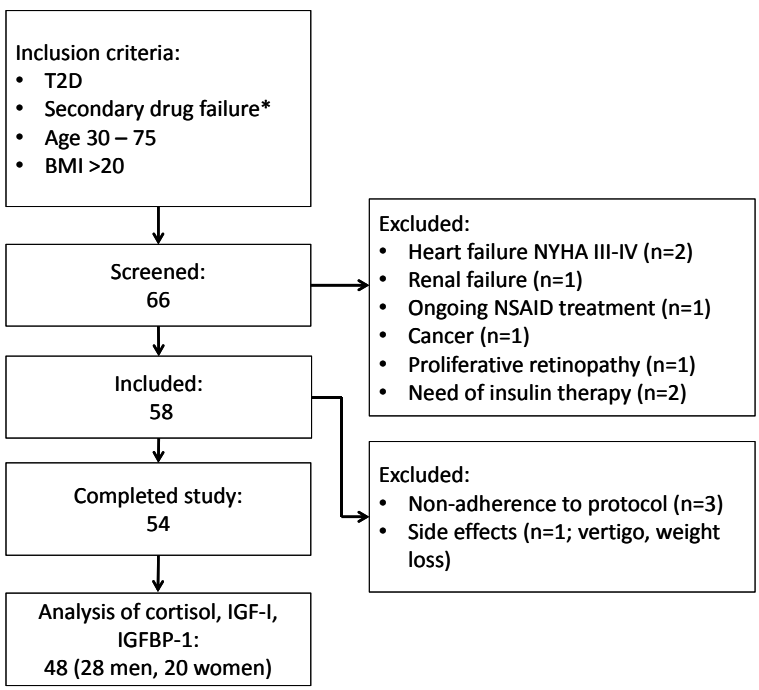

Figure 10. Flow chart of subject recruitment in **Study III**. BMI = body mass index, NSAID = non-steroidal anti-inflammatory drug, NYHA = New York Heart Association (scale of degree of symptoms of heart failure), T2D = type 2 diabetes. *Secondary drug failure = HbA_{1c} >6.5% (Mono-S) despite treatment with metformin and sulphonylurea.

3.1.4 Study IV – Is sitagliptin effect related to cortisol or hepatic insulin sensitivity?

Aims

a) To test the hypothesis that acute stress (ACS) increases cortisol levels in patients with newly diagnosed glucose abnormalities

b) To test the hypothesis that improved glycemic control with sitagliptin treatment in patients with glucose abnormalities diagnosed after ACS is related to decreased cortisol and/or increased hepatic insulin sensitivity

Subjects

Patients participating in the screening process of the Beta-cell function in patients with Glucose Abnormalities and Myocardial Infarction (BEGAMI) study (*Clinicaltrials.gov: NCT00627744*) were eligible. This was a multicenter, double-blind, randomized parallel group controlled study in which patients with newly discovered T2D or IGT after ACS were randomized to sitagliptin 100 mg daily or placebo.

As previously described (273), patients were selected from those admitted to the coronary care unit at Karolinska University hospital or Danderyd hospital due to ACS as defined according to the guidelines of the European Society of Cardiology and the American College of Cardiology Committee (279). Patients were eligible for inclusion if OGTT revealed previously undiagnosed disturbances in glucose tolerance (280). Exclusion criteria were previously known T1D or T2D, admission plasma glucose >12 mmol/L, age ≤18 years, impaired renal function (serum creatinine >130 µmol/L), congestive heart failure (NYHA III-IV), admission for planned coronary revascularization and inability to follow study protocol.

174 patients were screened with OGTT (for flow chart of patient recruitment and dropout see fig. 11). 99 were diagnosed with T2D (n = 36) or IGT (n = 63). Seven failed randomization due to difficulty obtaining venous access (n=4), PCI on the day of randomization, unwillingness to continue in the study after a vasovagal reaction, and not being fasting on the morning of the OGTT (n=1 for each). 34 were ultimately included in the sitagliptin group, and 37 in the placebo group.

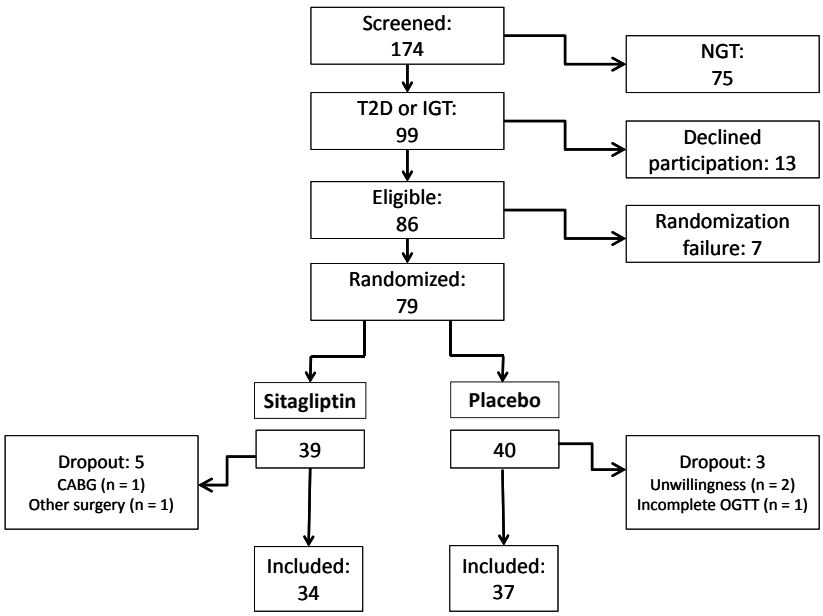

Figure 11. Flow chart of subject recruitment in **Study IV**. CABG = coronary artery bypass graft, IGT = impaired glucose tolerance, NGT = normal glucose tolerance, OGTT = oral glucose tolerance test, T2D = type 2 diabetes. Adapted from (273) with permission from the author.

Protocol
At baseline, all patients were given lifestyle advice and were started on treatments as needed after their ACS according to international guidelines, such as aspirin, beta-blockers, blood pressure medication, and statins (273). No other glucose-lowering drugs were given aside from the study drug.

OGTT was performed at baseline and after twelve weeks (see 3.2.2). Demographic data including weight and waist circumference were also recorded at these time points. Randomization to sitagliptin 100 mg or placebo once daily occurred in a 1:1 ratio and block size of four via a computer-generated randomization sequence. The last tablet of sitagliptin was ingested on the day prior to the twelve-week OGTT.

3.2 STUDY PROCEDURES

3.2.1 Evaluations of the HPA axis

3.2.1.1 The low-dose ACTH test (studies I and II)

Subjects rested throughout the test. A cannula was inserted into an antecubital vein and blood was drawn for the analysis of blood glucose and serum cortisol, insulin, IGF-I and IGFBP-1. The low-dose ACTH solution was prepared by removing 1 ml from a 50 ml bottle of NaCl 9 g/L, and then adding to the 50 ml bottle 1 ml of 0.25 g/L solution

synthetic ACTH (Synacthen; Novartis, Basel, Switzerland), resulting in a concentration of 250 µg/50 ml= 5 mg/L. A 1 µg injection was prepared by drawing up 0.2 ml of the 5 mg/L solution, and then 0.8 ml of pure NaCl solution. The injection was administered at 8 a.m. Blood was drawn at 30, 60 and 90 minutes after the injection, for repeated analyses of glucose, cortisol, insulin and IGFBP-1. The cannula was flushed with physiological NaCl solution after each sampling.

3.2.1.2 Control test with NaCl (study I)

The purpose of the placebo test was to exclude confounding by activation of the HPA axis due to the stress caused by the testing environment. The same test protocol was followed as for the ACTH test described above, but 10 ml physiological NaCl solution was injected instead of Synacthen.

3.2.1.3 The low-dose dexamethasone (DEX) inhibition test (study I)

For patients who had previously undergone ACTH and NaCl tests, new basal cortisol was drawn; for patients included at this time, the ACTH-test provided basal cortisol. After basal sampling, all subjects were given a capsule of DEX 0.25 mg, which they were instructed to take between 10 and 11 p.m. on the evening prior to their second visit. On that occasion they returned to the testing facility at 8 a.m., and blood was drawn for measurement of serum cortisol.

3.2.2 OGTT (study II and IV)

The OGTT was performed in the morning after an overnight fast. A venous catheter was inserted into an antecubital vein, and a basal blood sample (0 minutes) was drawn for analysis of blood glucose, serum insulin, C-peptide, cortisol, IGF-I and IGFBP-1. 75g of glucose were dissolved in 200 ml of water and given as a drink directly after sampling.

Study II
Sampling was repeated every 30 minutes, up to 120 minutes, for blood glucose, serum insulin, C-peptide (**Study II**) or proinsulin (**Study IV**), cortisol and IGFBP-1. Microdialysis of the SAT was performed in parallel with the OGTT (see *3.2.3.2*).

Study IV
The OGTT was performed under stable conditions on the day of discharge (earliest 4 days after admittance to the coronary care unit, or latest at an outpatient visit no more than 3 weeks after initial admission (range 4 – 23 days, median 7 in the sitagliptin group and 6 in the placebo group). After the basal test, sampling was repeated at 30, 60 and 120 minutes for blood glucose, serum insulin and proinsulin. Tests from 0 and 120 minutes (8 and 10 a.m.) were also analyzed for serum cortisol. Blood was collected in EDTA tubes for immediate analysis of glucose (see *3.3.1*). The remaining samples were kept on wet ice until centrifugation within one hour (2000 g for 20 minutes). Plasma for later analyses was stored at -70 °C.

3.2.3 Microdialysis in subcutaneous adipose tissue during OGTT (study II)

3.2.3.1 Protocol

Before the OGTT, a venous catheter was inserted into an antecubital vein and two wetted microdialysis catheters, connected to microdialysis pumps, were inserted in paraumbilical SAT. Every 30 minutes, venous blood samples were drawn and the vials were changed. After a wash-in period of one hour, subjects were given 75 g glucose dissolved in 200 mL water to drink. Sample collection continued every 30 minutes for an additional 150 minutes. Due to lag time before interstitial fluid is transported through the microdialysis catheter to the collection vial, dialysate samples collected at any given time point were taken to reflect interstitial concentrations 30 minutes previously (281). Hence, fasting levels were analysed from vials collected 30 minutes after glucose ingestion. Results from the catheter with the lowest lactate levels and least erroneous measurements were used in statistical analyses.

3.2.3.2 Materials and methods

The microdialysis catheter consisted of a 30 mm long dialysis membrane with a 20 kilo-Dalton molecular weight cut-off and an outer diameter of 0.6 mm (CMA Microdialysis AB, Solna, Sweden). The microdialysis catheters were connected to sterile syringes, which were placed in microinfusion pumps (CMA/107; CMA Microdialysis AB). Sterile Ringer's solution (Perfusion fluid, CMA Microdialysis AB) was perfused through the catheters and collected in microvials.

Interstitial glucose, pyruvate, lactate and glycerol were analysed with an enzymatic photometric assay (282), using a CMA 600 analyser (CMA Microdialysis AB). Glucose, lactate and glycerol are assayed with Trinder type reagents, using glucose oxidase, L-lactate oxidase and glycerol phosphate oxidase respectively. Glycerol was analysed by the latter reagent, after conversion to glycerol phosphate by the enzyme glycerol kinase.

3.3 BIOCHEMICAL ANALYSES

3.3.1 Blood glucose

Study I – II: Blood glucose was analysed from whole blood within 30 minutes from sampling, directly in the research laboratory, using YSI 2300 Stat Plus apparatus (Yellow Springs, Ohio, USA).

Study III: Plasma glucose was analysed using a glucose oxidase method, with a Beckman glucose analyzer II (Beckman Instruments, Fullerton, California, USA).

Study IV: Venous plasma glucose was analysed using a photometric technique (Hemocue® AB, Ängelholm, Sweden). The coefficient of variation (CV) was 5% at 4.0 mmol/L and 3% at 14.0 mmol/L.

3.3.2 HbA_{1c}

Study I: HbA_{1c} was analysed using spectrophotometric technique (DCA Vantage, Siemens, Munich, Germany). CV was 2% at both 34 and 96 mmol/mol.

Study II – IV: HbA_{1c} was analysed using the Variant II chromatographic method (high performance liquid chromatography, HPLC; BioRad Laboratories Inc., Hercules, California, USA). CV was 3.0% at HbA_{1c} 4.4 – 8.4% (ref. 4.0 – 5.3%) measured by the Swedish Mono-S method at the time of **Study III**; 2.5% at the time of **Study IV.** HbA_{1c} values were recalculated to SI (IFCC) from NGSP (DCCT) (283).

3.3.3 Serum cortisol

Serum cortisol was analysed with chemiluminescence technique, using Roche Modular apparatus (Roche Diagnostics Scandinavia, Bromma, Sweden). The total CV was 2.5% at 544 nmol/L and 2.1% at 855 nmol/L.

3.3.4 Serum insulin

Study I-II: Tests were centrifuged for 15 minutes at 2000 rpm, 15°C, and the supernatant stored at -80°C until the completion of the study. Serum insulin was measured by radioimmunoassay (RIA) method (Pharmacia insulin RIA 100, Pharmacia Diagnostics, Uppsala, Sweden). The interassay CV was <5.8% and the intraassay CV <5.4%.

Study III – IV: Insulin concentrations were measured with a double antibody enzyme-linked immunoassorbent assay (ELISA; DakoCytomation, Cambridgeshire, United Kingdom). Intraassay CVs were 7.5% at 39 and 269 pmol/L and 5.1% at 1240 pmol/L. Interassay CV were 9.3% at 40 pmol/L, 8.9% at 287 pmol/L and 4.2% at 1240 pmol/L.

3.3.5 Serum C-peptide (Study II - III)

Study II: Tests were centrifuged for 15 minutes at 2000 rpm, 15°C, and the supernatant stored at -80°C until the completion of the study. Serum C-peptide was analyzed using human C-peptide RIA kit HCP-20K (Millipore, Billerica, Maryland, USA). The detection level was 0.1 μg/L and the performance level was ED_{50} at 1.1 ± 0.1 μg/L and ED_{80} at 0.3 ± 0.1 μg/L.

Study III: C-peptide concentrations were measured using RIA (Linco, Missouri, USA). The lower limit of detection for C-peptide was 0.03 nmol/L. The intraassay CV was 4.5% and the interassay CV 3.2%.

3.3.6 Serum proinsulin (Study III - IV)

Study III: Serum proinsulin was analysed using ELISA (Total Proinsulin; DakoCytomation, Cambridgeshire, United Kingdom) with an interassay CV of 7%.

Study IV: Serum proinsulin was analysed by ELISA (kit 10-1118-01, Mercodia AB, Uppsala, Sweden). The sensitivity was 0.5 pmol/L. Total CV was 5.1% at 7.3 pmol/L, 6.1% at 20.7 pmol/L, and 5.0% at 65.6 pmol/L.

3.3.7 Serum adiponectin (Study III)

Adiponectin was analysed using RIA (Research Adiponectin Assay, Linco, St. Charles, Missouri, USA). The limit of sensitivity for the assay was 1 ng/ml and both the inter- and intraassay coefficients of variation were < 10%.

3.3.8 IGF-I

Total serum IGF-I was determined by an in-house RIA after separation of IGFs from IGFBPs by acid ethanol extraction and cryoprecipitation, described previously (284). The detection level of the RIA was 3.0 mg/L. Cross-reactivity with IGFBP-2 and IGFBP-3 was less than 0.5 and 0.05%, respectively. To minimize interference of remaining IGFBPs, des(1-3) IGF-I was used as radioligand. Serum levels of IGF-I decrease with age, and are thus expressed as standard deviation (SD) score = [(10logIGF-I-observed + 0.00693 * age) - 2.581] / 0.120 (133). The intra- and interassay CV were 4% and 11%, respectively.

3.3.9 IGFBP-1

Serum IGFBP-1 was analysed using in-house RIA (285). The sensitivity of the RIA was 3 μg/l and the intra- and interassay CV were 3% and 10%, respectively.

3.3.10 Other biochemical analyses

Lipid profile (**Study II**), complete blood count, electrolytes, ALAT, γ-GT and urinary albumin (**Study II**), serum HbA_{1c} (**Study II, IV**), creatinine (**Study II**), and serum insulin and proinsulin (**Study III**) were analysed using standard, accredited methods at the Central Chemistry Laboratory at the respective university hospitals.

3.4 CALCULATIONS

BMI was calculated as weight (kg)/height (m^2).

HOMA-IR was calculated as (serum insulin x blood glucose)/22.5, using fasting values of glucose and insulin (**Study I – II, IV**) (48).

HOMA-β (%) was calculated as (20 x insulin)/(glucose – 3.5), using fasting values of glucose and insulin (**Study IV**) (48).

IGI during the OGTT was calculated as the increment in insulin during the first 30 minutes of the test, divided by the increment in glucose (Δinsulin/Δglucose; **Study IV**) (286).

3.5 STATISTICS

Statistical analyses were carried out using STATISTICA software, version 10 (StatSoft, Tulsa, OH, USA). Data is presented as mean ± standard error of the mean (SEM) unless otherwise specified. P-values <0.05 were considered statistically significant. In multiple regression models variables were included if $p < 0.10$. Normality of variables was tested using the Kolmogorov-Smirnov and Lilliefors tests. Differences between variables that were normally distributed were analysed using paired and unpaired t-tests, whereas variables that were not normally distributed were analysed using the Wilcoxon and Mann-Whitney tests. In **Study II**, repeated measures ANOVA was used to study the baseline period of microdialysis, i.e. from -30 to +30 minutes from oral ingestion of glucose (see *3.2.4.2*). Correlations between pairs of continuous variables were analysed using Pearson's correlation coefficient, while multiple linear regression was performed with selected sets of continuous and categorical variables.

3.6 ETHICAL CONSIDERATIONS

The studies were all approved by the local ethics committee, and signed informed consent was obtained from all patients prior to inclusion. The studies were conducted in accordance with the ethical standards of the Declaration of Helsinki and Good Clinical Practice guidelines.

4 RESULTS

4.1 COMPARISON OF STUDY SUBJECTS

Study	I		II		III	IV	
Subjects	T2D	Healthy	T2D	IGT	T2D	T2D	IGT
N	21	38	10	10	48	24	47
Gender (n men/women)	10/11	20/18	10/0	10/0	28/20	18/6	40/7
Age (years)	62 (54 - 70)	58 (41 - 67)	54 (42 - 64)	61 (53 - 68)	61 (45 – 76)	66 (46- 81)	68 (49 – 87)
Years since T2D diagnosis	9 (1-19)	na	4 (0-9)	na	11 (3 – 30)	0 (0 – 0)	na
BMI (kg/m^2)	26.6 (19.0-32.4)	26.7 (20.3-38.6)	31.0 (28.7-36.0)	29.5 (27.7-31.6)	26.6 (23.0-40.3)	26.6 (21-2-32.0)	27.3 (20.7-33.7)
Waist circum-ference (cm)	99 (85 - 120)	94 (71 - 189)	111 (97 - 122)	106 (97 - 114)	111 (88 - 136)	99 (81 - 111)	102 (86 - 127)
Intervention (weeks)	None	None	Pio (12)	Pio (12)	Pio (26)	Sita (12)	Sita (12)
Baseline medications:							
Metformin* (n)	16	na	6	na	48	0	na
SU / Repa* (n)	0	na	1	na	48	0	na
Metformin and SU/Repa (n)	8	na	1	na	48	0	na
Pioglitazone (n)	1	na	0	na	0	0	na
Liraglutide (n)	1		0		0		
Sitagliptin (n)	3	na	0	na	0	0	na
Acarbose	1	na	1	na	0	0	na
No OADs	4	na	4	na	0	24	na

Table 1. Subjects' clinical characteristics and OADs at baseline. Data presented as means (range) unless otherwise specified. DPP-4 = dipeptidyl peptidase-4, IGT = impaired glucose tolerance, na = not applicable, OAD = oral antidiabetic drug, pio = pioglitazone, repa = repaglinide, Sita = sitagliptin, SU = sulphonylurea, T2D = type 2 diabetes, * = in monotherapy.

Study	**I**		**II**		**III**	**IV**	
Subjects	T2D	Healthy	T2D	IGT	T2D	T2D	IGT
HbA_{1c} (mmol/mol)[1]	49 ± 2 (36 - 68)	na	57 ± 4 (36 - 80)	41 ± 12 (35 - 52)	70 ± 7 (57-89)	41 ± 10 (35-46)	40 ± 0 (33-48)
Fasting glucose (mmol/L)[2]	6.5 ± 0.3 (4.2–8.8)	4.9 ± 0.1 (4.0-6.0)	8.7 ±0.8 (4.7-12.8)	5.0 ± 0.2 (3.1-5.9)	9.4 ± 0.0 (6.0 - 15.1)	5.7 ± 0.2 (4.1 – 6.9)	5.3 ± 0.1 (4.2 - 6.1)
Serum insulin (mU/L)[3]	22 ± 3 (11 - 59)	17 ± 1 (8 - 36)	21 ± 2 (11 - 36)	27 ± 7 (10 - 75)	8 ±1 (2 - 23)	11 ± 2 (4 - 41)	10 ± 1 (3 - 27)
Serum C-peptide (µg/L)	na	na	1.6 ± 0.3 (0.6 – 3.5)	2.8 ± 0.4 (0.6 - 4.2)	na	na	na
Serum proinsulin (pmol/L)	na	na	na	na	44 ± 6 (8 - 167)	17 ± 3 (5 - 71)	14 ± 1 (4 - 38)
HOMA-IR (mmol x mU)	7 ± 1 (3 - 20)	4 ± 0 (2 – 9)	8 ± 1 (2 - 18)	6 ± 2 (2 - 13)	23 ± 0 (8 – 57)	2.9 ± 0.4 (1.1 – 9.8)	2.3 ± 0.2 (0.6 – 6.8)
Serum cortisol, basal before ACTH[4] (nmol/L)	424 ± 27 (272-735)	385 ± 16 (193-584)	400 ± 30 (238-517)	402 ± 21 (326-532)	451 ± 24 (163-882)	483±26 (263-904)	446±17 (209-686)
Serum cortisol, peak after ACTH (nmol/L)	693 ± 31 (437-960)	624 ± 18 (364-863)	599 ± 32 (460-764)	643 ± 39 (472-863)	na	na	na
Δ cortisol from basal to peak after ACTH (nmol/L)	269 ± 26 (11-459)	239 ± 15 (30-437)	199 ± 34 (0-356)	241 ± 39 (0-482)	na	na	na
Serum cortisol, basal before DEX[5] (nmol/L)	448 ± 29 (216-715)	404 ± 15 (233-584)	na	na	na	na	na
Serum cortisol, after DEX (nmol/L)	283 ± 25 (90-516)	261 ± 15 (93-479)	na	na	na	na	na
Δ cortisol from basal after DEX (%)	-36 ± 5 (-68 - 14)	36 ± 3 (-65 - 10)	na	na	na	na	na
Serum IGF-I (SD)	0.3 ± 0.3 (-3.9 - 2.4)	0.3 ± 0.1 (-1.4 - 2.1)	-1.4 ± 0.5 (-4.8 - -0.1)	0.4 ± 0.3 (-1.2 – 2.2)	0.5 ± 0.2 (-4.5 - 3.6)	-0.1±0.3 (-3.6 – 2.7)	0.7 ± 0.2 (-1.6 – 2.9)
Serum IGFBP-1 (µg/L)	36 ± 3 (12 - 60)	35 ± 3 (7 - 81)	18 ± 4 (8 - 44)	18 ± 2 (6 - 27)	35 ± 3 (9 - 76)	26 ± 2 (6 - 49)	28 ± 2 (5 - 69)

Table 2. Biochemical analyses at baseline. All analyses are basal levels, unless otherwise stated. Data presented as mean ± SEM (range). ACTH = adrenocorticotropic hormone, 1 µg, DEX = dexamethasone, 0.25 mg, na = not applicable. 1-3 = recalculated to IFCC from Mono-S (1), from plasma to whole blood equivalent level (2)(287) and from pmol/L to mU/L (3)(288) in **Study III - IV**. 4 = basal cortisol in **Study I – IV**. 5 = repeated evaluation of basal cortisol before DEX in **Study I** for 32 subjects in whom ACTH and DEX tests were performed separately.

			Serum cortisol, basal (nmol/L)		Serum cortisol, peak (nmol/L)		Serum IGF-I (SD)		Serum IGFBP-1 (µg/L)	
Study	Group	n	Baseline	After	Baseline	After	Baseline	After	Baseline	After
I	T2D men	11	451±43	na	691 ±42	na	0.4 ±0.5	na	37±4	na
	T2D women	10	391 ±26	na	696 ±47	na	0.2 ±0.3	na	34±5	na
	Healthy men	20	368 ±22	na	582 ±21	na	0.2 ±0.2	na	32±4	na
	Healthy women	18	403±22	na	675 ±26	na	0.3 ±0.2	na	38±5	na
II	T2D men	10	400±30	312±25	599±32	511±43	-1.4±0.5	-0.5±0.4	18±4	21±5
	IGT men	10	402±21	461±35	643±39	713±37	0.4±0.3	0.4±0.3	18±2	22±4
III	T2D men	28	493±36	542±29	na	na	0.4±0.2	0.9±0.2	35±4	39±3
	T2D women	20	390±26	484±32	na	na	0.6±0.3	1.2±0.3	34±4	40±5
IV	T2D+IGT, baseline	71	460±15	na	na	na	0.4±0.2	na	27±2	na
	T2D+IGT, sitagliptin	34	na	425±24	na	na	na	0.1±0.3	na	33±3
	T2D+IGT, placebo	37	na	471±21	na	na	na	0.7±0.3	na	30±3
	Sitagliptin, men	29	na	429±25	na	na	na	0.2±0.4	na	33±3
	Sitagliptin, women	5	na	405±69	na	na	na	-0.3±1.0	na	39±9
	Placebo, Men	29	na	470±22	na	na	na	0.5±0.4	na	28±3
	Placebo, women	8	na	478±54	na	na	na	-0.6±0.6	na	34±5

Table 3. Cortisol and IGF-I before and after interventions, subdivided by gender. Data presented as mean ± SEM. After = at the end of intervention, baseline = before intervention, IGF-I = insulin-like growth factor-I, IGFBP-1 = IGF binding protein-1, IGT = impaired glucose tolerance, na = not applicable, T2D = type 2 diabetes. Data for **Study IV** pooled at baseline, subdivided after treatment (for separately reported baseline data see paper IV).

4.2 STUDY I – IS ADRENAL SENSITIVITY TO ACTH AFFECTED BY T2D OR GENDER?

4.2.1 Clinical characteristics and medications

Subjects' clinical characteristics and medications are summarized in table I. As expected, T2D patients had higher basal blood glucose ($p < 0.001$), serum insulin ($p = 0.012$) and HOMA-IR ($p = 0.007$) compared with healthy controls. There were no

gender differences in HbA_{1c} or duration of T2D. Four women, but no men, were without any pharmacological treatment for their T2D. All patients were middle aged, although men with T2D were slightly older than male controls (p = 0.009). All of the groups were matched for BMI. Although waist circumference in the healthy women was statistically lower than the other groups, their mean waist circumference was still high (88 ± 4 cm) and their fasting insulin levels did not differ from those of women with T2D.

Among the healthy women, one still had regular menstruation, and one less regularly than previously. The remaining women in the study were post-menopausal, based on patient history. Smoking (n = 2), menstruation and treatment with hormone replacement therapy (n = 2) or pioglitazone (n = 1) did not affect the outcome of the analyses.

4.2.2 Effect of ACTH injection compared with NaCl

After both ACTH and NaCl injection, glucose levels were unaffected whereas serum insulin decreased (0 vs. 90 min. measurements; p = 0.033 for ACTH and p = 0.044 for NaCl in T2D; p <0.001 for ACTH and p = 0.012 for NaCl in healthy subjects). Unlike after ACTH injection, serum cortisol decreased after NaCl injection (for T2D basal = peak 409 ± 34 nmol/L, after 90 min. 260 ± 37 nmol/L, p = 0.008 basal vs. 90 min.; healthy subjects basal = peak 394 ± 23 nmol7L, after 90 min. 244 ± 15 nmol/L, p <0.001 basal vs. 90 min).

4.2.3 The HPA axis in T2D - increased adrenal sensitivity to ACTH and eradicated gender differences

Basal fasting cortisol did not differ between subjects with T2D and controls or between men and women. Subjects with T2D had higher peak cortisol after ACTH injection (p = 0.043; tables 2 and 3, fig. 12). Among healthy subjects, peak cortisol was higher in women compared with men (p = 0.014) whereas there were no gender differences among those with T2D. This was due to higher peak cortisol in men with T2D compared to healthy men (p = 0.024). Among women, peak cortisol was equally high regardless of having T2D or not. Basal cortisol correlated with BMI (r = -0.461, p = 0.004) and waist circumference (r = -0.467, p = 0.003) in healthy subjects but not in T2D.

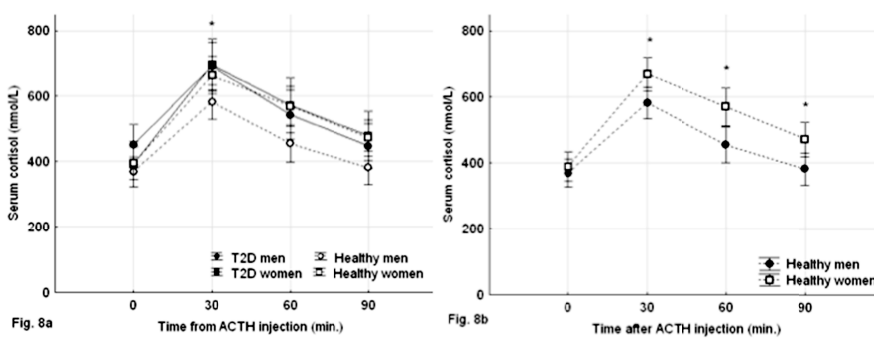

Figure 12. Serum cortisol before and after 1 µg ACTH injection, in all subjects (a) and in healthy men versus healthy women (b). Error bars = 95% confidence interval. * p <0.05 for healthy men compared with all other groups in 12a; for healthy women compared with healthy men in 12b.

4.2.4 Hormonal responses to DEX

Cortisol decreased in all four subgroups after DEX (table 2), but the cortisol levels after DEX did not differ between the T2D and healthy groups. Unlike for peak cortisol, healthy and T2D women did not differ in cortisol after DEX, nor were there any gender differences.

4.2.5 Factors affecting peak cortisol after ACTH injection

Multiple linear regressions were performed to assess the effects of gender, disease status (T2D or healthy), BMI, waist circumference and basal serum insulin on peak cortisol. Waist circumference had the lowest impact, and also neared multi-collinearity with BMI (correlation of regression co-efficients 0.778). After removal of waist circumference, only gender was significant ($p = 0.045$). Finally, in a model including basal insulin, gender and BMI, gender remained the only significant independent variable ($p = 0.048$), although BMI also showed a trend toward affecting peak cortisol negatively ($p = 0.063$). However, none of these models had a high r^2 (0.128 – 0.172), indicating that other factors not measured also affected peak cortisol.

4.2.6 Correlations between IGFBP-1 and cortisol

T2D and healthy subjects did not differ in basal serum IGF-I or IGFBP-1. Metformin did not affect IGF-I or IGFBP-1 among the T2D patients. Higher basal serum IGFBP-1 was associated with higher basal serum cortisol ($r = 0.506$, $p = 0.001$) in healthy subjects but no correlation existed in T2D (fig. 13). Correlations were performed between basal insulin and basal cortisol to see if insulin levels might be a confounder affecting both IGFBP-1 and cortisol levels, but no such correlation was found in either group.

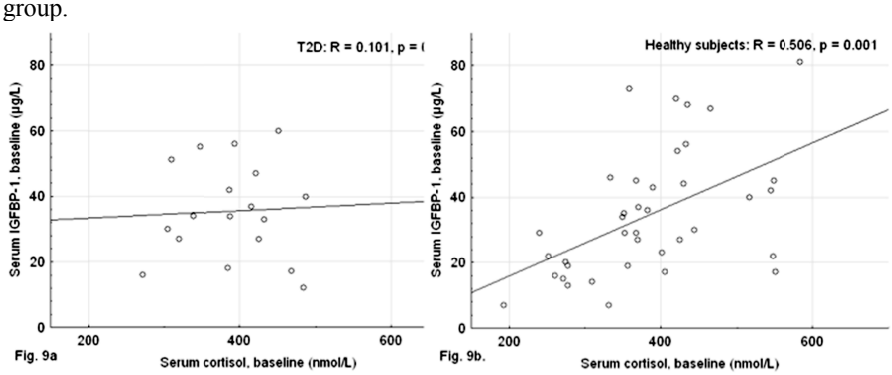

Figure 13. Correlation between basal serum cortisol and basal serum IGFBP-1 in subjects with T2D (a) and in healthy subjects (b).

4.3 STUDY II – EFFECT OF PIOGLITAZONE ON CORTISOL AND IGF-I IN T2D AND IGT

4.3.1 Clinical characteristics before and after twelve weeks of pioglitazone treatment

Baseline characteristics and OADs are summarized in table I. Subjects with T2D and IGT were matched for BMI and waist circumference, and did not differ in lipid levels.

Twelve weeks of treatment with pioglitazone did not significantly change HbA_{1c}, weight, BMI or WHR in either group. Lipid levels did not differ between the groups before or during treatment, although fasting serum triglycerides decreased in the T2D group ($p = 0.013$), as did serum γ-GT ($p = 0.021$).

4.3.2 Increased insulin sensitivity and β-cell function

Fasting glucose, C-peptide, and HOMA-IR decreased after twelve weeks of pioglitazone treatment in T2D ($p = 0.007$, 0.008 and 0.005, respectively) and IGT ($p = 0.028$, 0.009 and 0.038, respectively), while D-AUC for glucose and insulin were unaffected. Fasting serum insulin decreased in T2D ($p = 0.011$) and D-AUC for insulin decreased in IGT ($p = 0.028$). The decreases in glucose despite decreases in insulin reflect improved insulin sensitivity.

The IGI was higher in IGT compared with T2D both before ($p = 0.007$; data not reported in **Study II**) and after ($p = 0.016$) pioglitazone treatment, reflecting better β-cell function. After treatment, it increased in T2D ($p = 0.037$) but was unchanged in IGT.

4.3.3 Reduced fasting lipolysis in adipose tissue in T2D, despite lower insulin

Fasting interstitial glucose and pyruvate were higher in the T2D group before treatment ($p = 0.005$ and 0.010, respectively) but not afterwards. Fasting glucose, pyruvate and lactate were unchanged in both groups. D-AUC for interstitial glucose was higher in the T2D group both before and after treatment ($p = 0.008$ and 0.011, respectively), while no differences were noted in D-AUC for pyruvate or lactate. In the IGT group D-AUCs were unaffected, save for an increase in interstitial lactate ($p = 0.043$).

Fasting glycerol decreased in the T2D group ($p = 0.038$), despite lower insulin levels (see 4.3.2), without any changes in D-AUC. No effect was seen in subjects with IGT.

4.3.4 Peak cortisol decreased in T2D but increased in IGT

Before treatment, there were no significant difference in fasting or peak serum cortisol between T2D and IGT. In the T2D group, fasting serum cortisol decreased significantly during treatment ($p = 0.041$) and peak cortisol also showed a trend towards a decrease (table 3). Among the patients with IGT, basal cortisol instead increased ($p = 0.044$), resulting in a difference between the groups at week 12 ($p = 0.007$; table 3). An upward trend was also seen for peak cortisol, which was therefore higher among subjects with IGT during treatment ($p = 0.007$; fig. 14). There were no differences between the groups in the increases in cortisol from baseline to peak (measured in nmol/L or %) before or after treatment (data not reported in **Study II**).

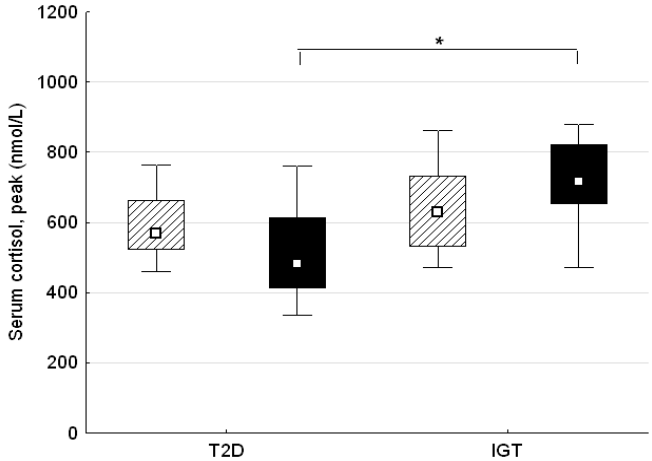

Figure 14. Peak serum cortisol (after 1 µg ACTH) before and after twelve weeks of pioglitazone treatment in subjects with type 2 diabetes (T2D) and impaired glucose tolerance (IGT). Striped bars = before treatment, black bars = at end of treatment period. *p = 0.007.

4.3.5 Increased IGF-I in T2D without changes in IGFBP-1

Before treatment serum IGF-I was lower in the T2D group compared to the IGT group (p = 0.006; tables 2 and 3, fig. 15). It increased after twelve weeks in the former (p = 0.017) to a level nearer the population mean, whereas it was unchanged in the latter. Basal IGFBP-1 did not differ between T2D and IGT before or after treatment. Serum IGFBP-1 decreased during the OGTTs in both groups (p <0.001 in both), without any impact of pioglitazone treatment on the degree of this decrease.

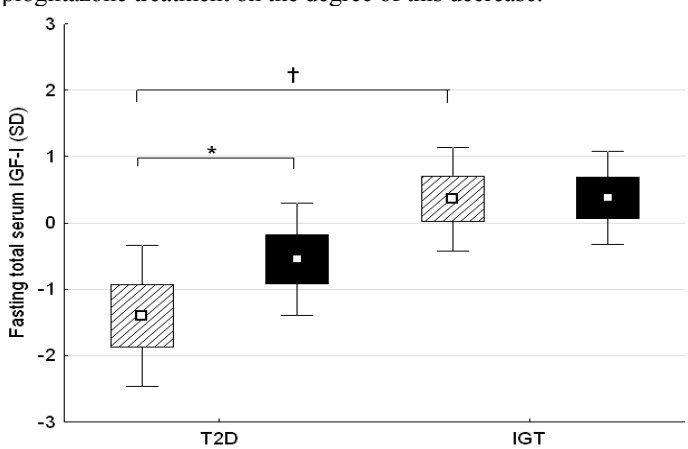

Figure 15. Fasting serum IGF-I before and after twelve weeks of pioglitazone treatment in subjects with type 2 diabetes (T2D) and impaired glucose tolerance (IGT). Striped bars = before treatment, black bars = at end of treatment period. † p = 0.006, * p = 0.017.

4.4 STUDY III – ARE THERE GENDER DIFFERENCES IN THE NON-GLYCEMIC EFFECTS OF PIOGLITAZONE IN T2D?

4.4.1 Clinical characteristics before and after pioglitazone treatment

Clinical characteristics and laboratory analyses at baseline are summarized in tables 1-3. There was no significant difference between the men and women in the study in age, duration of disease, BMI, fasting plasma glucose or PI/I ratio at baseline.

4.4.2 Improved metabolic control and β-cell function, without gender differences

As previously reported, pioglitazone improved HbA_{1c} despite an increase in weight, BMI and hip circumference in both genders (278). Triglycerides decreased only in women. There was no change in serum insulin levels, but proinsulin and PI/I decreased significantly ($p < 0.001$ for both). Adiponectin increased in both men and women ($p < 0.001$ for both); levels did not differ between the gender groups before or after treatment.

4.4.3 Gender differences in serum cortisol

At baseline, men had higher serum cortisol ($p = 0.045$; fig. 16). Serum cortisol increased among women ($p = 0.020$), whereas it was unchanged among men. However, Δ serum cortisol did not differ between men and women (paper III, table 3). The increase among the women was within the physiological range, and after 26 weeks of treatment, serum cortisol no longer differed between men and women.

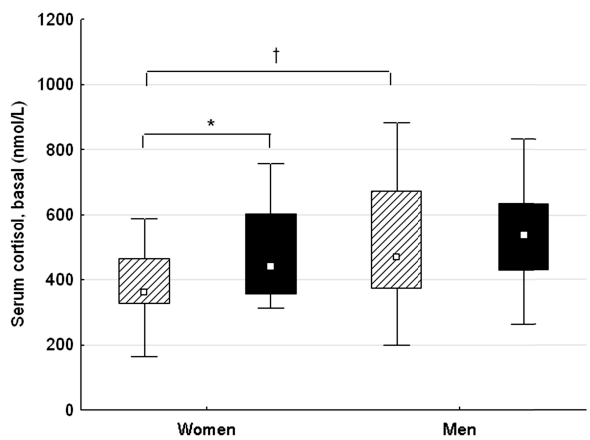

Figure 16. Fasting serum cortisol at baseline and after 26 weeks of pioglitazone treatment in patients with type 2 diabetes. Striped bars = before treatment, black bars = at end of treatment period. † $p = 0.020$, * $p = 0.045$.

4.4.4 Pioglitazone increased serum IGF-I in men and women

As in the men with T2D in **Study II**, serum IGF-I increased in both women and men ($p < 0.001$ in both; fig. 17). There were no gender differences in IGF-I levels before or during treatment, or in the magnitude of the increase.

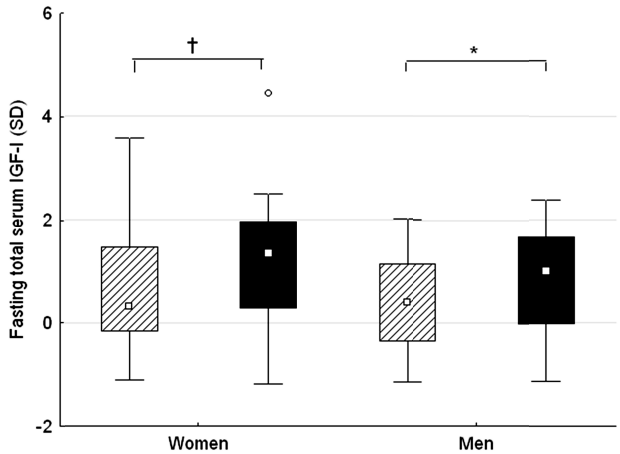

Figure 17. Fasting total serum IGF-I at baseline and after 26 weeks of pioglitazone treatment in patients with type 2 diabetes. Striped bars = before treatment, black bars = at end of treatment period, circles = outliers. † $p = 0.001$, * $p < 0.001$.

4.4.5 Serum IGFBP-1 increased, without decrease in serum insulin

When all subjects were analyzed together, IGFBP-1 increased during pioglitazone treatment ($p = 0.033$). However, when men and women were analyzed separately, neither increase reached statistical significance (fig. 18). The levels did not differ between the genders before or during treatment.

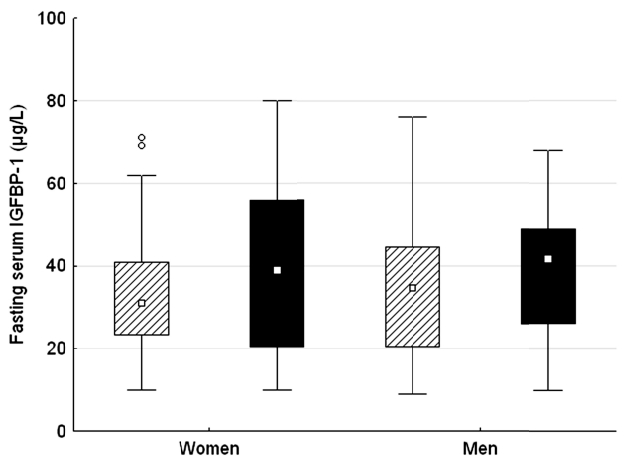

Figure 18. Fasting serum IGFBP-1 at baseline and after 26 weeks of pioglitazone treatment in patients with type 2 diabetes. Striped bars = before treatment, black bars = at end of treatment period, circles = outliers.

4.4.6 Correlations

Neither baseline nor Δcortisol correlated with ΔHbA_{1c}. Neither the increase in weight nor the decrease in WHR correlated with fasting cortisol or IGF-I in either group. ΔIGFBP-1 correlated positively with Δadiponectin (r = 0.600, p = 0.005) and Δcortisol (r = 0.458, p = 0.049) in women only.

4.5 STUDY IV – IS SITAGLIPTIN EFFECT RELATED TO CORTISOL OR HEPATIC INSULIN SENSITIVITY?

4.5.1 Clinical characteristics of subjects before and after treatment

Clinical characteristics of the subjects and laboratory analyses other than cortisol, IGF-I and IGFBP-1 are only reported briefly here (see tables 1-2), as they have been published previously (273). Among the 34 subjects who were randomized to sitagliptin, 10 had T2D and 24 IGT as determined by the OGTT. The placebo group was similar with 14 with T2D, 23 with IGT. Both groups consisted predominantly of men; 29 men in each group, with 5 women in the sitagliptin group and 8 in the placebo group. The groups were matched at baseline for age, BMI and waist circumference, and did not differ in baseline metabolic or hormonal analyses including those during the OGTT.

As previously reported (273), proinsulin was lower after treatment both in fasting and postprandially despite lower postload glucose in both the sitagliptin and placebo group. This reflects improved glucose tolerance. The sitagliptin group also displayed improved fasting blood glucose, HbA_{1c}, PI/I, glucose AUC and IGI during the OGTT.

4.5.2 Serum cortisol decreased twelve weeks after ACS; no effect of sitagliptin

Serum cortisol was measured at the start of the OGTT (around 8 a.m.) and after (10 a.m.), both at baseline and twelve weeks after ACS (table 2 and 3). All data is displayed with the patients in the sitagliptin and placebo groups pooled at baseline, and separated after the twelve-week treatment period.

Serum cortisol did not differ between subjects with T2D compared with IGT, before or after treatment. While no difference was seen in cortisol at 8 a.m. after twelve weeks, the level at 10 a.m. decreased to 278 ± 14 nmol/L (138 – 441) within the sitagliptin group, p = 0.038, and to 302 ± 15 nmol/L (132 – 479) in the placebo group, p = 0.017 (fig. 19).

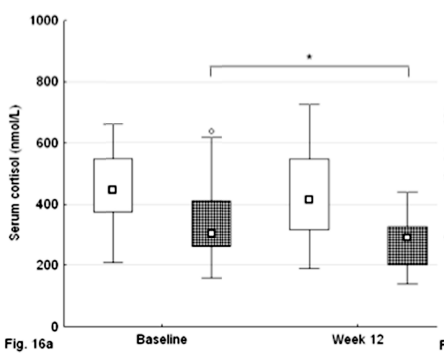

Fig. 16a

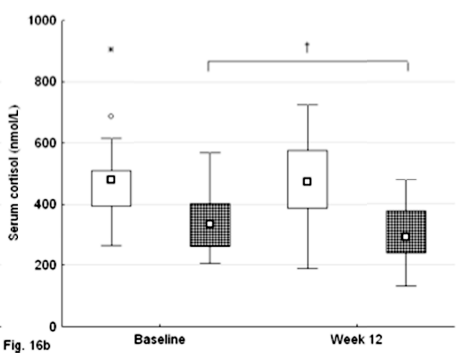

Fig. 16b

Figure 19. Serum cortisol at 8 and 10 a.m., at baseline and after twelve weeks of treatment with sitagliptin (a) and placebo (b). White bars = 8 a.m., checkered bars = 10 a.m., circles = outliers, stars = extreme outliers. * p = 0.038, † p = 0.017.

4.5.3 Sitagliptin had no effect on IGF-I or IGFBP-1

Both serum IGF-I and IGFBP-1 were unaffected after twelve weeks in the sitagliptin group as well as in the placebo group (table 3). Already at baseline, serum IGFBP-1 decreased >50% during OGTT in both groups. This indicates they had normal hepatic insulin sensitivity from the start, which was unchanged after the treatment period.

In the sitagliptin group, there was a positive correlation between the changes in 8 a.m. cortisol and log IGFBP-1 after treatment ($r = 0.407$, $p = 0.017$). Neither baseline levels, or the changes after treatment, in fasting cortisol or IGFBP-1 correlated with the changes in the measured parameters of glycemic control (fasting plasma glucose, HbA_{1c}, HOMA-IR) and β-cell function (PI/I and IGI) in either treatment group.

4.6 EFFECT OF METFORMIN AND SULPHONYLUREA/REPAGLINIDE (STUDIES I – II)

The effects of metformin and SU/Repa were studied to investigate potential confounders in the results, despite the subgroups being small (table 1).

In **Study I**, fasting blood glucose was lower among those treated with metformin ($p < 0.001$) and SU/repa ($p = 0.041$) compared to those without OADs. Those on metformin also had lower fasting serum insulin ($p = 0.010$) and HOMA-IR ($p < 0.001$). In **Study II**, HOMA decreased in the T2D patients with and without metformin, from 9.5 ± 2.0 to 5.3 ± 0.8 in the metformin group, $p = 0.028$; and from 6.3 ± 1.5 to 4.0 ± 0.7 in the non-metformin group, $p = 0.068$.

Fasting and peak serum cortisol did not differ between patients with and without metformin in **Study I**, or at baseline in **Study II**. Combination therapy with metformin and SU/repa was associated with higher cortisol after ACTH-injection ($p = 0.014$) and higher serum cortisol after DEX ($p = 0.041$) in **Study I**.

As SU/repa increases insulin levels, and insulin per se has a stimulatory effect on the HPA axis (289), analyses were performed to see if higher insulin levels could account for the increased peak cortisol level in patients on such therapy in **Study I**. While lower insulin levels were found in patients on metformin compared to those without ($p = 0.010$), no differences in insulin levels existed between the subgroups of T2D patients with and without SU/repa. However, even after exclusion of subjects treated with SU/repa, peak cortisol was still higher among men with T2D (700 ± 49 nmol/L) than among healthy men (582 ± 21; $p = 0.027$).

After pioglitazone treatment in **Study II**, fasting serum cortisol decreased both in patients with metformin (from 383 ± 37 to 300 ± 41 nmol/L, $p=0.016$) and without (from 426 ± 53 to 330 ± 13 nmol/L, $p=0.144$). Peak cortisol after ACTH decreased in the patients with metformin (from 613 ± 43 to 449 ± 27 nmol/L, $p = 0.028$) but not in those without (from 578 ± 55 to 605 ± 87 nmol/L, $p = 0.715$).

In **Study II**, IGF-I did not differ at baseline and increased in both the metformin-treated patients (from -1.8 ± 1.7 to -0.9 ± 1.3 SD, p = 0.068), and in the non-metformin group (from -0.8 ± 1.0 to 0.0 ± 0.8 SD, p = 0.144). In both cases, the increase occurred from levels that were low compared to the mean in a healthy population (0 SD), towards more normal levels. There was no trend toward a change in IGFBP1 in either subgroup.

Baseline glycerol decreased among patients treated with metformin in **Study II** (p = 0.036) but not in those without (p = 0.570).

5 GENERAL DISCUSSION

5.1 MAIN FINDINGS

Study I – The gender difference in adrenal sensitivity to ACTH, with higher sensitivity in healthy women compared to men, is abolished in T2D due to increased reactivity in men.

Study II – Improved insulin sensitivity during pioglitazone treatment is associated with reduced basal cortisol and increased IGF-I in obese men with T2D. This may indicate amelioration of disturbances in the HPA- and somatotropic axes.

Study III – Improved glycemic control during pioglitazone treatment in T2D is associated with increased IGF-I, and possibly IGFBP-1, in both men and women. Basal cortisol was lower in women compared with men at baseline, but increased during treatment in women while it was unaffected in men.

Study IV – Basal cortisol, measured at 10 a.m., decreased 12 weeks after ACS in patients with newly discovered glucose abnormalities. Treatment with sitagliptin had no effect on basal cortisol or IGF-I/IGFBP-1, despite improved glucose tolerance and β-cell function.

5.2 REGULATION OF THE HPA AXIS IN T2D AND IGT (STUDY I – II)

Study I confirmed the hypothesis that ACTH-stimulated cortisol differs between patients with T2D and healthy controls, while feedback inhibition was unaffected. However, there were no differences in basal cortisol between patients with T2D and healthy controls in **study I**, or in basal or stimulated cortisol between patients with T2D and IGT in study **II**.

The lack of differences in basal cortisol between patients with T2D and the other groups corroborates the results in most studies of obese subjects compared with normal-weight controls (93, 144-148). Few studies have compared basal cortisol between patients with T2D and controls matched for BMI, but one has shown T2D patients to have higher levels (158).

While adrenal sensitivity to low dose ACTH was similar between patients with T2D and IGT in **study II**, it was higher in patients with T2D compared with healthy controls in **study I**. Cortisol levels decreased during the control test with NaCl in both groups in **study I**, indicating the difference was not caused by differences in stress or other factors affecting the HPA axis.

Separate analyses of men and women revealed increased peak cortisol after ACTH in men with T2D compared with male controls, resulting in peak levels equal to those of women both with and without T2D. In other words, the study confirmed previous findings that adrenal sensitivity to ACTH is higher in women compared to men among

healthy subjects (226), but revealed that this gender difference is abolished in T2D due to increased sensitivity in men. Gender differences are discussed further in 5.4.

Factors that differed between the male groups with and without T2D in **study I**, and therefore may explain the increased reactivity in the former group, were age, body composition, insulin levels and medications. The potential impact of OADs is discussed in 5.7.

Our results do not support that age or body composition explained the difference in reactivity to ACTH between the groups. Although the men with T2D were slightly older than the healthy men in **study I**, and younger than the men with IGT in **study II** (table 1), the absolute differences were small and previous studies have shown HPA axis reactivity to be unaffected by ageing (290). Abdominal (visceral) obesity has a significant impact on regulation of the HPA axis (see 1.4.1), but neither BMI nor waist circumference differed between the male groups in **study I** (see paper I) **nor II**.

Hyperinsulinemia stimulates the HPA axis (289). Basal insulin levels were higher in men with T2D compared with healthy controls in **study I** (25 ± 4 vs. 16 ± 1 mU/L, $p < 0.001$), while they did not differ between T2D and IGT in **study II** (21 ± 2 vs. 27 ± 7 mU/L, $p = 0.912$). Multiple regression analyses in **study I** showed no significant impact of basal insulin on the adrenal response to ACTH, despite the levels differing between the groups (see 4.2.5). Basal insulin and HOMA-IR provide only a rough estimate of the degree of actual hyperinsulinemia and of insulin sensitivity (49), so it can not be excluded that differing degree of hyperinsulinemia between groups contributed to differences in adrenal sensitivity to ACTH. IGFBP-1 levels were similar between patients with T2D and healthy controls in **study I** despite higher insulin levels in T2D, indicating hepatic insulin resistance.

Serum testosterone was not measured in the study, but we hypothesize that altered testosterone levels could be an underlying cause of the changes seen in the HPA axis in T2D. Testosterone has an inhibitory effect on the HPA axis (73). Low testosterone levels are more often found in men with the metabolic syndrome or T2D compared with healthy men (291). Measurement of serum testosterone levels in relation to activity of the HPA axis in men with T2D would be of interest in a future study.

The degree of feedback inhibition of cortisol after 0.25 mg of DEX in **study I** was of comparable magnitude to that in previous studies (101). Sensitivity to feedback inhibition of the HPA axis did not differ between men and women, or between patients with T2D compared with controls. The T2D patients had good glycemic control and moderate insulin resistance, as suggested by elevated (but not excessively high) insulin levels and IGFBP-1 in relation to insulin. Studies by Björntorp et al suggest that blunted feedback inhibition is not seen until more advanced stages of metabolic disease (98).

IGF-I levels did not differ between subjects with T2D and controls in **study I**, suggesting that the somatotropic axis was unaffected in the T2D group. This is in line with the finding of retained sensitivity to feedback inhibition, as both of these changes are found in later stages of metabolic disease (98). In study II, IGF-I was lower in

subjects with T2D than with IGT at baseline. Compared to the men with T2D in **study** I, the T2D patients in **study II** were more overweight and had higher HbA_{1c} (table 1), and may therefore have had some degree of suppression of the somatotropic axis (see 5.2). Unfortunately, no DEX test was performed in **study II**.

A correlation existed between basal cortisol and fasting serum IGFBP-1 in healthy subjects, but not among patients with T2D, in **study I**. It is known that cortisol stimulates IGFBP-1 synthesis in healthy individuals with low insulin levels, but that the loss of correlation in subjects with T2D may be due to this effect being masked by hyperinsulinemia (137). The spread in cortisol and IGFBP-1 levels appeared to be higher among the healthy subjects (see fig. 9).

5.3 EFFECT OF IMPROVED INSULIN SENSITIVITY DURING PIOGLITAZONE TREATMENT ON THE HPA AXIS AND IGF-I/IGFBP-1 IN T2D AND IGT (STUDY II AND III)

Studies II and III were in essence mechanistic in design. The purpose of using pioglitazone was not to evaluate its effect on insulin sensitivity per se, but to see if improved insulin sensitivity during treatment is associated with changes in basal and ACTH-stimulated cortisol levels. The studies did indeed reveal such changes, but not in all cases the decreases that we hypothesized would occur.

Subjects in study III had higher HbA_{1c} and lower fasting serum insulin compared with those in study II (table 2). Both studies showed improved insulin sensitivity during pioglitazone treatment reflected by HOMA-IR or adiponectin. Insulin sensitivity improved in adipose tissue in the T2D group in **study II**, with decreased basal and stimulated glycolysis and basal lipolysis despite decreased C-peptide and insulin levels. HbA_{1c} decreased during treatment in **study III** but not in **study II**. However, study III was longer (26 compared to 12 weeks) and the patients had higher HbA_{1c} at the initiation of the study (table 2). In **study II** fasting C-peptide decreased in both T2D and IGT, and fasting insulin decreased as well in the T2D group. In **study III** PI/I decreased, indicating reduced β-cell stress (292). The original study on the patients in **study III** confirmed previous reports of increased adiponectin during pioglitazone treatment (22, 44, 278), without gender differences.

Basal serum cortisol decreased among patients with T2D in **study II**, in line with previous findings in rodents and patients with Cushing's syndrome (238, 239, 264). However, despite similar basal cortisol levels before pioglitazone treatment, the level instead rose in subjects with IGT. Basal cortisol also rose during treatment in the women with T2D in **study III**, from levels lower than those in men to a similar level after treatment. Basal cortisol was unaffected in the men with T2D in **study III**, even though they were higher than in the men with T2D in **study II** before pioglitazone treatment (table 3). Changes in peak cortisol levels were not significant within either group in **study II**, but a trend toward a decrease in the T2D group and an increase in the IGT group resulted in significantly higher peak cortisol in the IGT group during treatment (table 3). Gender differences in **study III** are discussed in 5.4, and the impact of medication in **study II** in 5.6.1.

Due to limitations in study design (see 5.6), we can only speculate on the reason for the discrepant reactions in basal cortisol during pioglitazone treatment. The men with T2D in **study III** had higher baseline HbA_{1c} than the men in **study II**, and had secondary drug failure indicative of β-cell failure; in short, they had more severe metabolic disturbances. The lack of effect on baseline cortisol may reflect reduced sensitivity to some effect that occurred in the men in **study II**. However, as baseline cortisol gives incomplete information on the regulation of the HPA axis and cortisol metabolism, this difference may also have arisen by chance.

Differences between the T2D and IGT groups in **study II** in the cortisol response to ACTH during pioglitazone treatment must also be interpreted cautiously, as the difference in peak level after treatment only arose from trends towards differences within the groups. Lower peak cortisol in the T2D group during treatment may simply have resulted from the basal level being lower. Alternately, reduced inflammation during pioglitazone treatment (258, 260, 261) may have lowered the drive on the HPA axis. An increased drive on the HPA axis, as was seen in the IGT group, may occur secondarily to down regulation of 11βHSD1 (see 5.8). Finally, the trends seen may not reflect an actual change in the HPA axis; one study on the effect of pioglitazone in women with polycystic ovary syndrome (PCOS) saw no effect on the ACTH or cortisol response to CRH (293).

IGF-I increased from a low level towards the mean of a healthy population (=0 SD) in the men with T2D in **study II**. The men with IGT in **study II** and both groups in **study III** had IGF-I nearer 0 SD at baseline, but levels still increased in both groups in **study III** while it was unaffected in the IGT group in **study II**. Low IGF-I in T2D patients in **study II** may be an indicator of suppressed function of the somatotropic axis (98); however, the patients in **study III** had signs of more severe metabolic disease that those in **study II**, and still had higher IGF-I levels at baseline.

CRH has an inhibiting effect on GH (207), and CRH effects were reduced by rosiglitazone in one animal study (238). Reduced CRH would theoretically release inhibition on GH synthesis, resulting in increased IGF-I.

FFA also inhibit the somatotropic axis, and in **paper II** we hypothesized that the increase in IGF-I in subjects with T2D resulted from reduced FFA (105, 240). This was based on reduced TG levels and basal lipolysis in the T2D group, not seen in the IGT group. TG also decreased in the female group in **study III**, but not in the male group although IGF-I increased among both women and men. However, more men than women were treated with fibrates, and baseline TG levels were lower in men (paper III, table 2) even if the difference was not significant. This may explain why no decrease occurred among the men. Studies have repeatedly shown that FFA decrease after TZD treatment (258), so it is possible that FFA decreased in all of the T2D groups including the men in **study II** despite fasting serum TG being unaffected.

Two studies on the effect of pioglitazone in women with PCOS have reported increased GH; however, one found a simultaneous increase in IGF-I without effects on cortisol (294), while the other found increased IGFBP-1 without effects on total or free IGF-I (295).

While these studies raise interesting questions, we can only speculate on the role of CRH, GH and FFA in the increases in IGF-I as these factors were not analysed.

Our findings are in contrast to studies by Lecka-Czernik et al, which reported decreased IGF-I during rosiglitazone treatment in mice and in healthy post-menopausal women (296). There may be differences in effects on IGF-I between rosiglitazone and pioglitazone, just as their effects on lipids differ (251, 256). Another possibility is that increased IGF-I is only seen in patients with T2D, as the mouse models and women in Lecka-Czernik's studies were not diabetic.

In **study II**, fasting serum IGFBP-1 was in the lower normal range before treatment, and remained low during treatment despite decreased fasting serum insulin and C-peptide in the T2D group. This indicates an increase in free serum IGF-I, and improved hepatic insulin sensitivity as insulin's inhibiting effect on the liver is the main factor regulating IGFBP-1 (297). In **study III**, IGFBP-1 increased significantly when men and women were pooled but not when they were analyzed separately, probably due to small sample size. Basal insulin was unaffected during treatment, but both IGFBP-1 and insulin levels were much higher in **study III** compared with in **study II** (table 1), underscoring the more severe insulin resistance in the subjects in the former. IGFBP-1 is a marker of hepatic insulin sensitivity (188), and the increase may reflect that this improved. A direct effect of pioglitazone could also contribute in both studies. PPARγ agonists bind to PPRE of the *IGFBP-1* gene, increasing transcription (298).

Adiponectin increased in both men and women in **study III**, reflecting improved insulin sensitivity (43). However, ΔIGFBP-1 correlated with Δadiponectin only in women. Gender differences in this correlation have previously been reported in healthy controls by our group (299). Adiponectin is a marker of adipocyte differentiation (300), which pioglitazone is known to increase (245). Greater improvement in adipose tissue metabolism in women than im men, suggested by the decrease in TG only in women, could explain the stronger correlation between changes in IGFBP-1 and adiponectin in women. As with adiponectin, ΔIGFBP-1 correlated with Δcortisol in women alone. Cortisol stimulates *IGFBP1* gene transcription (301), but as an increase in cortisol only occurred in women, this is unlikely to explain the increase in IGFBP-1 that occurred in both genders.

5.4 GENDER DIFFERENCES IN THE HPA AXIS AND EFFECTS OF PIOGLITAZONE IN T2D (STUDY I, III)

The original study on the patient material in **study III** indicated a greater improvement in metabolic control in women compared with men after pioglitazone treatment (278). After **study II** showed clear effects on the HPA axis in men, **study III** was performed to elucidate if gender differences may also exist in the non-glycemic response to pioglitazone. **Study III** confirmed such differences, while **study I** showed gender differences in ACTH-stimulated cortisol levels.

As discussed above, peak cortisol was higher after low-dose ACTH in healthy women compared with men in **study I**. The stimulatory effect of estrogen on the HPA axis has been hypothesized to be an underlying cause of higher adrenal sensitivity in women

(226). As the women in **study I** were post-menopausal, it can be hypothesized that estrogen levels did not differ significantly between the gender groups and did not account for this effect. However, post-menopausal status was only determined based on patient history and neither FSH nor estrogen were measured, so further studies would be necessary to confirm this assumption. The findings nonetheless underline the importance of accounting for gender in future studies of the HPA axis.

At baseline in **study III**, cortisol levels were lower in women than in men. One previous study found the same in healthy women, even after correction for body surface area (227). Treatment left basal cortisol unaffected in men, while in women it rose to become equal to those in the male group. However, this increase was within the bounds of normal levels and the magnitude of the increase (Δ) was not significantly larger than in men (paper III, table 3). Thus the clinical implications of this increase must be interpreted with caution. Glycemic control and lipid profile improved in the female group, despite increased cortisol that is normally associated with insulin resistance and dyslipidemia (302). This suggests that the improved metabolic parameters were due to factors other than cortisol. The cause for the increase in cortisol is speculated on briefly in **paper II**, but it is beyond the scope of the study.

Treatment with TZDs decreases bone density (303) and increases the risk of fractures, especially in distal extremities and more so in women than men (304). This is partly attributed to stimulation of pluripotent bone marrow stem cells to differentiate into adipocytes rather than osteoblasts; PPARγ are expressed in bone (296). Exogenous glucocorticoid treatment is associated with similar bone loss and increased fracture risk (305), just as in women on TZD treatment. Although the data are not strong enough to draw conclusions, it is interesting to speculate whether increased cortisol contributes to the increased fracture risk in women during TZD therapy.

5.5 EFFECT OF SITAGLIPTIN NOT RELATED TO CORTISOL OR HEPATIC INSULIN SENSITIVITY

The aim of **study IV** was to test the hypothesis that the positive glycemic effects during sitagliptin treatment in patients with glucose abnormalities and recent severe stress (ACS) are due to decreased cortisol and/or hepatic insulin resistance. To the best of our knowledge, this was the first study to examine the effect of a DPP-4 inhibitor on cortisol levels. The results disproved our hypothesis, as cortisol decreased in both the sitagliptin and placebo group, and there were no differences in IGF-I or IGFBP-1.

Fasting serum cortisol levels at 8 a.m. shortly after ACS were within the normal range (200 – 800 nmol/L) for the majority of subjects with T2D (483 ± 26 nmol/L, median 469, range 263 - 904) and all those with IGT (446 ± 17, median 465, range 209 - 686). However, these values tended to be higher than in **study II and III** (table 2-3), reflecting that ACS is a state of acute stress.

Regardless of treatment with sitagliptin or placebo, parameters reflecting glycemic control (basal proinsulin; post-load glucose, proinsulin and insulin) improved (273). This was likely due to lifestyle advice, medication and that the stress following ACS had subsided. However, a greater number of parameters reflecting glycemic control

(fasting plasma glucose, HbA_{1c}, PI/I, glucose AUC) and β-cell function (IGI) improved in the group treated with sitagliptin. The beneficial effects of sitagliptin treatment on glycemic control and β-cell function were not related to reduced cortisol levels, or to improved hepatic insulin sensitivity as measured by serum IGFBP-1 before and after OGTT.

Elevated levels of stress hormones such as cortisol, adrenaline, and glucagon counteract counteract insulin and raise serum IGFBP-1 (135, 139, 140). We hypothesized that fasting serum IGFBP-1 would be elevated in these subjects with glucose abnormalities after recent ACS, and that the reduction in serum IGFBP-1 during OGTT would be decreased indicating hepatic insulin resistance. Moreover, we expected fasting IGFBP-1 to decrease due to increased insulin production in the group treated with sitagliptin. However, inhibition of serum IGFBP-1 during the OGTT was normal already at baseline, indicating that the subjects had adequate hepatic insulin sensitivity. Basal IGF-I and IGFBP-1 were also unchanged at follow-up, regardless of treatment arm. IGF-I levels were normal already at baseline (near 0 SD), which indicates a normally functioning somatotropic axis. This may also be the reason for the lack of change in fasting serum IGFBP-1. Another factor is that sitagliptin primarily increases serum insulin levels after meals, when GLP-1 is released (306). Fasting serum IGFBP-1, however, reflects mean insulin levels over the past 24 hours (187), which was perhaps not increased as substantially in these subjects on sitagliptin.

The subjects in this study had newly discovered glucose abnormalities, and almost normal HbA_{1c}. Inclusion of a third group of subjects with a longer duration of T2D and higher $HbA_{1c,}$ along with measurement of glucagon, may have clarified this issue.

Finally, it is possible that sitagliptin does not raise GLP-1 levels to a sufficient extent to affect the HPA axis. A study including measurement of serum GLP-1 levels and comparison with a group treated with a GLP-1 analogue would be needed before completely discarding the idea that incretin-based medications exert their effects partially via affecting cortisol levels.

5.6 LIMITATIONS IN STUDY DESIGN

The heterogeneity of the study groups must be accounted for in the interpretation of the results. **Studies II and IV** would have benefitted from a control group matched for BMI and WHR, in order to ascertain whether the changes seen in the HPA axis were related to disturbed glucose metabolism per se or other to factors. The groups in **study II** were small, and the study aught to be repeated with larger groups including women in order to confirm the results. However due to risk of side effects, e.g. edema, inclusion of a healthy control group in **study II** did not outweigh the risks.

As no OGTTs were performed in **study I**, it can not be excluded that some of the subjects in the control group had IGT. However, they all had normal fasting glucose and fasting serum IGFBP-1 in relation to insulin, indicating that insulin sensitivity was normal. Measurement of β-cell antibodies in the patients with T2D (**studies I-IV**) would have excluded the possibility of misdiagnosed latent autoimmune diabetes of the adult (LADA). Fasting serum insulin levels were in the higher normal range, or

elevated, in the face of elevated fasting plasma glucose (reference range 2 – 25 mU/L, with levels 14-25 indicating insulin resistance (307)) indicating these patients were insulin resistant.

5.6.1 Effect of oral antidiabetic drugs (OADs)

Although the heterogeneity of medications in **studies I and II** make interpretation of the results more difficult, positive aspects in study design were that no patients on insulin treatment were included in any of the studies, as well as OADs other than the study drug being standardized in **studies III and IV**. Metformin and SU did not appear to affect the results regarding IGF-I/IGFBP-1 in **study I and II**, but may have influenced those regarding the HPA axis (see 4.6).

Basal and peak cortisol did not differ between those with and without metformin in **study I** or at baseline in **study II**. In **study I**, SU/repa was associated with higher cortisol after ACTH as well as DEX. More men than women were treated with SU/repa. However, peak cortisol remained higher among men with T2D compared with controls even after exclusion of those on SU/repa, and peak cortisol did not differ between the men and women with T2D. Nor was fasting serum insulin higher among patients with SU/repa, which suggests that fasting hyperinsulinemia was not responsible for the increased adrenal reactivity. However, it is possible that insulin levels were higher over the day and that this contributed to elevated adrenal sensitivity and reduced sensitivity to feedback inhibition.

After pioglitazone treatment in **study II**, peak cortisol after ACTH decreased only in those not on simultaneous metformin therapy. While this is important to note, the subgroups were too small to draw certain conclusions and other studies have reported metformin both to decrease ACTH-stimulated cortisol (308) or to lack effect(309, 310).

5.6.2 Methods of studying cortisol production and metabolism

In order to obtain a complete picture of cortisol production and metabolism, studies must involve stimulation of all levels of the HPA axis, testing of feedback inhibition, analyze peripheral metabolism and excretion, separate between free and total cortisol, and account for GR sensitivity. Results vary depending on whether cortisol is measured in blood, urine or saliva, the time of day at which sampling takes place, study methods, sample size, and whether body composition, gender, phase of the menstrual cycle, and psychological or pharmacological factors affecting the HPA axis are accounted for (99, 145, 226, 230, 311). For practical reasons most studies do not examine all of these parameters. The following section discusses the methods used to study the HPA axis in this thesis, as well as information that is lacking and how it may have affected the results.

5.6.2.1 The HPA axis

Basal serum cortisol varies considerably between individuals, but is stable over time within individuals and correlates well with feedback inhibition with low-dose DEX (101). However, dynamic testing always provides more information. This is

exemplified in **study I**. Lack of dynamic tests in studies **III and IV** are a drawback in study design, but the studies do still provide new data on the effects of pioglitazone and sitagliptin on cortisol levels.

In **studies I and II**, the 1 μg ACTH test was chosen for its sensitivity. Its use provided new information on the loss of gender differences in T2D, and the effect of improved insulin sensitivity with pioglitazone, on adrenal sensitivity to ACTH. Adding a CRH test with measurement of ACTH and cortisol to study the hypothalamic and adrenal response, and a stress test to study hypothalamic reactivity, would have optimized study design (see 1.2).

The low-dose DEX test used in **study I** is more sensitive than the standard-dose test, and incorporating it is a strength of the study (see 1.2.5). However the DEX test regardless of dose has the limitation of primarily examining the function of GR-II. DEX can bind to both GR-I and –II, but preferentially binds to the latter whereas endogenous glucocorticoids preferentially bind to GR-I (73). Feedback inhibition via both GRs is suppressed in obese men (312), and use of hydrocortisone, which acts as an endogenous glucocorticoid, may have provided a more physiological test of feedback inhibition (73).

5.6.2.2 Peripheral cortisol metabolism

Assessment of 11βHSD1 expression or activity may have helped clarify our results. Women have lower activity in 11βHSD1 compared with men (148, 313), which may secondarily increase the drive on the HPA axis as seen in healthy women in **study I**. GH inhibits hepatic 11βHSD1 through an IGF-I mediated effect (314). IGF-I increased in patients with T2D during pioglitazone treatment in **studies II and III**, and may have inhibited 11βHSD1 in the liver. This could in theory lead to reduced basal cortisol levels as seen in T2D patients in **study II.** PPARγ may have had direct effects by downregulating 11βHSD1 as seen in adipocytes in vitro and in db/db mice and humans treated with rosiglitazone (263, 315).

5.6.2.3 Measurement of free cortisol

Free (i.e. active) cortisol can either be assessed by measuring CBG levels, or the level in saliva in which only free cortisol is found (65). The benefit of saliva cortisol is that it can be collected non-invasively and repeatedly under field conditions, and is strongly correlated with serum cortisol (316). Some studies have shown strong correlations between free and total cortisol, implying no need to correct for CBG levels (317). As the women in the study were predominantly post-menopausal, gender differences in CBG ought not to have been large. Still, measurement of either saliva cortisol, or both CBG and urinary free cortisol, would have provided more robust data on the active cortisol levels.

5.6.2.4 GR sensitivity

The cellular response to glucocorticoids is determined by GR sensitivity, i.e. how effectively the GR-mediated signals are transferred to the DNA (318). Even if total or free cortisol is measured, one can not be certain how well this correlates with the intracellular effects (319). Variants of the *GR* gene have been linked to disturbances in metabolism and activity of the HPA axis (143, 320). It can not be ascertained from our

data if reduced GR sensitivity contributed to elevated cortisol levels in women in **study I**, or during pioglitazone treatment in **study III**.

5.6.2.5 Exogenous and psychological factors affecting the HPA axis
Stress and many other factors affect regulation of the HPA axis, and are potential confounders in studies on this subject. Depression is associated with visceral obesity, metabolic disturbances and dysregulation of the HPA and somatotropic axis (321, 322). Stress may also cause weight gain (323). Smoking and alcohol abuse both raise cortisol levels (324, 325). In **study I** analyses were performed with and without the two patients who were smokers, and including them did not affect the results. However information is lacking on nicotine use, stress, depressive symptoms and alcohol intake in our studies.

5.6.3 Are disturbances in the HPA axis a cause or consequence of obesity and T2D?

The only way to answer this question with certainty would be to follow a cohort of individuals prospectively, a study which to our knowledge has not been performed. One can only speculate on the answer, based on previous studies.

Much evidence indicates that obesity (especially visceral obesity) triggers disturbances in the HPA axis, which in certain individuals (perhaps those genetically predisposed) can initiate development of T2D (98). Visceral obesity often arises before the development of glucose abnormalities, and is a strong risk factor for T2D regardless of gender (326) as well as being associated with dysregulation of the HPA axis (98).

On the contrary, disturbances of the HPA axis could also result in (or exacerbate) insulin resistance. Acute elevation of cortisol levels causes secondary hyperinsulinemia in healthy subjects (327). Cortisol inhibits insulin secretion from the β-cells in vitro and in vivo, ultimately resulting in T2D in mice (328, 329).

5.7 FUTURE PERSPECTIVES

The purpose of this thesis was to test the hypothesis that disturbance in the HPA axis exists in T2D, and that improved insulin sensitivity or ß-cell function are associated with normalization of the disturbance. Other aims were to evaluate if gender differences exist in these parameters, and to characterize them in relation to IGF-I and IGFBP-1. Although the present studies could not answer these questions completely, they provided important pieces of the puzzle and raised several questions for future studies.

Although the physiological relevance is not clear, the gender difference in adrenal reactivity to ACTH lost in T2D may have an influence on the pathogenesis or metabolic control in T2D. It underscores the importance of accounting for gender in future studies both on the function of the HPA axis, and in T2D in clinical trials.

If disturbances in the HPA axis do precede and perhaps contribute to the development of T2D, what possibilities are there of targeting these disturbances in order to prevent

or treat T2D? Physical activity has positive effects in reducing VAT and insulin resistance (330), although it is not clear whether this is associated with changes in the HPA axis (331, 332). GR blockade has been evaluated, but not been as effective as hoped (Ottosson 1995 Obes Res). At later stages, a dysregulated HPA axis may suppress activity of the somatotropic and sex steroid axes, and substitution of IGF-I or sex steroids may improve insulin sensitivity (110, 291). This is not a solution for all patients, but may be of benefit in those in whom IGF-I or sex steroid levels are low. 11ßHSD1 blockers have been developed in recent years, and are showing potential in the treatment of T2D (333).

T2D is a multifaceted disease. Some patients have severe insulin resistance, others display ß-cell failure without being severely overweight. Studies indicate that the same may be true for pathology of the HPA axis, with several phases of dysregulation (98). In order to develop treatments for T2D with focus on the HPA axis, studies will need to follow subjects at risk for T2D prospectively to fully elucidate the impact of the HPA axis' activity, and to learn whether it plays a role especially in certain subgroups.

6 CONCLUSIONS

The main conclusion of this thesis is that differences exist in the regulation of the HPA axis between groups with different degrees of glucose intolerance, but that the nature of the changes in the HPA axis depends on the characteristics of the group.

1. Adrenal sensitivity to ACTH was higher in men with T2D compared with healthy controls, despite excellent metabolic control. IGF-I was suppressed in overweight men with T2D and higher HbA_{1c}, compared to patients with IGT. Further studies will be needed on the reasons for the differences between the different groups of abnormal glucose tolerance, but the results add to the evidence that more advanced T2D is associated with suppression of the somatotropic axis.

2. Improved insulin sensitivity and ß-cell function during pioglitazone treatment was associated with changes in cortisol levels both in patients with T2D and IGT, although in different ways in different groups. IGF-I increased in all of the groups with T2D, reflecting normalization of the somatotropic axis that we hypothesize is due to improved lipid metabolism.

3. Our data confirmed that adrenal sensitivity to ACTH is higher in healthy women than men. This gender difference was abolished in T2D, due to increased reactivity in men. Basal cortisol was lower in women with T2D before pioglitazone intervention, and increased during therapy. However, this increase was small and metabolic control improved both in men and women. Hence, improved glycemic control was most likely improved by other mechanisms than reduced cortisol levels

4. Improved glycemic control and ß-cell function during sitagliptin treatment were not related to reduced cortisol levels, or to improved hepatic insulin sensitivity, in patients with glucose abnormalities diagnosed after acute stress (ACS).

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