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## Assessment and Treatment of Impaired Insulin- Secretion and Action in Type 2 Diabetes

Dorkhan, Mozghan

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PO Box 117  
221 00 Lund  
+46 46-222 00 00



Lunds Universitet, Institutionen för kliniska vetenskaper, Endokrinologiska kliniken,  
Universitetssjukhuset MAS, Malmö

## **Assessment and Treatment of Impaired Insulin- Secretion and Action in Type 2 Diabetes**

*Mozhgan Dorkhan, M. D.*



### **Akademisk avhandling**

Som med vederbörlig tillstånd av Medicinska Fakulteten vid Lunds Universitet för  
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*To the memory of Lennart Fredstorp*

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Cover picture: © [unite](#) for diabetes, a global symbol for diabetes



*Some times the more I think, the more there is no real answer.*

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## ABBREVIATIONS

ADA	American Diabetes Association
ALT	alanine-aminotransferase
ATP	adenosine 5'-triphosphate
BNP	brain natriuretic peptide
BMI	body mass index
CV	coefficient of variation
CVD	cardiovascular disease
DI	disposition index
DTSQ	diabetes treatment satisfaction questionnaire
IDF	the International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IVGTT	intravenous glucose tolerance test
FPG	Fasting plasma glucose
FPIR	first phase insulin response
GO	Graves ophthalmopathy
HbA <sub>1c</sub>	glycosylated (glycated) haemoglobin
HDL	high-density lipoprotein
HOMA	homeostatic model assessment
ITT	insulin tolerance test
MODY	maturity onset diabetes of the young
NGT	normal glucose tolerance
NGSP	the National Glycohemoglobin Standardisation Program in the United States
Nt-proBNP	N-terminal pro brain natriuretic peptide
NYHA	New York heart association
OGTT	oral glucose tolerance test
PPAR	peroxisome-proliferator-activated receptor
PROactive	the prospective pioglitazone clinical trial in macrovascular events
SU	sulfonylurea
T2D	type 2 diabetes mellitus
TAO	thyroid-associated ophthalmopathy
TSH	thyroid stimulating hormone
TZD	thiazolidinedione
UKPDS	United Kingdom Diabetes Prospective Study
WHO	The World Health Organization
WHR	waist-to-hip-ratio

## LIST OF PAPERS

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

- I Dorkhan M, Tripathy D, Malm G, Asgharian H, Tuomi T, Groop L. Independent measures of insulin secretion and insulin sensitivity during the same test; the glucagon-insulin tolerance test (GITT). *J Intern Med* 2008; [Epub ahead of print].
  
- II Dorkhan M, Magnuson M, Frid A, Grubb A, Groop L, Jovinge S. Glycaemic and non-glycaemic effects of pioglitazone in triple oral therapy of patients with type 2 diabetes. *J Intern Med* 2006; 260:125-133.
  
- III Dorkhan M, Lantz M, Frid A, Groop L, Hallengren B. Treatment with a thiazolidinedione increases eye protrusion in a subgroup of patients with type 2 diabetes. *Clin Endocrinol* 2006; 65:35-9.
  
- IV Dorkhan M, Frid A, Groop L. Effects and side effects of adding insulin glargine or pioglitazone to oral anti-diabetic therapy in patients with type 2 diabetes. Submitted.

Permission to reprint papers I-III has been granted from the Blackwell Publishing Ltd.

## BACKGROUND

### GENERAL INTRODUCTION

Diabetes mellitus is a condition of chronically raised blood glucose concentration caused by an absolute or relative lack of insulin in parallel with varying degrees of insufficient insulin action (Lillioja et al., 1993, Gerich, 1998, Weyer et al., 2001b). The two main types of diabetes are type 1 and type 2. Type 2 diabetes (T2D) is the most common type of diabetes, representing more than 90% of cases in most western countries. The classification of diabetes will be discussed in the following sections but the focus of this thesis is on T2D.

T2D is a progressive disease associated with several complications affecting all body organs. The major complications of diabetes are caused by affection of small blood vessels (microangiopathy) in the eyes, kidneys and nerves and large blood vessels (macroangiopathy or atherosclerosis) in the heart, brain and legs. These complications occur generally after several years of diabetes but it has been estimated that T2D may be present for up to 12 years before clinical diagnosis. During this period of undiagnosed and untreated diabetes, micro- and macrovascular disease progress and by the time of diagnosis a considerable proportion of patients have already developed retinopathy, nephropathy, neuropathy, and up to 50 % have cardiovascular disease (CVD) (Jones, 1997). The frequency of coronary heart disease and stroke is about 2-4 times higher in patients with diabetes and CVD is the most common cause of death in patients with T2D, accounting for about 2/3 of all-cause mortality (Haffner et al., 1998).

Diabetes is now a global problem with devastating human and social consequences and the costs for the care of diabetes and its complications have an overwhelming economic impact globally. Even though the disease is not curable, in many cases T2D and its complications can be prevented. T2D is such a major public health problem that there is now a UN resolution aiming to improve knowledge and increase awareness about the disease and to unify the world in the fight against diabetes (Silink, 2007).

In this thesis we have presented a method for assessing insulin secretion and action in clinical practice. We have also evaluated effects and side effects of modern treatment alternatives in T2D with emphasis on potential effects on markers of CVD.

## **THE PREVALENCE OF TYPE 2 DIABETES**

Today more than 240 million people worldwide suffer from diabetes and each year another 7 million people develop the disease. T2D accounts for 90-95% of all cases with diabetes in developed countries and for an even higher percentage in developing countries (Amos et al., 1997). The prevalence of T2D varies widely in different populations ranging from over 50% in Pima Indians (Bennett et al., 1971, Knowler et al., 1978, Knowler et al., 1990) to about 2.5% in some tribes in rural Africa (McLarty et al., 1989). European populations show a moderate prevalence of 5-10%. The prevalence in Scandinavian countries is relatively low, about 4-5% in Sweden (2002), but the prevalence is increasing dramatically worldwide (King and Rewers, 1993, King et al., 1998, Zimmet et al., 2001, Dunstan et al., 2002) and a steady increase has also been observed in Scandinavia (2003). Numbers of new cases are reaching epidemic proportions with the most striking increase in developing countries. The factors contributing to the rise in the prevalence of T2D are sedentary lifestyle with lack of physical activity and obesity, as a result of urbanisation.

## **THE CLASSIFICATION AND DIAGNOSIS OF DIABETES**

The term diabetes mellitus covers a wide spectrum of diseases and several attempts have been made to present an international consensus on classification taking into consideration etiological types and clinical stages. The first widely accepted classification system of diabetes mellitus published by The World Health Organization (WHO) was in 1980 (1980) and, modified in 1985 (1985). This system classified diabetes into two major types of insulin dependent diabetes mellitus (IDDM) or type 1 diabetes and non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes. In 1985, WHO recommended that the diagnosis of diabetes should be based on a 2-hour venous plasma glucose concentration of  $\geq 11.1$  mmol/l during an oral glucose tolerance test (OGTT), and/or a fasting plasma glucose (FPG) level of  $\geq 7.8$  mmol/l. In 1997, American Diabetes Association (ADA) introduced new criteria for diagnosis of diabetes with a lowered fasting plasma glucose of 7 mmol/l (1997). For simplicity ADA recommends the measurement of FPG while WHO recommends measuring both FPG and the 2-hour glucose. As more data on aetiology and the importance of intermediate non-diagnostic glucose values emerged, new diagnostic criteria and classification system was introduced by WHO in 1999 (Alberti and Zimmet, 1998).

**Table 1.** Comparison of 1999 WHO and 2003 ADA diagnostic criteria

	WHO 1999	ADA 2003
<b>Diabetes</b> Fasting glucose 2-h glucose*	≥7.0mmol/l <b>or</b> ≥11.1mmol/l	≥7.0mmol/l <b>or</b> ≥11.1mmol/l
<b>IGT</b> Fasting glucose 2-h glucose	<7.0mmol/l (if measured) <b>and</b> ≥7.8 and <11.1mmol/l	<b>Not required</b>  ≥7.8 and <11.1mmol/l
<b>IFG</b> Fasting glucose 2-h glucose	6.1 to 6.9mmol/l <b>and (if measured)</b> (measurement recommended)	5.6 to 6.9mmol/l  Measurement not recommended (but if measured should be <11.1 mmol/l)

\* Venous plasma glucose 2-h after ingestion of 75g oral glucose load

The major change recommended in these diagnostic criteria for diabetes mellitus was the lowering of the diagnostic value of fasting venous and capillary plasma glucose from  $\geq 7.8$  to  $\geq 7.0$  mmol/l based on several studies showing increased risk of micro- and macrovascular disease in persons with fasting plasma glucose concentration above this cut off (McCance et al., 1994, Charles et al., 1996). These most recent classifications by WHO and ADA use the terms type 1 and type 2 diabetes and have dropped the earlier used terms of insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) since these terms were confusing and resulted in misclassification of patients based on their treatment rather than on pathogenesis. Type 1 diabetes refers to a disease characterized by autoimmune destruction of  $\beta$ -cells and defect insulin secretion. T2D results from the interaction of genetic and environmental factors and the underlying pathophysiology is varying degrees of defects in insulin secretion and action. Impaired glucose tolerance (IGT) was considered a class in the earlier WHO classification. Impaired fasting glycaemia or impaired fasting hyperglycaemia

(IFG) was also introduced in the 1999 WHO classification, including individuals with fasting plasma glucose values of 6.1-6.9 mmol/l. The spectrum of diabetic disorders also includes other specific forms like MODY (maturity onset diabetes of the young), which will not be further addressed in this thesis (Alberti and Zimmet, 1998). In 2003, ADA reviewed its diagnostic criteria and recommended a plasma glucose level of 5.6 mmol/l as a new diagnostic threshold for IFG. Obviously the existing diagnostic cut off values for glucose are arbitrary and there are studies demonstrating increased risk for microvascular and cardiovascular disease at levels below those diagnostic of diabetes (McCance et al., 1994, Charles et al., 1996, Gerstein et al., 1999). In 2005 a joint WHO and International Diabetes Federation (IDF) Technical Advisory Group reviewed and updated the current WHO guidelines and recommended to maintain current fasting plasma glucose levels for definition of IFG because of lacking evidence for the benefits of lowering the FPG level for risk of diabetic complications (<http://www.who.int/diabetes/publications>)(Table 1).

Lately, newer hypothesis concerning classification of diabetes have been presented. The difference between the two types of diabetes may be more apparent than real and the accelerator hypothesis proposed by Wilkin (Wilkin, 2001, Wilkin, 2007) suggests that type 1 and 2 diabetes represent the extremes of a continuum and the difference lies in their rate of progression determined by accelerators in terms of constitution, adiposity and autoimmunity.

## **DETERMINANTS OF TYPE 2 DIABETES**

### **Genetic factors**

A genetic susceptibility to T2D is well recognized. Twin studies demonstrate a higher concordance rate for T2D in identical twins ranging from 50 to 90% (Kaprio et al., 1992, Medici et al., 1999, Poulsen et al., 1999) than in dizygotic twins ranging from 25 to 40% (Lo et al., 1991). The lifetime risk for an offspring of a parent with T2D is about 40%, and higher if both parents have diabetes. The relative risk to a sibling of patients with T2D is about 3-fold increased (Poulsen et al., 1999, Weijnen et al., 2002). There are also monogenic forms of diabetes e.g. MODY which account for < 5% of all cases with diabetes. Although they show some similarities to classical T2D, they develop at earlier age and show a strong dominant penetrance (Fajans, 1989, Karlsson and Groop, 2007). The new classifications of diabetes call

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them genetically defined subgroups of diabetes. However, T2D is a paradigm for a complex, multifactorial polygenic disease where multiple genes interact with the environment to cause the disease. The rapid development of large-scale genotyping technologies has led to a breakthrough in the knowledge of genetics of T2D in recent years (Saxena et al., 2007).

### **Environmental factors**

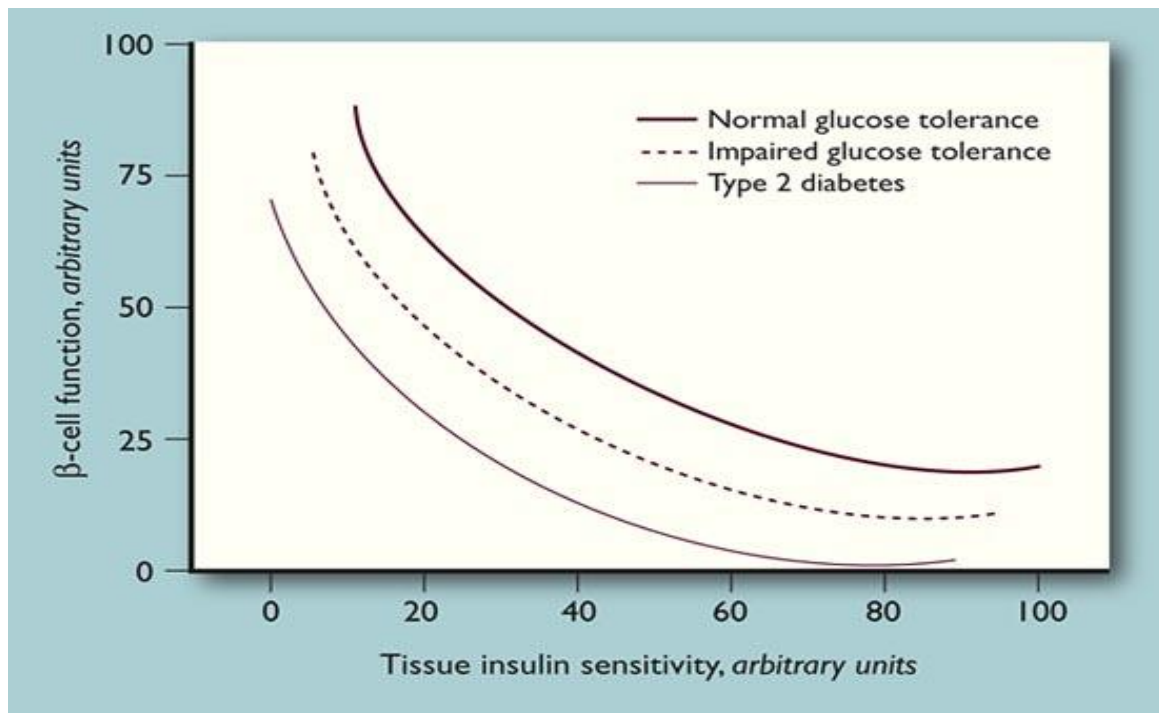
Although there is a strong genetic component to T2D, not all people who are genetically at risk develop the disease. This reflects the important role of the environment in the course of disease development. This fact is supported by the observation of rapid changes in the prevalence of diabetes in certain populations. The most striking changes have been observed in Pima Indians after a change to Western lifestyle. The prevalence of T2D increased by more than 40% over a ten years period in parallel with an increase in obesity (Knowler et al., 1990). T2D was also more common in Japanese migrants to US and their offspring than in their counterparts in Japan (Kawate et al., 1979).

The strongest environmental risk factors for developing T2D are obesity (Knowler et al., 1981, Bennett, 1992) and physical inactivity. Studies in several ethnic groups have shown that the prevalence of diabetes in the physically inactive subjects is about two to three times higher than among physically active people (Kriska et al., 1993). Consequently, several studies have demonstrated the effect of weight loss and physical activity in prevention of T2D (Pan et al., 1997, Tuomilehto et al., 2001, Knowler et al., 2002).

### **METABOLIC DEFECTS IN THE DEVELOPMENT OF TYPE 2 DIABETES**

The fundamental defects in T2D are impaired insulin secretion and action in peripheral tissues. This defective sensitivity to insulin is expressed as increased gluconeogenesis in the liver and reduced glucose uptake under insulinized conditions in muscles and adipose tissue. In 1981 Bergman suggested (Bergman et al., 1981) that in subjects with normal glucose tolerance (NGT) insulin secretion is inversely related to insulin sensitivity so that the product of these both variables is a constant (disposition index, DI) in order to maintain normal glucose levels. This has been confirmed in several cross-sectional studies of individuals with

NGT (Kahn et al., 1993). In other words, the degree of insulin secretion is tightly regulated by the level of insulin sensitivity.



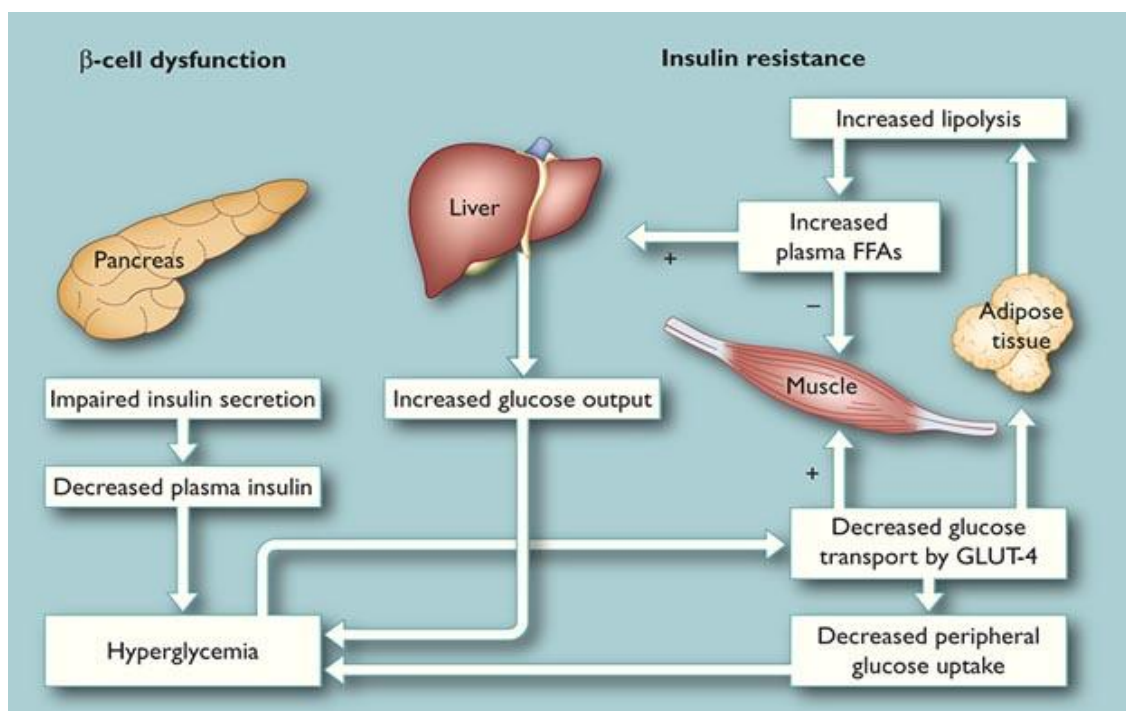
**Figure 1.** Reciprocal relationship between  $\beta$ -cell function and tissue insulin sensitivity.

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Patients who develop IGT or T2D have an inadequate  $\beta$ -cell compensation for insulin resistance in that for any degree of reduced tissue insulin sensitivity, the  $\beta$ -cell response is below normal (Weyer et al., 1999, Kahn, 2001, Pratley and Weyer, 2001)(figure 1). In non-diabetic individuals, insulin suppresses hepatic glucose production, stimulates glucose uptake into muscle and adipose tissue, and suppresses lipolysis in adipose tissue. When these tissues become insulin resistant, hepatic glucose production increases, glucose uptake is decreased, and lipolysis is enhanced. Increased free fatty acids (FFAs) from lipolysis stimulate cellular uptake of FFAs and lipid oxidation. In muscle, the increased FFA availability accelerates fat oxidation, resulting in decreased insulin-mediated glucose uptake and disposal. In the liver, elevated FFAs stimulate gluconeogenesis and increase hepatic glucose output. When  $\beta$ -cell

dysfunction is also present, insulin resistance in the target tissues leads to hyperglycaemia and the development of T2D (figure 2).



**Figure 2.** Defects in the pancreas and target tissues for insulin action in type 2 diabetes

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### Insulin secretion

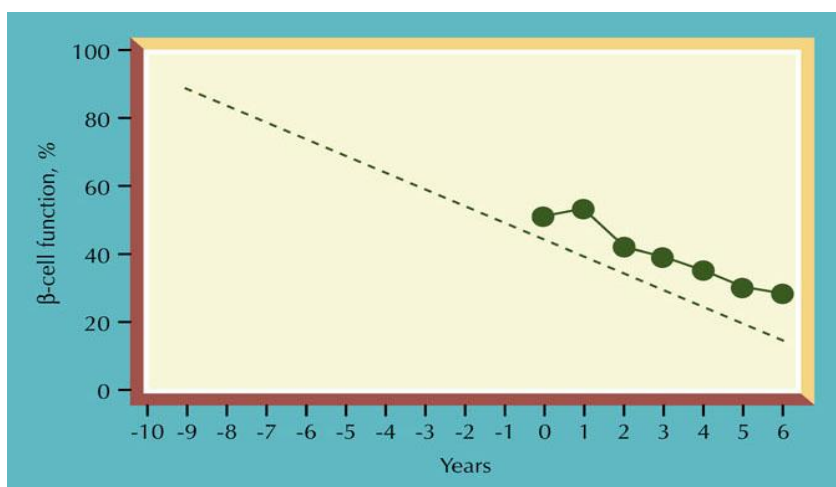
Insulin secretion is regulated by several factors consisting of nutrients, endocrine peptides, gastrointestinal hormones and autonomous nervous system (Ahren, 1999). The major nutrient stimulating insulin secretion under normal conditions is glucose. In response to an acute elevation of blood glucose concentrations, insulin is secreted in a biphasic manner. The early response or first phase insulin secretion (FPIR) lasts for approximately 10 minutes and is believed to represent the release of insulin from mature secretory granules in close proximity to the  $\beta$ -cell plasma membrane, i.e. a rapidly releasable pool. This phase is followed by a slower, second phase of insulin secretion that can last for hours. This response is due to

prolonged stimulation of  $\beta$ -cells and represents partly mobilizing insulin granules from a storage pool to the rapidly releasable pool as well as increased *de novo* insulin synthesis (DeFronzo et al., 1979, Elahi, 1996, Rorsman, 1997).

### *Impaired insulin secretion in T2D*

The complex of  $\beta$ -cell dysfunction and insulin resistance is present well before the development of hyperglycaemia and clinical diagnosis of T2D (Eriksson et al., 1989). There has been a long debate regarding the relative contribution of  $\beta$ -cell dysfunction and insulin resistance to the pathogenesis of T2D (Taylor et al., 1994, Cerasi, 1995, Gerich, 1998, Ferrannini, 1998). Patients with T2D often appear to have normal  $\beta$ -cell function, which usually is a corollary of the existing feedback connections between insulin secretion and insulin action and that in many studies, these parameters have not been evaluated together.

$\beta$ -cell dysfunction in T2D affects different components. The FPIR is decreased or lost (Brunzell et al., 1976) along with a reduced maximal secretory capacity (Ward et al., 1984, Van Haeften et al., 1991). Also the pulsatility of insulin secretion is deranged (Lang et al., 1981, Polonsky et al., 1988) and finally there is an abnormality in the conversion of proinsulin to insulin (Ward et al., 1987, Yoshioka et al., 1988, Saad et al., 1990, Kahn and Halban, 1997, Nagi et al., 1998). A progressive loss of  $\beta$ -cell function has been demonstrated to exist many years before the debut of clinical T2D (1995, Lyssenko et al., 2005). This deterioration continues during the course of different therapies (figure 3).



**Figure 3.** Progressive decline of  $\beta$ -cell function in the UKPDS. Year 0 represents time of diagnosis of T2D.

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Several mechanisms have been proposed to contribute to this progressive deterioration of  $\beta$ -cell function, the most important ones being glucose desensitization or glucose toxicity (Leahy et al., 1986), elevated levels of free fatty acids (FFA) or lipotoxicity (Unger, 1995) and the effects of amyloid fibril formation by islet amyloid polypeptide (IAPP)(Clark et al., 1990).

#### *Methods for estimation of insulin secretion*

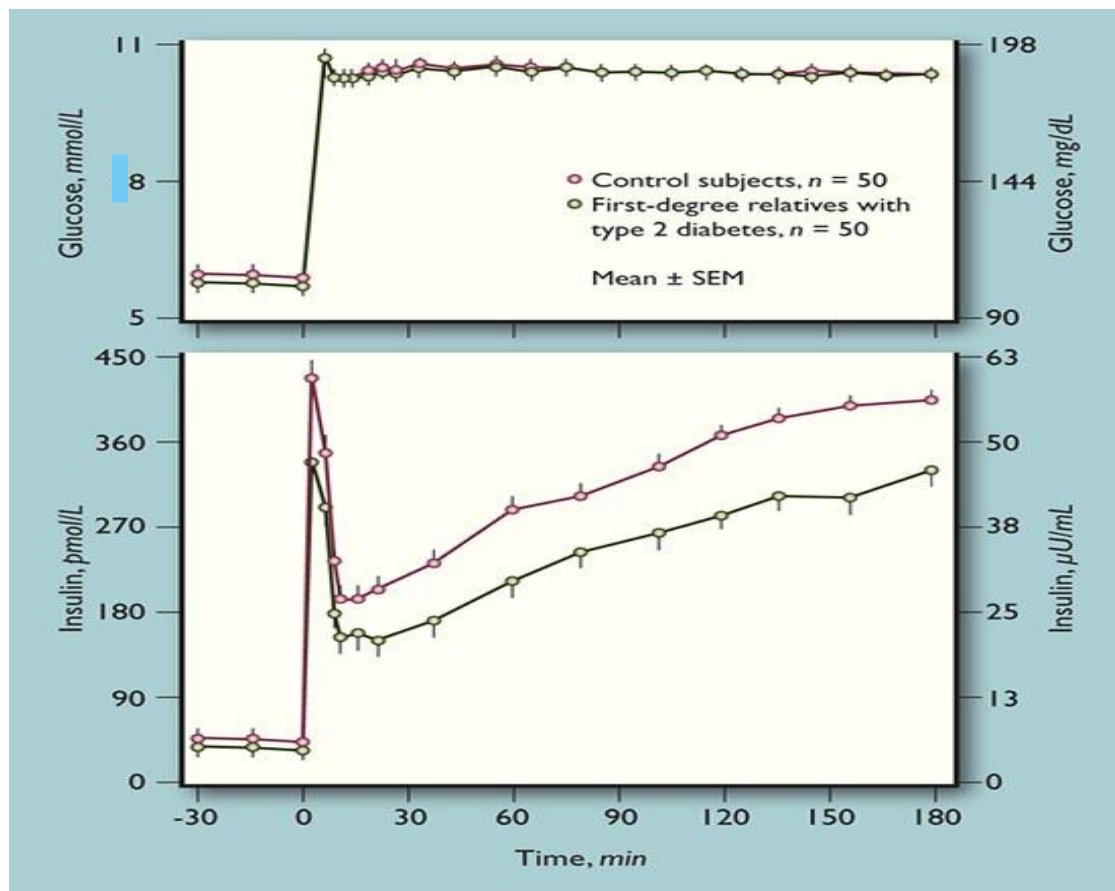
Evaluation of  $\beta$ -cell function is crucial for the understanding of the pathogenesis of T2D since an absolute or relative defect in insulin secretion is one of the major defects in T2D. However, this is not simple since several factors affect  $\beta$ -cell function and should be taken into account. For instance, insulin secretion is affected by the degree of insulin sensitivity as well as by the level of glucose at which the measurements take place along with differences in insulin secretion in response to different mode of administration of glucose. The most commonly used methods for estimation of  $\beta$ -cell function are discussed below. The glucagon-stimulated C-peptide test has been employed in this thesis to measure the residual  $\beta$ -cell capacity and is discussed more in detail in the methods section.

#### Hyperglycaemic clamp

This method is the golden standard for the measurement of  $\beta$ -cell function and provides estimates of both first- and second phase insulin response at glucose levels that can be matched between groups (DeFronzo et al., 1979). A bolus dose of glucose is injected intravenously followed by a constant infusion of glucose to achieve any desired glycaemic level. During the following three hours the plasma insulin levels are monitored. The area under the curve during the first ten minutes reflects FPIR and the area under the curve during the following clamp period reflects the late insulin response. These measures have also been used to characterize pre-diabetic states (figure 4). The problem is that the hyperglycaemic clamp is time- and personnel consuming and cannot be applied in large-scale studies. Therefore alternative methods have been searched for epidemiological studies and for use in clinical practice.

## Test meals

The idea with the meal tests is to measure  $\beta$ -cell function under normal life and physiological conditions. In the multiple test meal model subjects receive three standardized meals over a period of 14-15 hours and blood samples are drawn for measurement of glucose, insulin and C-peptide. The insulin secretory profile and the indices of  $\beta$ -cell function are derived from glucose and C-peptide dynamics (Mari et al., 2002).



**Figure 4.** Plasma glucose and insulin concentrations in hyperglycaemic clamp experiment, demonstrating the biphasic insulin response to elevation of plasma glucose concentration. A reduction in both early (first-phase) and late (second-phase) insulin release is demonstrated in this figure in subjects with normal glucose tolerance and a family history of T2D relative to a matched group without a family history of T2D.

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### Oral glucose tolerance test (OGTT)

In subjects with T2D and IGT there is a decrease in early insulin response after an oral load of 75-gram glucose while insulin values after two hours can be normal or elevated. The ratio of insulin to glucose 30 minutes after administration of oral glucose load is called ÷insulinogenic indexö and has been used as a surrogate marker of insulin secretion and has been shown to predict future development of diabetes (Phillips et al., 1994, Haffner et al., 1995, Hanson et al., 2000). The usefulness of OGTT is limited because of great intra-individual variation, partly due to day-to-day variability in gastrointestinal function and a poor reproducibility (Ko et al., 1998, Sievenpiper et al., 2001).

### Intravenous glucose tolerance test (IVGTT)

This method measures ÷cell response to 25 gram of glucose injected intravenously. The area under the curve during the first ten minutes following glucose injection represents FPIR and is impaired in conditions of abnormal glucose tolerance (Perley and Kipnis, 1967, Brunzell et al., 1976, Bourey et al., 1993). Loss of FPIR is one of the earliest defects seen in T2D (Lundgren et al., 1990, Skarfors et al., 1991, Lillioja et al., 1993).

### Glucose-dependent arginine stimulation test

The early insulin response to glucose stimulation is impaired when ÷cells still can respond to other stimuli such as glucagon, arginine, secretin and tolbutamide suggesting a defective glucose sensing (Robertson and Porte, 1973, Pfeifer et al., 1981). Elevated glucose levels stimulate the ÷cell response to non-glucose secretagogues causing an increased insulin response or so called glucose potentiation (Halter et al., 1979). The arginine stimulation test measures the insulin and glucagon responses to intravenously administered arginine at three different glucose levels; fasting, 14mmol/l and > 25 mmol/l glucose, thereby giving information on several aspects of ÷cell function such as the FPIR to arginine at fasting glucose, the maximal ÷cell secretory capacity, the ÷cell sensitivity to glucose and the glucose potentiation of insulin secretion (Ward et al., 1984, Larsson and Ahren, 1998). This method is also time-consuming and labour-intensive and unsuitable for large-scale studies.

### Glucagon-stimulated C-peptide test

Intravenous bolus injection of 1 mg glucagon has been widely used to assess endogenous insulin secretion for clinical or research purposes. Plasma C-peptide level is usually measured immediately before and six minutes after glucagon injection. The incremental C-peptide response is a measure of residual  $\beta$ -cell function and shows good intra-individual reproducibility (Faber and Binder, 1977). Measuring C-peptide rather than insulin has also the advantage of circumventing the hepatic extraction of insulin and has been shown to be more reproducible than insulin measurements (Gjessing et al., 1987, Gottsater et al., 1992). Dose-response studies have shown an equal effect of 0.5 mg glucagon but with reduced side effects in form of nausea (Ahren et al., 1987). In this thesis we have chosen the low dose glucagon-stimulated C-peptide test to measure residual  $\beta$ -cell function.

### **Insulin resistance**

The isolation of insulin in 1921 was followed by its use in the treatment of diabetes. There were consequently observations of ineffectiveness of insulin to regulate blood glucose in some individuals and this lack of insulin effect was termed *insulin resistance*. In 1936 Himsworth suggested that some people with diabetes were hyperglycaemic due to resistance to the action of insulin rather than to lack of insulin (Himsworth, 1949). This has been later confirmed by the observation of hyperinsulinaemia in obese and T2D subjects (Yalow and Berson, 1960). After the introduction of the clamp technique, several studies showed an increased insulin resistance in patients with T2D (DeFronzo et al., 1979).

#### *Insulin resistance in type 2 diabetes*

Even though insulin has a wide variety of effects on glucose-, lipid and protein metabolism as well as on vascular function, electrolyte balance and autonomic nervous system, the term *insulin resistance* has been considered mainly in the context of glucose metabolism. Insulin resistance is a key feature in the pathogenesis of different stages of glucose intolerance and T2D (Haffner et al., 1997, Arner et al., 1991). In the general population, insulin resistance tends to cluster with obesity, glucose intolerance, hypertension and dyslipidaemia (Ferrannini, 1993, Ferrannini et al., 1997). It is also being increasingly recognized that insulin resistance is important in the progression of cardiovascular disease (Reaven, 1988). Insulin resistance

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manifests as 1) decreased insulin mediated glucose transport and metabolism in skeletal muscles and adipocytes and 2) impaired suppression of hepatic glucose output. Insulin resistance is associated with adiposity (Ferrannini et al., 1997), especially visceral adiposity (Banerji et al., 1995, Boyko et al., 1996). Adipose tissue has in recent years been viewed as a major endocrine organ, secreting multiple metabolically active proteins termed adipokines (Chandran et al., 2003). Adiponectin, a protein synthesized by adipose tissue has gained attention for its role in glucose and lipid metabolism. Also, multiple studies have shown relationship between adiponectin and insulin sensitivity (Weyer et al., 2001a, Steffes et al., 2004). Low plasma adiponectin levels have been associated with coronary artery disease (Pischon et al., 2004, Dekker et al., 2008). One group of recently developed anti-diabetes drugs, namely thiazolidinediones (TZDs) target insulin resistance component of T2D. In this thesis, the effect of a TZD (pioglitazone) and a long-acting insulin (glargine) has been investigated on insulin sensitivity and on plasma adiponectin levels.

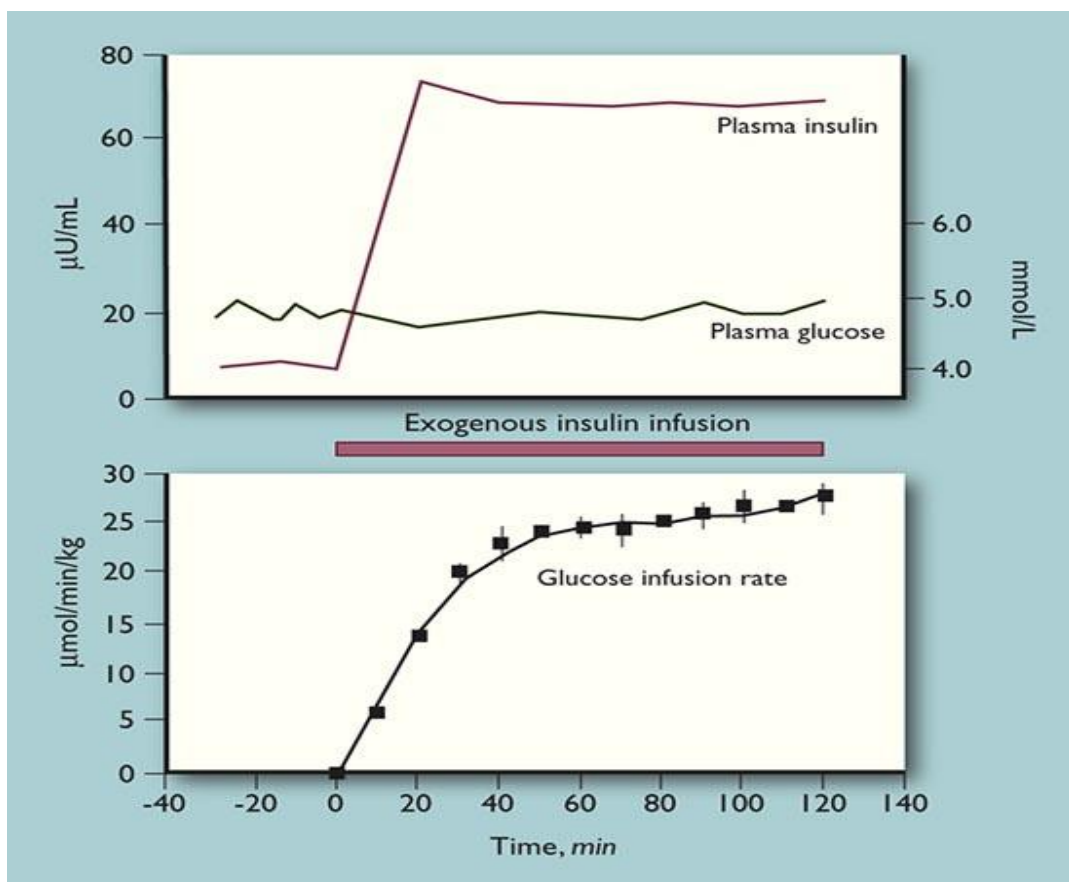
#### *Methods for estimation of whole body insulin sensitivity*

In the studies of pre-diabetic and diabetic state, qualitative and quantitative assessment of insulin resistance is required. The most commonly used methods for estimation of insulin sensitivity are discussed below. The insulin tolerance test is employed in this thesis to measure the degree of insulin sensitivity and is further discussed in the methods section.

#### Euglycaemic, hyperinsulinaemic clamp

This method is regarded as the "golden standard" for the measurement of insulin sensitivity in vivo (DeFronzo et al., 1979) and all other methods are validated against it (figure 5). Exogenous insulin is infused in a primed-constant format to raise plasma insulin concentrations to any desired level. As peripherally infused insulin is cleared rapidly from the plasma, a stable hyperinsulinaemic plateau is reached within 20 minutes. Exogenous glucose is infused simultaneously to prevent insulin-induced hypoglycaemia. The glucose infusion rate is adjusted every 5 to 10 minutes under the guidance of on-line plasma glucose measurements and plasma glucose concentration is clamped at the normal fasting or any other desired levels. During the second hour of a 2-hour clamp study, endogenous glucose release generally is suppressed, and the glucose infusion rate equals the total amount of glucose taken up by all tissues in the body. Glucose infusion rate during the clamp is an index of insulin

sensitivity (M-value). Because insulin suppresses endogenous glucose output and increases glucose disposal, the infusion rate is equal to the sum of increase in glucose disposal (peripheral insulin sensitivity) and suppression of endogenous glucose production (hepatic insulin sensitivity) under euglycaemic conditions. The clamp can be combined with a number of other procedures to enhance the information content of the study. For instance, if labelled glucose ( $^3\text{H}$ -3-glucose) is infused, then the amount of glucose produced by the liver can be calculated, allowing assessment of the hepatic insulin resistance.



**Figure 5.** Euglycaemic, hyperinsulinaemic clamp.

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The euglycaemic, hyperinsulinaemic clamp is highly reproducible (CV $\leq$ 10%). Similarly to the hyperglycaemic clamp, the euglycaemic clamp is also very costly, technically advanced and labour-intensive method and therefore not suitable for studies of large patient groups.

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### The minimal model

While the euglycaemic clamp method measures the effect of insulin on glucose uptake at steady state, the minimal model or frequently sampled intravenous glucose tolerance test (FSIGT), proposed by Bergman (Bergman et al., 1979) measures insulin action from a dynamic relationship between glucose and insulin. This model is a development of the IVGTT accounting for both insulin and glucose concentrations during the IVGTT by using a simplified mathematical representation of the glucose-insulin relationships. After basal sampling, glucose is injected and frequent sampling is begun. In the modified protocols, at 20 minutes a bolus of insulin or tolbutamide is injected and the frequent sampling for measurement of plasma glucose and insulin concentrations is continued until three hours. Using the program MINMOD, a set of values expressing insulin sensitivity and  $\beta$ -cell function are calculated. The measure of insulin sensitivity derived from this model shows a good correlation with insulin sensitivity derived from euglycaemic clamp (Bergman et al., 1987). The model has been widely used in a large number of clinical investigations. However, the main limitation of this method is that this technique requires endogenous insulin production and is therefore not applicable in subjects with T2D who have defective insulin secretion. Giving exogenous insulin or tolbutamide modifies the technique and allows it to be used in subjects with T2D (Finegood et al., 1990).

### Insulin tolerance test (ITT)

This was the first test developed to measure insulin sensitivity *in vivo* (Horgaard, 1929). The ITT consists of a bolus intravenous injection of rapid acting insulin and measurements of plasma glucose during following minutes. Both the amount of insulin given per kg bodyweight (usually 0.05-0.15 IU/kg) and the length of the test (10-40 minutes) can vary in different protocols. The plasma glucose concentrations after insulin injection are plotted on a semi logarithmic scale and, in most cases, a linear decline is observed. A greater slope of this linear decline ( $K_{ITT}$ ) represents better insulin sensitivity, which is simply calculated from the first order rate constant for disappearance rate of glucose estimated from the slope of the regression line of the logarithm of blood glucose against time ( $K_{ITT} = 0.693 / \text{plasma glucose half-time}$ ). The measure of insulin sensitivity derived from ITT ( $K_{ITT}$ ) shows close correlation with the values for glucose disappearance rate from euglycaemic clamp (M-value) in normal and diabetic subjects (Bonora et al., 1989). The main problems with the ITT are the risk of

hypoglycaemia and the possibility of secretion of counter-regulatory hormones in response to it. In this thesis the insulin tolerance test is used for assessment of insulin sensitivity.

#### Surrogate measures of insulin sensitivity

Several surrogate measures of insulin sensitivity have been developed. The most frequently applied methods are those measuring insulin sensitivity based on fasting levels of glucose and insulin, and those based on the OGTT.

The frequently used homeostasis model assessment (HOMA) (Matthews et al., 1985) represents insulin resistance as proportional to the inverse of the product of fasting insulin and fasting glucose  $[(\text{insulin} * \text{glucose})/22.5]$ . The model assumes that the basal insulin levels are directly proportional to insulin resistance. These interpretations may not be applicable when comparing groups with different  $\beta$ -cell function.

An insulin sensitivity index (ISI) can be calculated from the insulin and glucose values during an OGTT. This index has been shown to correlate with the euglycaemic clamp-derived insulin sensitivity (M-value) (Gutt et al., 2000). The OGTT derived indices of insulin sensitivity have own limitations and estimates of insulin sensitivity and  $\beta$ -cell function derived by the OGTT do not reproduce the hyperbolic relationship (Albareda et al., 2000). The measures are less valid in subjects with impaired  $\beta$ -cell function, since low insulin levels reflect the defective  $\beta$ -cell secretory capacity rather than insulin sensitivity.

## TREATMENT OF TYPE 2 DIABETES

*The ultimate objective in diabetes treatment is prevention* (Mudaliar and Henry, 2001).

It cannot be enough emphasized that the corner stone of management of T2D is modification of lifestyle. The majority of patients with T2D are overweight and show metabolic features of insulin resistance. Since even modest weight loss is associated with a reduction in insulin resistance and perhaps even an improvement in  $\beta$ -cell function (Goldstein, 1992), it is generally accepted that total energy should be reduced in order to achieve weight reduction. While the composition of an optimal diet remains a subject for debate, there is now considerable evidence to support the suggestion that a diet low in fat and relatively high in

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wholegrain and fibre-rich carbohydrate is appropriate for achieving and maintaining weight reduction (Miller et al., 1990, Prewitt et al., 1991).

Regular physical activity enhances weight loss in addition to beneficial effects on insulin sensitivity, lipid profile and blood pressure (Hughes et al., 1993, Barnard et al., 1992) and is associated with a reduction in mortality (Moy et al., 1993). In this chapter only the pharmacologic treatment of T2D will be discussed (Figure 6).

There is plenty of evidence that achieving specific glycaemic goals can substantially reduce morbidity (1999). Maintaining glycaemic levels, as close to normal range as possible can reduce microvascular complications (1998b, 1998a) but the beneficial effect on macrovascular complications is not as clear and under investigation (2005, Buse et al., 2007). T2D is associated with other abnormalities such as dyslipidaemia, hypertension, obesity, insulin resistance and hypercoagulability and these problems also need to be addressed to reduce complications. Different glucose lowering agents vary in their mechanisms of action thereby targeting different underlying defects of T2D but they also show differences in safety profile and tolerability as discussed below. Also cost is an important factor in the choice of therapy.

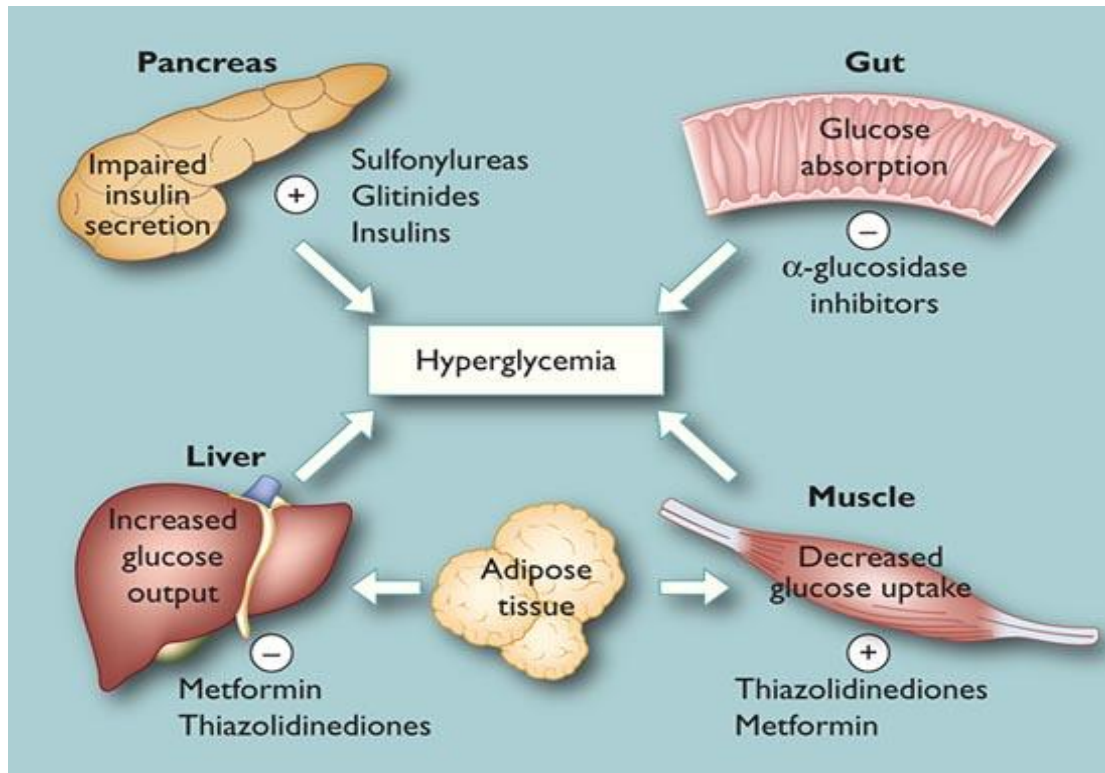
T2D is a progressive disease and there is a need for escalation of therapy by sequentially adding new glucose lowering agents to achieve and maintain glycaemic control. In recent years, several classes of drugs have been developed that target new pathways of potential importance for T2D. The efficacy and safety of these drugs is still under intensive research.

In this thesis, the thiazolidinedione pioglitazone and the long-acting insulin analogue glargine have been investigated for their roles in the treatment of T2D.

### *Biguanides*

Metformin is currently the only available drug belonging to the family of biguanides in most parts of the world. The exact cellular mechanism of action of metformin is not well known. There are evidences suggesting that activation of the enzyme adenosine monophosphate-activated protein kinase (AMPK) in hepatocytes, an important enzyme in the metabolism of

FFA and glucose may partly explain the mechanism of action of metformin (Zhou et al., 2001).



**Figure 6.** Anti-diabetic drugs and their mechanisms of action

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Metformin is effective in lowering fasting glycaemia. In monotherapy metformin lowers HbA<sub>1c</sub> levels by approximately 1.5%. Metformin is shown to improve insulin sensitivity in the liver, skeletal muscle and adipose tissue by reducing gluconeogenesis in the liver and enhancing glucose uptake by the peripheral tissues and liver. Metformin is specially effective in obese individuals and associated with significant weight reduction (DeFronzo et al., 1991, Stumvoll et al., 1995). The main side effect of metformin is gastrointestinal discomfort. There is no hypoglycaemia induced by metformin. Lactic acidosis is a quite rare but potentially fatal side effect that occurs mostly in patients with concurrent illnesses (Brown et al., 1998, Misbin et al., 1998) and limits the use of it in patients with T2D and nephropathy, hepatic dysfunction, congestive heart failure and advanced age.

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The UKPDS demonstrated improvement of insulin resistance with metformin and in a subgroup analysis metformin was associated with a significant reduction in diabetes-related macrovascular endpoints and all-cause mortality (1998a). These and other results caused a substantial increase in the use of metformin worldwide and metformin is now the drug of choice for the first-line treatment of T2D. Metformin is also the most cost-effective glucose-lowering agent available.

### *Sulfonylureas*

Sulfonylureas (SUs) act by enhancing endogenous insulin secretion. They exert their insulin-releasing effect mainly by binding to the sulfonylurea receptor (SUR) of the pancreatic  $\beta$ -cell, inhibiting ATP-sensitive potassium channels (Ashcroft and Rorsman, 1989). The insulin release is not modified by prevailing plasma glucose and there is a substantial risk for hypoglycaemia, the main side effect with this class of drugs. Newer SUs are more short acting and have a relatively lower risk for hypoglycaemia (Tessier et al., 1994, Holstein et al., 2001). SUs are as effective as metformin in decreasing HbA1c (Groop, 1992, Campbell and Howlett, 1995) but a weight gain of about 2 kg is common with the initiation of SU therapy. University Group Diabetes Program (UGDP) raised concerns about an increase in CVD mortality in patients with T2D treated with SUs (Prout, 1971, Karam et al., 1975). These results were not confirmed by UKPDS (1998b). The European Diabetes Policy Group of the International Diabetes Federation (IDF) has recognized metformin or SUs as the first choice of drug therapy in T2D. Considering the different mechanisms of action of metformin (primarily affecting insulin resistance) and SUs (primarily affecting defective insulin secretion) this combination may be rational. SUs are also the least expensive hypoglycaemic agents available after metformin.

### *Meglitinides or glinides*

Meglitinides are very short-acting insulin secretagogues, which like SUs stimulate insulin secretion by binding to a different site within the SUR (Malaisse, 2003). Meglitinides act to some extent in a glucose-dependent way and thereby exert a lower risk for hypoglycaemia. The efficacy of meglitinides in lowering hyperglycaemia is comparable to that of metformin and SUs. The weight gain associated with this class of drugs is similar to that of SUs. While

SUs lower glucose values even between meals meglitinides have a better effect on postprandial hyperglycaemia (Gerich et al., 2005).

#### *-Glucosidase inhibitors*

Normally, carbohydrates are rapidly absorbed in the first half of the small intestine. -Glucosidase inhibitors reduce the rate of digestion of polysaccharides in the proximal small intestine, lowering postprandial glucose levels. Consequently, increased transport of carbohydrates to colon results in increased gas production and gastrointestinal symptoms. This side effect is the reason why up to 45% of patients treated with -Glucosidase inhibitors discontinue medication. The effect of -Glucosidase inhibitors on HbA<sub>1c</sub> is more moderate, causing a mean decrease of about 0.7%. There is no weight gain and no hypoglycaemia associated with these drugs (van de Laar et al., 2005).

#### *Thiazolidinediones*

Thiazolidinediones (TZDs) or glitazones are synthetic agonists of peroxisome-proliferator activated receptor (PPAR) (Lehmann et al., 1995, Henry, 1997). PPAR are nuclear receptors, composed of three major isoforms (α, β and γ). PPAR α is a transcription factor, which when activated by TZDs (or other unknown ligands), promote transcription of a number of genes involved in fatty acid and glucose metabolism and thereby enhancing action of insulin (Saltiel and Olefsky, 1996). PPAR α is also essential for normal adipocyte differentiation and proliferation (Lemberger et al., 1996). PPAR α is expressed in key target tissues for insulin action, most abundantly in adipose tissue but also in liver, skeletal muscle, pancreatic β-cells, vascular endothelium and macrophages (Dubois et al., 2000, Willson et al., 2001). The glucose-lowering effect of TZDs is attributed to increased peripheral glucose disposal (Nolan et al., 1994, Miyazaki et al., 2001, Miyazaki et al., 2002a) and decreased hepatic glucose output (Miyazaki et al., 2001, Miyazaki et al., 2002a). There is also evidence of increased insulin secretory response in subjects with T2D following treatment with TZDs (Miyazaki et al., 2002b, Wallace et al., 2004). TZDs have been shown to cause redistribution of fat from hepatic and visceral adipose tissue to subcutaneous adipose tissue, and thereby possibly protecting liver, skeletal muscle and pancreatic β-cells from lipotoxicity (Miyazaki et al., 2002a, Shadid and Jensen, 2003, Smith et al., 2005, Rasouli et al., 2005).

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Used in monotherapy TZDs cause a decrease in HbA<sub>1c</sub> of about 0.5-1.5% (Aronoff et al., 2000, Rosenblatt et al., 2001, Scherbaum and Goke, 2002, Herz et al., 2003) and the effect is comparable with that of metformin and/or SUs (Einhorn et al., 2000, Hanefeld et al., 2004). TZDs seem to have additional effect when combined with metformin and/or SUs (Kipnes 2001, Hanefeld 2004). It is important to keep in mind that these drugs enhance the effect of insulin but do not replace insulin. Therefore, the effect is limited in late stages of insulinopaenic T2D.

The most common adverse effects of TZDs are weight gain and fluid retention. The fluid retention manifests usually as peripheral oedema but development or worsening of heart failure is also attributed to the use of TZDs (Dormandy et al., 2005, Singh et al., 2007).

Two TZDs are currently approved for treatment of hyperglycaemia in T2D, rosiglitazone and pioglitazone, which are both potent and highly selective agonists for PPAR $\gamma$ . Pioglitazone has in addition shown some minor activation of PPAR $\alpha$  (Forman et al., 1995, Lehmann et al., 1995). This activation has been linked to lowering of plasma triglycerides (TG) levels and a more beneficial effect of pioglitazone on lipid profile (Goldberg et al., 2005). PPAR $\alpha$  activation also has anti-inflammatory and preventive effects on arteriosclerosis (Rubins et al., 1999, Duez et al., 2002). TZDs have also been shown to increase adiponectin levels (Forst et al., 2005, Pfutzner et al., 2005, Dorkhan et al., 2006), which are reduced in obesity and negatively correlated to insulin resistance and development of arteriosclerosis.

TZDs are relatively new drugs. Rosiglitazone and pioglitazone have been on the market in the United States since 1999 and their effects and side effects have been and continue to be subject for intensive research. Recent data suggest that rosiglitazone therapy may result in an increased risk of myocardial infarction (Nissen and Wolski, 2007). Therefore, caution is advised if prescribing rosiglitazone to patients with or at risk of ischaemic heart disease. In contrast, the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events) demonstrated a reduction in composite cardiovascular endpoints with pioglitazone therapy in T2D patients who had a high risk of macrovascular events (Dormandy et al., 2005). Notably, this was associated with an increase in prevalence of heart failure. The effect of pioglitazone on glycaemic control, measures of  $\beta$ -cell function and insulin sensitivity and cardiac load are being investigated in this thesis. Another less known side effect of TZDs, namely the effect on eye protrusion is also investigated in this thesis.

### *Insulins*

Insulin is the oldest of available treatment modalities for diabetes hyperglycaemia and clearly the most effective one. The most common side effects of insulin therapy are weight gain and hypoglycaemia. The need for injections is though a barrier for insulin therapy. The risk of hypoglycaemia is reduced but not absent using long-acting insulin analogues. In the treatment of T2D, long-acting insulin can be added to lifestyle and metformin or at any stage during the treatment when glycaemic goals cannot be achieved by other means. Eventually, with the progress of  $\beta$ -cell deficiency, patients may also require prandial therapy with rapid-acting insulins (Nathan et al., 2008). Insulin glargine is a very long-acting human insulin analogue that has been on the market in Sweden since 2003. The effect of insulin glargine on  $\beta$ -cell function, insulin sensitivity and cardiac load are compared to those of pioglitazone in this thesis.

### *Drugs affecting the incretin pathway*

A new class of drugs that have been approved for use in the US since 2005 and in Sweden since 2007 are glucagon-like peptide 1 (GLP-1) agonists (incretin mimetics) and dipeptidyl peptidase-4 (DPP- IV) inhibitors (incretin enhancers). These compounds stimulate endogenous insulin secretion in a glucose dependent manner by mimicking the effect of incretin hormones or by inhibiting the degradation of endogenous incretins. GLP-1 agonists need to be injected subcutaneously while DPP-IV inhibitors are administered orally (Ahren and Schmitz, 2004).

## **GLYCAEMIC GOALS IN TYPE 2 DIABETES**

Epidemiological data (Klein et al., 1988, Chase et al., 1989) along with results from UKPDS (1998b, 1998a) and Kumamoto study (Ohkubo et al., 1995) support that reducing glycaemia is an effective means to reduce long-term complications. ADA recommends that treatment should aim at lowering  $HbA_{1c} < 7\%$  ([care.diabetesjournals.org/cgi/content/full/31/Supplement\\_1](http://care.diabetesjournals.org/cgi/content/full/31/Supplement_1)). In essence,  $HbA_{1c}$  should be as close to normal ( $< 6\%$ ) as possible without significant hypoglycaemia. However, the upper limit of non-diabetic range is 6.1% NGSP (the National Glycohemoglobin Standardisation Program in the United States). In Europe the corresponding  $HbA_{1c}$  target is slightly lower,  $< 6.5\%$  ([www.easd.org](http://www.easd.org))- IDF ([www.idf.org](http://www.idf.org)). These goals may

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not be achievable without significant increased risk of hypoglycaemia in some patients and therapy always needs to be individually tailored. These goals have been questioned by the recent results from the ACCORD (Action to Control Cardiovascular Risk in Diabetes) trial (Buse et al., 2007), which aimed to reduce HbA<sub>1c</sub> to levels seen in healthy individuals. The National Heart Lung and Blood Institute terminated the intensive glycaemic control arm of the study due to a surprising 20% increase in the mortality rate irrespectively of mode of therapy. Data from other studies investigating the effect on cardiovascular risk of a more aggressive approach to blood glucose levels should also be taken into consideration (Mellbin et al., 2008, Gaede et al., 2008). The results of ADVANCE (Action in Diabetes and Vascular Disease) study (2005) are expected to be presented later this year.

## **COMBINATION THERAPY IN TYPE 2 DIABETES**

Given the progressive nature of T2D, glucose lowering therapy needs to be intensified by time in order to meet the glycaemic goals. The current consensus (Nathan et al., 2008) is that metformin therapy should be initiated together with lifestyle intervention at diagnosis. When HbA<sub>1c</sub> goal is not achieved by these means, one can consider to add SU, TZD or basal insulin. If this combination fails to achieve glycaemic goals, a third agent can be added i.e. either insulin, TZD or SU or the insulin regimen can be intensified. When prandial insulin injections are started, SU or glinides may be discontinued. The combination of glucose lowering agents with different mode of actions may have the best effect. However, even if the combination of insulin and TZD is very effective, this combination is currently not recommended in Europe due to its high risk of fluid retention and heart failure.

## **AIMS**

1. To test and validate a new method for independent assessment of  $\beta$ -cell function and insulin sensitivity at the same occasion for metabolic studies in clinical practice.
2. To investigate the effect of adding pioglitazone to metformin and SU/meglitinide in patients with T2D not achieving glycaemic goals.
3. To examine the effect of pioglitazone therapy on eye protrusion.
4. To compare the effects and side effects of adding pioglitazone or insulin glargine to metformin and SU/meglitinide in patients with T2D.

## SUBJECTS, STUDY DESIGN AND METHODS

### *Subjects*

Subjects in different protocols of **paper I** ranged from healthy control subjects with NGT to patients with T2D and inadequate glycaemic control during treatment with oral glucose-lowering agents. Subjects were classified into different stages of glucose tolerance according to the WHO 1999 criteria. The number of subjects is presented for each protocol in the study design section.

Subjects in **papers II-IV** are patients with T2D ( $n = 54, 36$  and  $36$  respectively), inadequately controlled on treatment with metformin and SU/meglitinide. Inadequate glycaemic control in **papers II & III** was defined as HbA<sub>1c</sub> values  $\geq 6.5\%$  as measured by the Swedish Mono-S method ( $\leq 7.2\%$  NGSP) (Miedema, 2004) based on the Swedish national guidelines at the study time. In **paper IV** the cut-off value was lowered to  $6.2\%$  ( $\leq 7\%$  NGSP) to make the study better comparable with similar international studies and also because the Swedish national guidelines were assumed to be revised to adopt lower glycaemic goals. The main exclusion criterion was signs of congestive heart failure according to NYHA (New York heart association) II-IV.

### *Study design*

**Study I** included the five protocols presented below:

1. Ten subjects with NGT underwent in random order: glucagon-stimulated C-peptide test, ITT, ITT followed by the glucagon-stimulated C-peptide test, and glucagon-stimulated C-peptide test followed by the ITT (GITT). The tests were carried out on four separate days at least one week apart in order to investigate if and how the two tests could be combined.
2. Nine patients with T2D participated in GITT twice at one week interval in order to assess the reproducibility of the test.
3. Twenty subjects (10 T2D, 2 IFG, 8 NGT) participated in a Botnia clamp and a GITT one week apart in order to compare measures of insulin sensitivity obtained from both tests.

4. Eight subjects with NGT were compared with six drug-naïve patients with T2D and 11 patients with T2D and drug failure, defined as  $HbA_{1c} \geq 6.5\%$  in spite of treatment with metformin and SU/meglitinide to assess the discriminative capacity of the GITT.
5. In 17 patients with T2D, insulin sensitivity ( $K_{ITT}$ ) was calculated using all time point samples and also using only a reduced number of samples during the GITT in order to see if the number of samples and the duration of the test could be decreased.

**Studies II & III** were open-label, with a 26-week prospective follow-up period. Patients served as their own controls by comparing with their pre-interventional values. Patients received 30 mg pioglitazone once daily in addition to ongoing therapy with metformin and SU/meglitinide. The pioglitazone dose was increased to 45 mg/day after 16 weeks if  $HbA_{1c}$  was  $\geq 6.5\%$ . At the end of the study one third of patients were randomized to be followed with  $HbA_{1c}$  measurements for another three months after withdrawal of pioglitazone but with unchanged previous medication.

**Study IV** was a randomized, open-label 26-week prospective study. Patients inadequately controlled on metformin and SU/meglitinide were randomized to receive add-on therapy with insulin glargine or pioglitazone for 26 weeks. Insulin was up-titrated to achieve fasting plasma glucose  $< 6$  mmol/l. Pioglitazone was increased to 45 mg/day after 16 weeks if  $HbA_{1c} > 6.2\%$ .  $\beta$ -cell function and insulin sensitivity were assessed by measuring proinsulin and adiponectin, and in a subgroup using a combined glucagon-stimulated C-peptide test and insulin tolerance test (GITT).

### *Methods*

#### Anthropometrics:

Height, weight and waist to hip ratio (WHR) were measured in all subjects at inclusion.

In **paper I** fat free mass (FFM) was measured on the first test day with a bioelectrical impedance method (Segal et al., 1988).

In **papers II-IV** weight was measured at 8, 16 and 26 weeks follow up visits. WHR was also measured after 26 weeks.

#### Blood sampling:

Blood samples for fasting concentrations at all the investigations were drawn after an overnight fast while anti-diabetic medication was postponed until the sampling and the tests

were completed. Blood samples for the measurement of BNP and NT-proBNP were collected after at least 10 minutes rest in the supine position. Samples of insulin, C-peptide, NT-proBNP and adiponectin were centrifuged immediately and stored at -80° C to be analysed at the end of the study.

**Botnia Clamp (paper I):** This test was earlier designed to obtain independent measures of insulin secretion and insulin sensitivity at the same occasion (Tripathy et al., 2003). In brief, 0.3g/kg body weight of a 20% glucose solution was given at time 0. Blood samples for the measurement of serum insulin and plasma glucose were obtained at 0, 2, 4, 6, 8, 10, 20, 40, 50, 60, 120 and 180 minutes. The incremental trapezoidal area during the first 10 minutes of the test was called the first phase insulin response (FPIR). After 60 minutes, a priming dose of insulin was given followed by an infusion (infusion rate 45mU/m<sup>2</sup>) of short acting human insulin (Actrapid, Novo Nordisk, Denmark) and continued for 120 minutes. Blood samples for the measurement of plasma glucose were obtained at 5 minutes intervals throughout the clamp. A variable infusion of 20% glucose was started to maintain plasma glucose concentration unchanged at 5.5 mmol/l with a CV of 6%. Insulin sensitivity (M-value) was calculated from the glucose infusion rates during the last 60 minutes of the euglycaemic clamp and expressed as mg/(kg FFM x minute).

**Glucagon-stimulated C-peptide test (paper I):** Following basal sampling (-5, 0 minute), 0.5 mg of intravenous glucagon was given at time 0, and plasma glucose, serum C-peptide and insulin values were measured at 0, 5, 10 and 60 minutes.

**ITT (paper I):** 0.05 U/kg of short acting insulin (Actrapid 100 IU/ml, Novo Nordisk, Gentofte, Denmark), diluted to 10 IU/ml with saline was given at time 0 and the plasma glucose concentrations were measured at 0, 1, 3, 5, 7, 9, 11, 13, 15 and 20 minutes.

**ITT followed by a glucagon-stimulated C-peptide test on the same day (Paper I):** The ITT was performed as described above, but at 20 min. the iv. Injection of glucagon was given. Blood samples for the measurement of plasma glucose were taken at 0, 5, 10, 13, 15 and 20 minutes and for serum C-peptide and insulin at 0, 20, and 26 minutes.

Glucagon-stimulated C-peptide test followed by ITT on the same day (GITT) (papers I & IV): A bolus of 0.5 mg glucagon was given intravenously at time 0 and a blood sample for the measurement of plasma glucose and serum C-peptide and insulin were taken 6 minutes later. At 30 minute, a bolus injection of 0.05 U/kg of Actrapid insulin was given and blood samples for the measurement of plasma glucose were taken at 30,31,33,35,37,39,41,43,45, 50 and 60 minutes. The incremental C-peptide response at 6 min was used as a marker of  $\beta$ -cell function. For insulin sensitivity, the first order rate constant for glucose disappearance, the  $K_{ITT}$ , was estimated from the slope of regression line of  $\ln$  plasma glucose compared with the time using the formula  $K_{ITT} = \ln 2/T_{1/2} \times 100$  from 0 to 30 minutes after the insulin bolus. Since a hyperbolic relationship between  $\beta$ -cell function and insulin sensitivity is a prerequisite for estimating disposition index (DI) we investigated the existence of the hyperbolic relationship between insulin sensitivity and  $\beta$ -cell function in GITT using complete GITT data from 24 Subjects with NGT. DI was then calculated as product of the  $K_{ITT}$  and the incremental C-peptide response at 6 minutes.

Exophthalmometry (paper III):

The degree of eye protrusion was measured at inclusion and at the end of study i.e. after 26 weeks of therapy with pioglitazone. The measurements were performed by the same investigator (MD), using the same Kahnø exophthalmometer. Also the same inter-eye distance of the instrument was used for both measurements in given patient. At the second measurement the investigator was not aware of the previous measures of eye protrusion. The CV for repeated measurements in the same patient for the investigator (MD) was 3.7% for the right eye and 3.4% for the left eye.

Assays:

HbA1c was analysed using the Variant II chromatographic method from Bio-Rad with a CV of 3.0% at HbA1c 4.4%- 8.8% (ref. 4-5.3%) measured by Swedish Mono-S (high performance ion-exchange liquid chromatography).

Insulin concentrations were measured with a double antibody ELISA assay (DAKO, Cambridgeshire, United Kingdom) with an inter-assay CV of 8 %. C-peptide concentrations were measured by radioimmunoassay (LINCO, Missouri,USA). The lower limit of detection for C-peptide was 0.03 nmol/l; the intra-assay CV was 4.5% and the inter assay CV 3.2%.

Proinsulin was analysed using an ELISA assay (DakoCytomation Total Proinsulin) with an inter-assay CV of 7%.

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

BNP was measured with an immunoassay system from Beckman (Biosite, Ca, USA) with a CV of 3.5%. NT-proBNP was analysed using a competitive Enzyme Immuno Assay (Biomedica laboratories, Vienna, Austria) with an inter-assay CV of 6.5% and intra-assay CV of 4.4% (**paper II**) and an immunometric method (Hitachi Modular-E, Roche) with a CV of 4.4% (**paper IV**).

Cystatin C was measured by an automated particle-enhanced turbidometric assay (Dade Behring) and a calibrator obtained from DakoCytomation (Glostrup, Denmark).

#### Statistical analyses:

Data are expressed as means  $\pm$  SD, unless otherwise stated. All statistical tests were two-sided and p-values < 0.05 were considered significant. Analyses were carried out using the SPSS statistical software, versions 12.02 (**papers I-III**) & 15 (**paper IV**), Microsoft office Excel 2003 (**papers I-IV**), Eviews version 4.0 statistical software (**paper II**) and NCSS statistical software (Number cruncher statistical system, version- 6, Cork, Ireland) (**paper I**).

In **paper I** statistical analyses included Mann Whitney's test for comparison between group means, and Spearman rank correlation for testing of relations. The coefficients of variation (CVs) for the measures of insulin sensitivity ( $K_{ITT}$ ) and insulin secretion (C-peptide) for each subject were calculated individually from each set of repeated tests. To investigate whether the relationship between C-peptide response and  $K_{ITT}$  was hyperbolic ( $X \times Y = \text{Constant}$ ), we estimated the  $\ln$  (C-peptide response) as a linear function of  $\ln$  ( $K_{ITT}$ ) using regression (Kahn et al., 1993, Utzschneider et al., 2006).

In **paper II** differences over time of normally distributed variables were tested by paired t-test and of non-normally distributed variables by Wilcoxon rank test. Differences between group means were tested by unpaired-test or Mann-Whitney rank sum test, where appropriate.

In **paper III** significance of differences in patients' characteristics (age, BMI,  $HbA_{1c}$ , adiponectin) between subgroups was analyzed by non-parametric Wilcoxon test. The Wilcoxon signed ranks test was used to compare increase in eye protrusion before and after

six months of treatment with pioglitazone. A  $\geq 2$  mm change in eye protrusion measured by the Krahn exophthalmometer was considered as a significant change (Mourits et al., 1989, Bartalena et al., 2000, Asman, 2003). Logistic regression analysis was used to determine factors that predicted a significant change in eye protrusion. Change in eye protrusion of  $\geq 2$  mm was considered as dependent variable and baseline characteristics (age, sex, BMI, smoking, presence of thyroid disturbance and adiponectin levels) as well as pioglitazone dose as independent variables.

In **paper IV** analysis of variance (ANOVA) was used to test differences in changes of measured variables between treatment groups. Since there was a slight difference in the effect on HbA<sub>1c</sub> between the two treatment arms, changes in HbA<sub>1c</sub> was used as a covariate in analysis of covariance (ANCOVA). Non-normally distributed data were log-transformed.

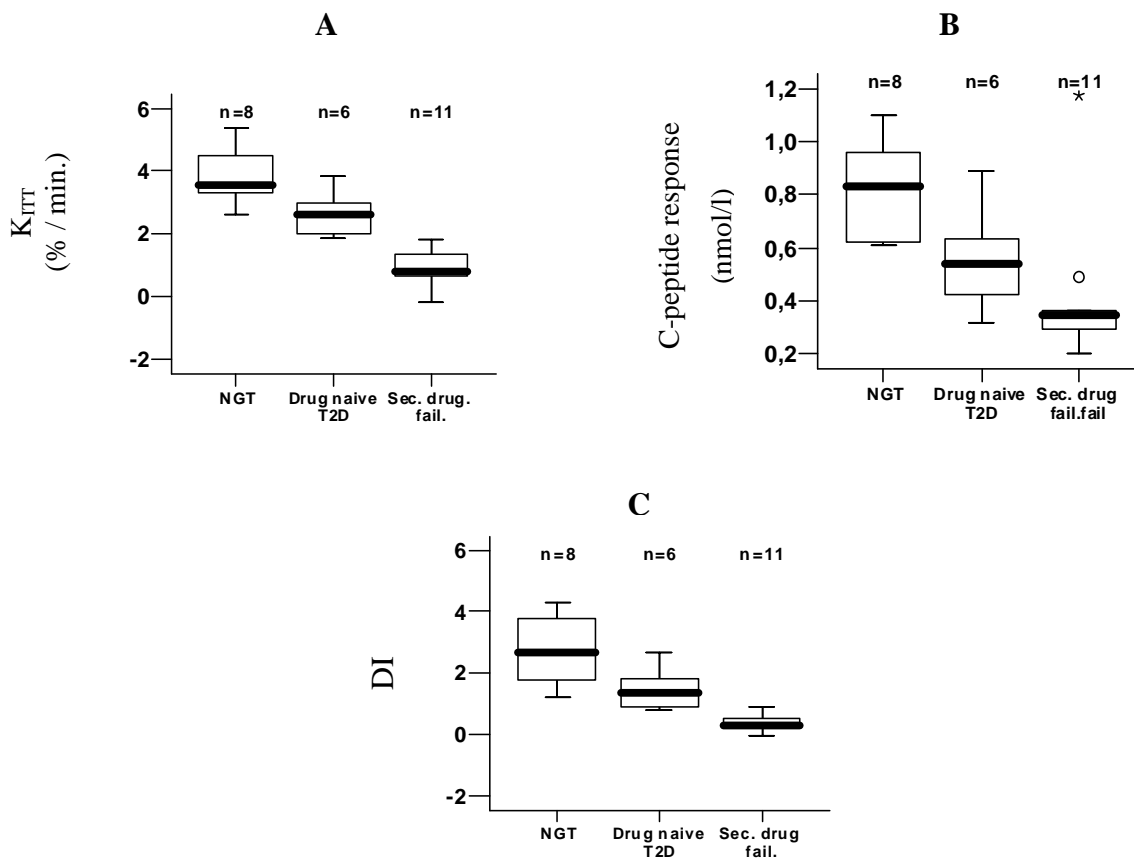
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## RESULTS

**Paper I:** Independent measures of insulin secretion and insulin sensitivity during the same test; the glucagon-insulin tolerance test (GITT)

1. The  $K_{ITT}$  measured on three different occasions in individuals with NGT did not differ regardless of whether the ITT preceded or followed the glucagon test. The mean CV for  $K_{ITT}$  calculated from 3 different days was 13%. On the other hand, C-peptide response to glucagon was significantly lower when it was preceded by the ITT.
2. The mean CV for  $K_{ITT}$  calculated from GITT at two different test days was 22% and for the C-peptide response 23% in patients with T2D.
3. The  $K_{ITT}$  correlated strongly with the M-value obtained during the Botnia clamp ( $r=0.87$ ,  $r^2 = 0.75$ ,  $p<0.001$ ) in both diabetic and non-diabetic subjects. The C-peptide response to glucagon did not correlate with the FPIR measured during the Botnia clamp ( $r=0.09$ ,  $p=ns$ ).
4. In 24 subjects with NGT the C-peptide responses to glucagon plotted against the  $K_{ITT}$  values showed the expected hyperbolic relationship as reported by Kahn et al. (Kahn et al., 1993). The correlation between the log-transformed C-peptide responses and the log-transformed  $K_{ITT}$  values was equal to  $-0.59$ ,  $p = 0.004$ . The slope of the curve, when we plotted the log-transformed C-peptide responses against the log-transformed  $K_{ITT}$ , was equal to  $-1.37 \pm 0.82$  and we could not reject the null hypothesis of the coefficient being equal to  $0$  ( $p = 0.366$ ), supporting a hyperbolic relationship between the two variables. We therefore used these measurements to calculate the DI as the product of  $K_{ITT}$  and C-peptide response at 6 minutes. The DI was significantly lower in patients with T2D and drug failure than in drug-naïve patients with T2D who in turn, had lower values compared with individuals with NGT (figure 7).
5. To test whether the number of glucose measurements needed during the test could be reduced, we recalculated the  $K_{ITT}$  values in 17 patients with T2D using different approaches.

$K_{ITT}$  values calculated based on measurements at 0,1,3,5,7,9,11,13,15, 20 and 30 minutes were compared to values calculated using fewer measurements. There was no benefit of extending the sampling beyond 20 minutes and the number of glucose samples could also be reduced to four without any significant changes of  $K_{ITT}$  values (0-30 vs 0,5,11,20 min. =  $1.18 \pm 0.15$  vs.  $1.18 \pm 0.16$ ,  $p=0.96$ ,  $CV= 11\%$ ).



**Figure 7.** (A)  $K_{ITT}$ , (B) C-peptide response and (C) Disposition Index calculated from GITT in 8 subjects with normal glucose tolerance (NGT), 6 drug-naïve patients with type 2 diabetes (T2D) and 11 patients with type 2 diabetes and secondary drug failure as a product of C-peptide response and  $K_{ITT}$ . The p-value for differences in DI between groups was significant after adjustment for age, sex and BMI (NGT vs. drug naïve T2D,  $p = 0.038$ , drug naïve T2D vs. drug failure,  $p = 0.022$ ).

In conclusion, the GITT provides simple, reproducible and independent measures of insulin-secretion and sensitivity on the same occasion for metabolic studies in clinical practice, especially in patients with T2D.

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**Paper II: Glycaemic and non-glycaemic effects of pioglitazone in triple oral therapy of patients with type 2 diabetes**

*Metabolic effects*

After 26 weeks of treatment with pioglitazone, HbA<sub>1c</sub> decreased from  $7.8 \pm 0.9$  to  $6.3 \pm 0.9$ ,  $p < 0.001$  with a greater decrease in women,  $p=0.029$ . While forty-three patients (80%) had at start HbA<sub>1c</sub>  $> 7\%$ , thirty-three (61%) reached the goal of HbA<sub>1c</sub>  $< 6.5\%$ . In the 18 patients, who were followed off-pioglitazone, HbA<sub>1c</sub> increased after three months from  $6.1 \pm 0.73$  to  $7.1 \pm 0.9$ ,  $p < 0.001$ . Triple therapy was associated with an increase in HDL ( $1.06 \pm 0.23$  to  $1.11 \pm 0.29$  mmol/l;  $p = 0.029$ ) and decrease in triglycerides ( $1.9 \pm 0.9$  to  $1.6 \pm 0.8$  mmol/l;  $p = 0.008$ ) concentrations whereas total cholesterol concentrations remained unchanged.

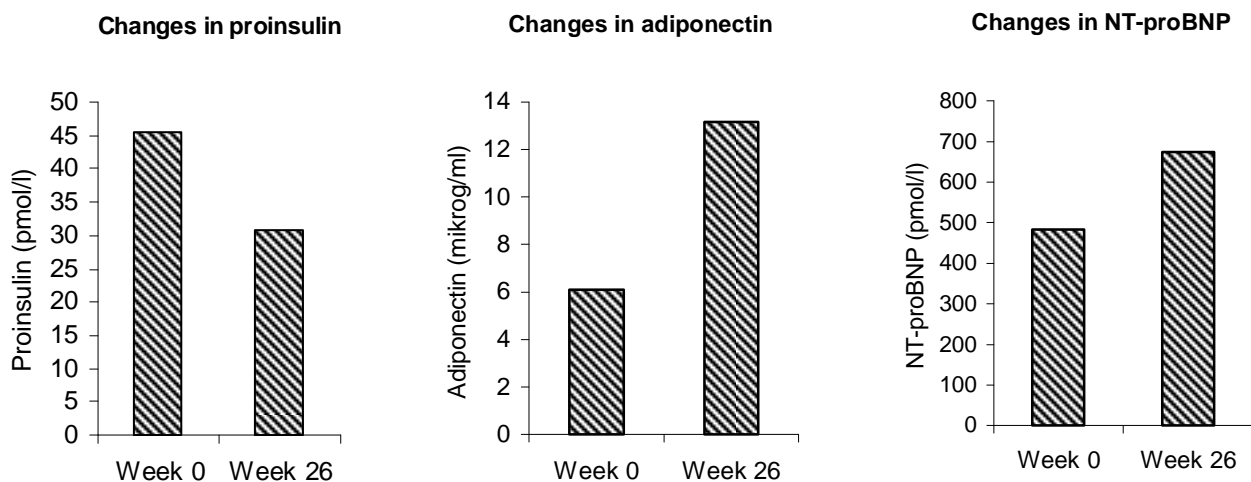
There was a highly significant decrease in proinsulin ( $45.7 \pm 38.7$  to  $30.9 \pm 27.2$  pmol/l,  $p = 0.001$ ) (figure 8) and proinsulin to insulin ratio ( $0.89 \pm 0.66$  to  $0.66 \pm 0.53$ ,  $p < 0.001$ ) without any significant changes in insulin levels. Adiponectin levels, which were used as surrogate markers of insulin sensitivity, increased more than two fold ( $6.1 \pm 2.8$  to  $13.2 \pm 5.8$   $\mu\text{g/ml}$ ,  $p < 0.001$ ) (figure 8) and the increase correlated with the decrease in HbA<sub>1c</sub> ( $r = -0.45$ ,  $p = 0.001$ ). Further analyses after publication revealed that the changes in adiponectin also correlated with the changes in HDL ( $r = 0.29$ ,  $p = 0.042$ ).

*Side effects*

During the study there was a significant weight gain from  $90 \pm 15$  to  $94 \pm 16$  kg ( $p < 0.001$ ) corresponding to an increase in BMI from  $31 \pm 4$  to  $32 \pm 4$  kg/m<sup>2</sup> ( $p < 0.001$ ). Despite the increase in BMI there was a significant decrease in waist-to-hip ratio (WHR) from  $1.03 \pm 0.08$  to  $1.00 \pm 0.06$  ( $p = 0.002$ ) that was due to smaller increase in waist ( $+1.6$  cm;  $p = 0.026$ ) than hip ( $+4.9$  cm;  $p < 0.001$ ) circumference. The most common side effect was oedema reported by ten patients (19%), in four (7%) of them transient. Twelve patients (22%) experienced mild hypoglycaemia; none of them required assistance but these patients had because of hypoglycaemic episodes to reduce the dose of sulfonylurea. Alanin-aminotransferase (ALT) was monitored as a safety parameter but there was actually a decrease in ALT levels ( $0.5 \pm 0.3$  to  $0.4 \pm 0.1$   $\mu\text{kat/l}$ ,  $p < 0.001$ ).

Nt-proBNP levels increased significantly after 26 weeks of treatment with pioglitazone from  $487.3 \pm 252.2$  to  $657.8 \pm 392.1$  pmol/l ( $p < 0,001$ )(figure 8). The elevation in Nt-proBNP was still significant after exclusion of patients with known cardiovascular disease from the analysis, from  $447.2 \pm 223.6$  to  $638.8 \pm 385.7$  ng/l ( $p = 0,002$ ). There was also a significant decrease in haemoglobin concentrations from  $139 \pm 11$  to  $131 \pm 13$  g/l ( $p < 0.001$ ). In the 37 patients without known cardiovascular disease the change in Nt-proBNP correlated negatively with the change in haemoglobin ( $r = -0.353$ ,  $p = 0.032$ ).

At the same time there was an increase in Cystatin C from  $0.96 \pm 0.20$  g/l to  $1.02 \pm 0.21$  g/l, ( $p = 0.004$ ) which corresponded to a  $6.8 \pm 0.18\%$  change in GFR as calculated from the cystatin C values (Grubb et al., 2005). The individual changes in GFR and Nt-proBNP did not correlate with each other ( $r^2 = 0.02$ ).



**Figure 8.** Changes in proinsulin, adiponectin and NT-proBNP during 26 weeks of therapy with pioglitazone. To be compared with figure 9.

In conclusion, pioglitazone was effective as add-on to metformin + SU/meglitinide in achieving glycaemic targets. Pioglitazone also improved measures of  $\beta$ -cell function as well as insulin sensitivity but there was evidences of fluid retention and increased cardiac load.

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**Paper III:** Treatment with a thiazolidinedione increases eye protrusion in a subgroup of patients with type 2 diabetes.

In all 36 subjects there was a median change in eye protrusion of 1 mm (interquartile range 2 mm) for the right eye,  $p < 0,001$  and 1 mm (interquartile range 1 mm) for the left eye,  $p = 0,011$ . Patients were divided into two subgroups based upon whether they showed an increase in eye protrusion of 2 mm or more (Group A,  $n=13$ ) or not (Group B,  $N=23$ ). The increase in eye protrusion (mean  $\pm$  SD) was significantly larger in group A ( $17.3 \pm 3$  to  $19.5 \pm 3.1$  mm in right eye and  $17.4 \pm 2.9$  to  $18.9 \pm 2.8$  mm in left eye) than in group B ( $17.5 \pm 2.5$  to  $17.7 \pm 2.3$  mm in right eye and  $17.6 \pm 2.5$  to  $17.6 \pm 2.5$  mm in left eye) ( $p_{\text{right}} < 0.001$ ,  $p_{\text{left}} = 0.001$ ). In 13 patients (group A) eye protrusion increased by 2 mm or more in right or left eye ( $p_{\text{right}} = 0.001$ ,  $p_{\text{left}} = 0.002$ ) while the change in eye protrusion in 23 other patients (group B) was not significant ( $p_{\text{right}} = 0.433$ ,  $p_{\text{left}} = 0.928$ ), ( $p_{\text{between groups}}=0.036$ ). There was a strong correlation between the degree of changes in eye protrusion between right and left eye ( $r = 0.66$ ,  $p < 0.001$ ). None of the patients noticed changes in the appearance of their eyes, nor did they report any other eye symptoms.

Group A and B did not differ regarding weight gain or glycaemic control measured as HbA<sub>1c</sub>. The group with the greatest increase in eye protrusion (group A) had lower adiponectin levels at baseline as well as after treatment with pioglitazone. The sex-adjusted increases in adiponectin levels in group A and B were similar after six months (118%). In group A, patients with previous or present thyroid disturbance were more frequent.

Logistic regression analysis, including change in eye protrusion of  $\geq 2$  mm as dependent variable and baseline characteristics (age, sex, BMI, smoking, presence of thyroid disturbance and adiponectin levels) as well as pioglitazone dose as independent variables was applied. Presence of thyroid disturbance, low adiponectin levels and pioglitazone dose were factors that predicted a significant change in eye protrusion.

In conclusion, treatment with pioglitazone induced an increase in proptosis in a subgroup of patients with T2D. This subgroup showed lower plasma concentration of adiponectin and more frequent thyroid disturbance, and was treated with higher doses of pioglitazone.

**Paper IV:** Effects and side effects of adding insulin glargine or pioglitazone to oral anti-diabetic therapy in patients with type 2 diabetes

*Metabolic effects*

After 26 weeks of treatment, the reduction in HbA<sub>1c</sub> was slightly greater in the insulin glargine than in the pioglitazone group (8.2±1.3 to 6±0.7 vs. 8.1±1.4 to 6.8±1.1, p= 0.050); therefore change in HbA<sub>1c</sub> was used as covariate when analysing changes in other variables. Baseline HbA<sub>1c</sub> correlated inversely with reduction in HbA<sub>1c</sub> in all subjects (r = -0,72) as well as in the glargine (r = -0.9) and pioglitazone (r = -0.61) group (all p < 0.01) separately. Pioglitazone, but not insulin glargine resulted in an increase in HDL concentrations (1.10±0.24 to 1.24±0.3 mmol/l, p<0.01 vs. 1.08±0.35 to 1.04±0.33 mmol/l, p=ns, p between groups <0.01).

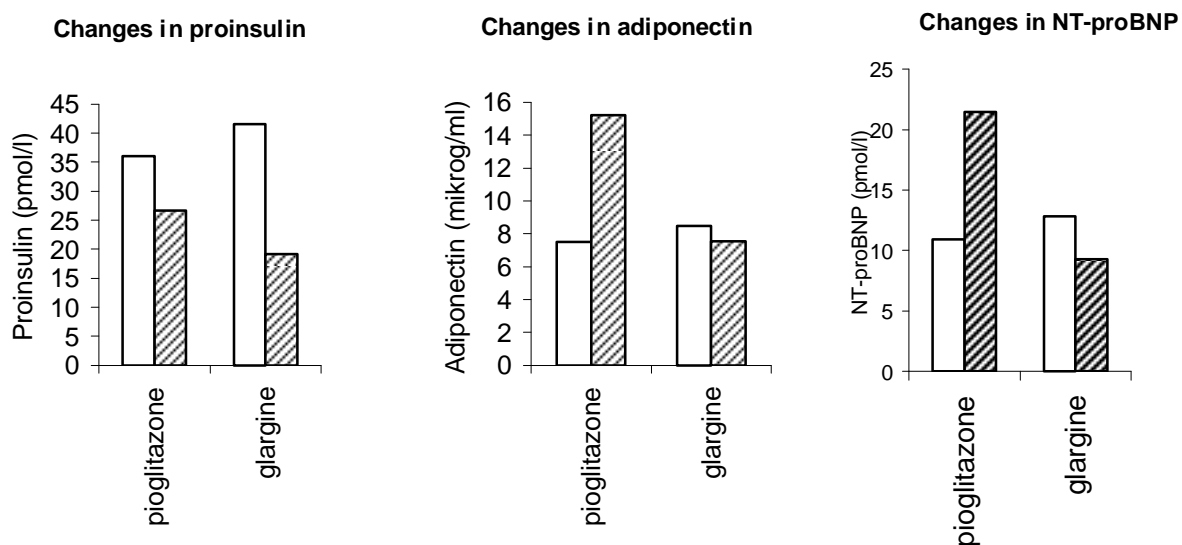
Insulin glargine resulted in a greater reduction in proinsulin concentrations than pioglitazone (-55 % vs. 625 %, p<0.01) (figure 9). This was accompanied by a decrease in fasting insulin concentrations in both groups. HOMA -cell also improved in the insulin glargine group but did not reach significance level in the pioglitazone group. However, there was no significant change in the C-peptide response to glucagon in any of the two treatment subgroups that underwent a second GITT at the end of the study.

Both treatments resulted in an improvement in insulin resistance as evidenced by a reduction in the HOMA-IR index without difference between the groups. There was a doubling of serum adiponectin levels in the pioglitazone group (7.5±3.7 to 15±10 g/ml, p<0.01) in contrast to a significant decrease in the insulin glargine group (8.7±4 to 7.6±3 g/ml, p=0.04), (p between groups <0.01) (figure 9), that correlated with changes in HDL in the whole group (r = 0.34, p = 0.045). There was a trend towards increased insulin sensitivity measured during the ITT (K<sub>ITT</sub>) in both subgroups without any significant difference between them.

*Side effects*

There was a similar weight gain in both groups. None of the subjects developed clinical heart failure. More hypoglycaemic episodes were reported in the insulin glargine than in the pioglitazone group (n = 5 vs. 1, p=0.053) but none of them was severe requiring assistance.

BNP and NT-proBNP correlated strongly both at start ( $r = 0.71$ ,  $p < 0.01$ ) and at the end of the study ( $r = 0.72$ ,  $p < 0.01$ ). There was a doubling of BNP and NT-proBNP concentrations in the pioglitazone group ( $6.6 \pm 5.2$  to  $13.7 \pm 16.1$  resp.  $27 \pm 45$  to  $52 \pm 102$  pmol/l) but no change in the glargine group ( $8.8 \pm 11.6$  to  $8.6 \pm 10.6$  resp.  $31 \pm 44$  to  $23 \pm 22$  pmol/l) ( $p$  between groups for BNP and NT-proBNP = 0.03) (figure 9) with large inter-individual differences between subjects. The changes in BNP and NT-proBNP were also correlated ( $r=0.79$ ,  $p<0.01$ ). The increase in BNP and NT-proBNP correlated inversely with the changes in haemoglobin ( $r = -0.34$ ,  $p = 0.045$  and  $r = -0.43$ ,  $p<0.01$ ) in the whole group. The inverse correlation between increase in NT-proBNP and the change in haemoglobin was even greater in the group treated with pioglitazone ( $r = -0.53$ ,  $p=0.03$ ) while there was no correlation in the group treated with insulin glargine ( $r = -0.003$ ,  $p = 0.99$ ). The NT-proBNP values at base line correlated strongly with changes from baseline during treatment with pioglitazone ( $r = 0.9$ ,  $p < 0.01$ ).



**Figure 9.** Changes in proinsulin, adiponectin and NT-proBNP during 26 weeks of therapy with pioglitazone vs. insulin glargine. To be compared with figure 8.

□ = Week 0, ▨ = week 26.

In conclusion, there are characteristic differences in the effects of insulin glargine versus pioglitazone on measures of  $\beta$ -cell function and insulin sensitivity as well as on cardiac load with some beneficial effects of each treatment alternative.

## DISCUSSION

### **How to measure $\beta$ -cell function and insulin sensitivity in clinical practice?**

It is still an open issue whether assessment of insulin secretion and insulin action would help in the choice of treatment in patients with T2D. One reason might be that there are few studies which have applied such measurements, most likely because available measurements are either not sensitive enough or too cumbersome for clinical practice.

For this purpose we have evaluated a modified and simplified combination of two established tests for independent measurements of residual  $\beta$ -cell function and insulin sensitivity at the same time, namely the combined glucagon-stimulated C-peptide test and the insulin tolerance test (GITT).

Our results demonstrates the expected relationship between  $\beta$ -cell function and insulin sensitivity when combining these two tests, allowing for evaluation of  $\beta$ -cell function adjusted for the degree of insulin sensitivity and calculating the disposition index (DI). Several studies have demonstrated the additive value of DI in the prediction- and definition of the pathology of T2D (Lyssenko et al., 2005, Nittala et al., 2006, Palmer et al., 2006). The test showed also good reproducibility.

One problem with  $\beta$ -cells of patients with T2D is that they do not longer respond to glucose, therefore tests using different glucose stimuli are not always informative in T2D. Glucagon, on the other hand, stimulates insulin secretion by bypassing glucose metabolism causing depolarisation of the  $\beta$ -cell (Ahren et al., 1987). This makes the GITT more useful in diabetic patients who have lost their early insulin response to iv glucose.

The main problem with the ITT has been the risk of hypoglycaemia and activation of counter regulatory mechanisms. A low dose of insulin in our study was chosen in order to prevent hypoglycaemia and none of the patients with abnormal glucose tolerance in our study developed hypoglycaemia. Also the counter regulatory mechanisms due to hypoglycaemia are shown to start after 20 minutes following insulin injection (Gerich et al., 1980). The shorter duration of the test enables us to prevent these unwanted interactions with the test results.

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In conclusion, GITT is a simple, reproducible and feasible method for independent assessment of  $\beta$ -cell function and insulin sensitivity at the same time in clinical practice. It takes about 50 minutes and five venous samples are needed. Our hope is that the manufacturers could produce a  $\beta$ GITT-kit including 0.5 mg of glucagon, diluted rapid acting insulin along with a protocol for registration of anthropometric data and the dose of insulin and results of glucose and C-peptide measurements. This could facilitate the performance of the test for instance at primary care centers and make it possible to obtain more standardized and comparable results from the future studies.

### **To substitute or sensitise when choosing add-on treatment in T2D**

The progressive nature of T2D is reflected in studies by a consistent and steady increase in HbA<sub>1c</sub> over time independent of the mode of treatment. This in turn is associated with enhanced risk of micro-and macrovascular complications. Combination therapy in order to meet glycaemic goals is inevitable. The failure to maintain glycaemic control is a consequence of deterioration of both insulin secretion and insulin sensitivity (hepatic and peripheral).

At the time our first interventional study started, the recommended treatment strategy of T2D was to start with metformin and add a SU/meglitinide (or vice versa) and when this combination therapy failed, to add or replace SU/meglitinide with insulin. Although TZDs had been tested in combination with metformin or SU/meglitinide, there were virtually no studies of their efficacy in triple therapy. Since metformin had been proposed to exert its effect mainly on hepatic insulin resistance and SU/meglitinide on insulin release from  $\beta$ -cells and TZDs were postulated to have their main effect on peripheral insulin resistance, the combination of these three classes of drugs seemed appealing. However, there was concern raised about fluid retention, increased cardiac load and risk of heart failure but there were no agreements on how to monitor the development of these side effects. Treatment with insulin is also accompanied by weight gain and fluid retention. In 2003, the first extra long acting insulin, insulin glargine, was commercially available in Sweden and we started our randomized study, comparing the effect of pioglitazone versus insulin glargine on glycaemic control,  $\beta$ -cell function, insulin sensitivity and surrogate measures of cardiac load.

Both pioglitazone and insulin glargine were effective in achieving glycaemic goals when used as part of triple therapy in patients with T2D who failed to maintain optimal glycaemic control during treatment with metformin and SU/meglitinide. The effect of pioglitazone on HbA<sub>1c</sub> was somewhat greater in study II than in study IV in spite of lower HbA<sub>1c</sub> levels at baseline of study II. This might have partly been due to a greater proportion of women in study II (43% vs. 24%), since TZDs are known to have better effect on HbA<sub>1c</sub> in women, most likely due to a greater amount of body fat. This was also confirmed in our study II. Treatment with insulin glargine resulted in a greater reduction in HbA<sub>1c</sub> levels than pioglitazone (**paper IV**). Studies comparing TZD and long acting insulins have yielded discrepant results (Rosenstock et al., 2006, Triplitt et al., 2006), which most likely can be ascribed to different titration schedules. While the maximum effect of TZD is limited to the maximum dose, there is no maximum dose for titration of insulin but this will in turn increase risk of hypoglycaemia. Also, the dose of insulin is easy to adjust based on fasting glucose levels, which is not the case with oral hypoglycaemic agents.

#### *Effect on $\beta$ -cell function*

In cross sectional studies, both proinsulin and the proinsulin to insulin ratio have been considered as markers for impaired  $\beta$ -cell function (Mykkanen et al., 1997, Mykkanen et al., 1999). Elevated proinsulin levels predict cardiovascular morbidity and mortality (Zethelius et al., 2002, Zethelius et al., 2005). We observed positive effects of pioglitazone on surrogate measures of  $\beta$ -cell function as measured by proinsulin/insulin (**paper II**) or proinsulin (**paper II & IV**). However, this effect was greater in the group treated with insulin glargine (**paper IV**). Whether the lower proinsulin concentrations seen after insulin glargine really represent an improvement in  $\beta$ -cell function as a result of replacement therapy, or suppression of endogenous insulin secretion by exogenous insulin cannot be deduced from the results. As measured by HOMA  $\beta$ -cell index, the improvement in the pioglitazone group did not reach significance while it did in the insulin glargine group. This could favour the explanation of an actual improvement in  $\beta$ -cell function but whether this is translated to long-term  $\beta$ -cell preservation is not known. In the subgroups that underwent a second GITT, there was no significant change in the C-peptide response to glucagon after six months treatment with pioglitazone or insulin glargine.

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In a study of diet-treated patients with T2D, treatment with pioglitazone caused a dose-dependent enhancement of  $\beta$ -cell function as measured by insulinogenic index during an OGTT (Miyazaki et al., 2002b). In another study of diet-treated patients with T2D, the improvement in HOMA  $\beta$ -cell index after treatment with pioglitazone was not accompanied by change in stimulated  $\beta$ -cell function as determined by hyperglycaemic clamp (Wallace et al., 2004). The subjects in our study had at baseline low residual  $\beta$ -cell function as measured by C-peptide response to glucagon ( $0.36 \pm 0.17$  nmol/l). According to older suggestions, a C-peptide response to glucagon  $< 0.6$  nmol/l predict insulin requirement (Madsbad et al., 1981, Gjessing et al., 1988, Hother-Nielsen et al., 1988). No other studies have compared the effects of an insulin sensitizer and insulin on proinsulin levels. Although both treatment regimes seem to reduce  $\beta$ -cell stress, insulin seems to be superior in this regard.

#### *Effect on insulin sensitivity*

Low adiponectin levels are associated with insulin resistance and cardiovascular disease (Weyer et al., 2001a, Steffes et al., 2004, Pischon et al., 2004, Dekker et al., 2008). Here we observed beneficial effects of pioglitazone on plasma adiponectin levels (**papers II & IV**) but surprisingly, there was a significant decrease in adiponectin levels in the group treated with insulin glargine (**paper IV**). Similar effects by insulin on adiponectin levels were proposed in a study by Basu et al using hyperglycaemic clamp (Basu et al., 2007). It has also been known that even if metformin exerts similar effects on insulin sensitivity as TZDs, metformin has no effect on adiponectin levels (Putz, 2004). An association has been shown between hepatic fat content and plasma adiponectin concentration (Bajaj et al., 2004, Kotronen et al., 2008, Juurinen et al., 2008). Therefore, the differences in the effect on adiponectin observed with different classes of drugs could potentially be attributed to their different effect on the hepatic fat content, as particularly TZDs have been shown to cause a redistribution of fat from viscera and liver to subcutaneous adipose tissue (Shadid and Jensen, 2003).

Insulin sensitivity as measured by HOMA-IR and  $K_{ITT}$  was enhanced after treatment with insulin glargine and pioglitazone but there was no significant correlation between changes in adiponectin, HOMA-IR and  $K_{ITT}$  in our study (**paper IV**). In a study of diet-treated patients with T2D (Wallace et al., 2004) there was a weak correlation between adiponectin and insulin-stimulated glucose uptake ( $M/I =$  quantity of glucose metabolised/unit of plasma

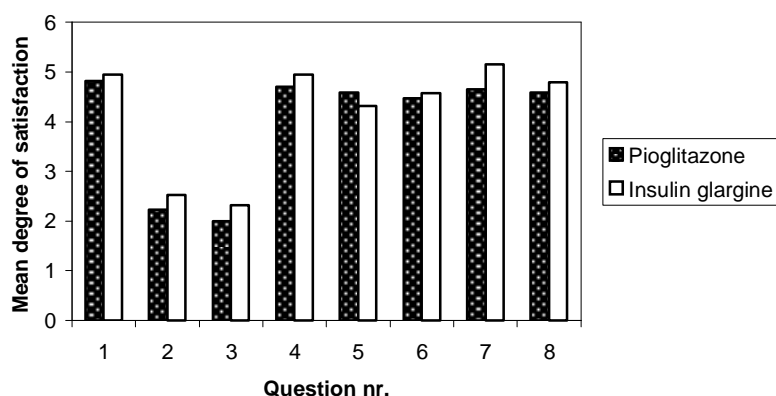
insulin concentration) assessed during hyperinsulinaemic clamp. Even though adiponectin is associated with insulin resistance, it is not known what component of insulin resistance it reflects. HOMA-IR reflects mostly changes in fasting plasma glucose and insulin whereas  $K_{ITT}$  reflects whole body insulin sensitivity. However, the small study size limits in-depth interpretations.

#### *Effect on natriuretic peptides*

Brain natriuretic peptide (BNP) is a peptide hormone released from the cardiac ventricles in response to pressure and volume overload. Among the various biomarkers applied to assess the risk of heart failure and coronary artery disease BNP, and the inactive, more stable N-terminal fragment of its prohormone (NT-proBNP) have generated a lot of attention in recent years. Both predict morbidity and mortality in patients with heart failure and acute coronary syndromes (Daniels and Maisel, 2007, Masson and Latini, 2008, Omland and de Lemos, 2008). NT-proBNP has also been shown to be independent risk marker for cardiovascular disease in patients with diabetes (Tarnow et al., 2005, Gaede et al., 2005). The circulating concentrations of BNP correlates with severity of heart failure assessed by echocardiography (Doust et al., 2004). Prior to our first study Ogawa et al had shown that pioglitazone could cause an increase in circulating BNP concentrations (Ogawa et al., 2003). In our studies (**papers I & IV**) there was a consistent increase in natriuretic peptides during treatment with pioglitazone. This was not the case in the group treated with insulin glargine even though the degree of weight gain and haemodilution (decrease in haemoglobin) seemed to be similar in both groups (**paper IV**). Patients with stages (II) III-IV of heart failure according to NYHA are not recommended treatment with pioglitazone due to the risk for induction or worsening of heart failure as a result of fluid retention. Accordingly, in most trials, including the PROactive study, the inclusion/exclusion of patients has been based on NYHA classification, which does not distinguish between symptoms due to coronary heart disease or heart failure. Diastolic dysfunction is present early in the course of T2D (Poirier et al., 2001) but hardly detected by the NYHA classification. Measurement of natriuretic peptides and haemoglobin in addition to monitoring of weight and clinical symptoms seems to be helpful in the monitoring of patients on TZD therapy. Echocardiography is however usually required for a proper diagnosis of heart failure. The high intra-individual variability in measurements of natriuretic peptides hampers their clinical use.

*Some additional aspects: effect on lipids, risk of fractures, patient satisfaction*

In keeping with previous studies pioglitazone was associated with a more beneficial lipid profile, particularly an increase in HDL (**papers II & IV**). Another important aspect to be taken into account is the patients' preferences. It is sometimes assumed that patients will rather take a pill than an injection. Our patients' response to DTSQ (diabetes Treatment Satisfaction Questionnaire) (Bradley, 1994, Bradley and Speight, 2002) showed an equal degree of satisfaction with both treatments (**paper IV**)(figure 10, original questions in Appendix 1).



**Figure 10.**  
The degree of satisfaction with treatment assessed by DTSQ

TZD therapy has also been associated with increased risk of fractures and osteoporosis (Schwartz et al., 2006). These aspects developed after initiation of our studies and were not monitored.

### **Thiazolidinedione Associated Retrobulbar Adipogenesis**

Thyroid-associated ophthalmopathy (TAO) or Graves' ophthalmopathy (GO) is an autoimmune disorder associated primarily with Graves' disease. TAO is the most common cause of unilateral or bilateral proptosis in adults and can seriously decrease quality of life. The signs and symptoms of TAO result from varying degrees of inflammation in the orbit and increased volume of the orbital contents, including adipose, connective and extra ocular muscle tissues. Orbital adipogenesis is a characteristic of TAO. *In vitro* studies have demonstrated that PPAR agonists contribute to the adipogenesis of orbital fibroblasts and

that TZDs can promote adipose tissue growth by activating the PPAR receptor in predpredominantly subcutaneous and orbital preadipocytes (Adams et al., 1997). In cultured retrobulbar preadipocytes, PPAR agonists caused a 2- to 13-fold increase, and a PPAR antagonist a 2- to 7- fold reduction, in adipogenesis (Starkey et al., 2003). As a result of these observations, concern has been raised about the use of PPAR agonists in patients with TAO (Smith et al., 2002).

With this notion and with the information from one case report showing worsening of ophthalmopathy after treatment with pioglitazone (Starkey et al., 2003) we decided to examine whether treatment with pioglitazone caused a systematic change in the degree of eye protrusion.

Our results demonstrated a significant increase of eye protrusion in a subgroup of patients treated with pioglitazone during six months. The predisposing factors for increased eye protrusion showed to be low adiponectin levels, thyroid disturbance and higher dose of pioglitazone. Our results were supported by another case report of a patient with congenitally prominent globes ó but without thyroid disease- who responded with increased proptosis after treatment with rosiglitazone for concomitant T2D (Levin et al., 2005). Also Lee et al. reported in 2007 another case of worsening of TAO after treatment with rosiglitazone (Lee et al., 2007).

The mechanism of TAO is complex and the pathogenesis still incompletely understood. Inflammatory processes are involved leading to expansion of retrobulbar structures. Anti-inflammatory treatment with steroids represents the main therapy for TAO in addition to surgery (Bartalena et al., 2000). TZDs are known to be involved in both modulation of inflammatory responses and adipocyte differentiation and growth.

Two other studies should be quoted in this context. The chemokine CXCL10 play an important role in the initial phases of autoimmune thyroid disorders. Human thyrocytes produce large amounts of CXCL10 when stimulated by IFN - and TNF . Antonelli et al. showed higher serum levels of CXCL10 in patients with Gravesø disease and Gravesø ophthalmopathy (GO) than matched controls. Treatment of thyrocytes and retrobulbar cell

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types with the PPAR agonist, rosiglitazone, dose-dependently suppressed IFN- $\gamma$  plus TNF- $\alpha$  induced CXCL10 release. The authors concluded that in GO, thyrocytes and retrobulbar cell types participate in the self-perpetuation of inflammation by releasing chemokines under the influence of cytokines and that PPAR activation plays an inhibitory role in this process (Antonelli et al., 2006). However, in this study, the cell cultures were incubated with rosiglitazone only for 24 hours. It may be necessary to expose the cells to TZDs for more than 10 days before their adipogenic effects can be seen.

Another recent study illustrates an antiinflammatory action of adiponectin in human monocyte-derived macrophages, suppressing T-lymphocyte chemoattractants such as CXCL10 (Okamoto et al., 2008).

Taken together, the results of these *in vitro* and *in vivo* studies may suggest that PPAR activation could modulate inflammation and stimulate retrobulbar adipogenesis. These findings have raised justified concerns about using TZDs in patients with TAO and stimulated studies of PPAR antagonists in the treatment of TAO (Vondrichova et al., 2007).

In conclusion, when considering TZD therapy in patients with autoimmune thyroid disease and particularly those with Graves disease or evidence of TAO, the potential risks of stimulation of orbital adipogenesis and increased proptosis should be considered.

### **Limitations of the studies**

The main limitation of **paper II** was the open study design but the results of the withdrawal test at the end of the study confirmed the additive effect of pioglitazone. These patients were inadequately controlled and needed intensified treatment; therefore placebo was not an option. The most logical comparator would have been insulin in this situation, which is not easy to study in a blinded fashion. This comparison was then carried out in **paper IV**. This study was however limited by the low number of patients, which makes it difficult to interpret all the results. Also the GITT should have been performed in all subjects at the end of the study to allow assessment of the effect of different treatments on changes in  $\beta$ -cell function and insulin sensitivity. Despite the small study size the clear differences in the effects of TZD and insulin on proinsulin, adiponectin and natriuretic peptides should stimulate to further studies. These

differences in the effects of pioglitazone versus insulin glargine may in turn have clinical implications.

The same limitation applies to **paper III**, i.e. the lack of a placebo arm and a low number of patients studied. Also the individuals with thyroid disturbance were few and had different diagnoses. However, the results are supportive of an adipogenic effect of TZDs.

## CONCLUSIONS AND IMPLICATIONS

- GITT is a new, simple and reproducible test for independent measurement of  $\beta$ -cell function and insulin sensitivity at the same time in clinical practice. Further exploration of the test may give us an applicable tool in the choice of treatment as well as the evaluation of effect of different interventions in T2D.
- Both pioglitazone and insulin glargine are effective in reducing hyperglycaemia when used as part of triple therapy. There are several differences in the effect of pioglitazone versus insulin glargine on  $\beta$ -cell function, insulin sensitivity and cardiac load. This knowledge may be of great value in the design of future intervention studies targeted to reduce the burden of cardiovascular disease in patients with diabetes.
- Treatment with TZDs may stimulate retrobulbar adipogenesis in patients with autoimmune thyroid disease or TAO and concurrent T2D. These results can be used in planning future studies of treatment in TAO.

*Scientific truth, which I formerly thought of as fixed, as though it could be weighed and measured, is changeable. Add a fact, change the outlook, and you have a new truth. Truth is a constant variable. We seek it, we find it, our viewpoint changes, and the truth changes to meet it.*

- William J. Mayo (1861-1939)



## SUMMARY IN SWEDISH

### Populärvetenskaplig sammanfattning på svenska

*Bakgrund:* Diabetes är en sjukdom som karakteriseras av förhöjt blodsocker och som obehandlad kan leda till svåra komplikationer i blodkärl, ögon, njurar, nerver mm. Typ 2 diabetes (åldersdiabetes) utgör ca 90-95 % av alla diabetes fallen. Typ 2 diabetes beror på kroppens oförmåga att producera tillräckligt med insulin samt oförmåga att svara på effekt av insulin (insulinresistens). Diabetes och dess komplikationer orsakar stort personlig lidande för drabbade patienter och orsakar stora kostnader för samhället. Förekomsten av diabetes ökar lavinartat globalt och har fått sådana epidemiska proportioner att Förenta Nationerna antog ett transatlantiskt konsensusdokument 2006 för att utöka kunskapen om och förena världen i kampen mot diabetes.

Diabetes sjukdomen har en multifaktoriell bakgrund där både ärftliga faktorer och miljö faktorer bidrar till utveckling av sjukdomen. Den västerländska livsstilen med minskad fysisk aktivitet, högt kaloriintag, övervikt och bukfetma är de viktigaste bidragande faktorer till sjukdomsutvecklingen.

Ett av dem viktigaste åtgärderna för att förebygga diabetes komplikationer är att sänka blodsockret. Behandlingen av typ 2 diabetes har varit föga framgångsrikt då patienter visar sig fortfarande ha kortare överlevnadstid och drabbas i mycket större utsträckning av komplikationer i multipla organ och av hjärtinfarkt och stroke även om komplikationer i njurar och ögon har reducerats betydligt. Trots behandling med de traditionella läkemedlen fortsätter patienter med typ 2 diabetes att stiga i blodsockervärden och kräver med tiden behandling med flera sorters läkemedel. Olika läkemedel vid behandling av diabetes riktar sig mot olika bakomliggande defekt. Det finns läkemedel som påverkar kroppens egen insulin produktion, de som ökar kroppens känslighet för insulin och slutligen insulin. På senare år har nyare läkemedel framtagits som bidrar till att kontrollera blodsockerhalterna genom att direkt minska insulinresistensen (glitazoner) och som eventuellt skulle kunna vara effektiva i att förebygga hjärta/kärl komplikationer. Dock har dessa nyare läkemedel även varit behäftade

med en del oönskade biverkningar. Samtidigt har vi fått tillgång till insulin som är extra långverkande och täcker dygnsbehovet med en injektion om dagen, vilket skulle innebära en förenkling när behov av insulinbehandling föreligger.

*Målsättning:* Avhandlingens mål har varit att:

- Ta fram en metod som lätt kan användas för att mäta graden av bristande insulinproduktion samt graden av insulinresistens i syfte att kunna lättare välja lämplig behandling för varje individ.
- Undersöka nya läkemedel avseende effektivitet, verkningsmekanism och biverkningsmönster.
- Undersöka om vissa enkla blodprovstagningar kan vara vägledande i att identifiera individer som riskerar att drabbas av allvarliga biverkningar i samband med behandling med dessa nya läkemedel.

*Resultat:* Patienter som har studerats är de med typ 2 diabetes som trots behandling med två sorters tabletter har fortsatt för höga blodsockervärden och är i behov av ytterligare behandlingstillägg för att uppnå behandlingsmålen. Avhandlingen har gett instrument för att i klinisk praxis på ett enkelt sätt kunna mäta graden av defekt insulinproduktion och insulinresistens inför val av behandling samt kunna utnyttja samma test för att undersöka olika behandlingsalternativs verkan på dessa parametrar. Avhandlingen har också gett oss en del förklaringar kring mindre vanliga biverkningar som skulle kunna undvikas genom att identifiera individer som riskerar att drabbas av dessa biverkningar. Dessa kunskaper ger oss bättre möjlighet att uppnå önskat behandlingsresultat hos patienter med typ 2 diabetes samtidigt som vi kan undvika en del oönskade, i vissa fall allvarliga biverkningar. Slutligen har vi jämfört två olika, moderna tilläggsbehandlingsalternativ och konstaterat vissa skillnader i deras effekt som tidigare inte har varit kända och som skulle kunna ha betydelse i val av behandling.

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