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Pathophysiology and Genetics of Impaired Insulin Secretion and Insulin Resistance

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UNIVERSITY OF
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ALENA STANČÁKOVÁ

Pathophysiology and Genetics of Impaired Insulin Secretion and Insulin Resistance

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ABSTRACT

Impaired insulin secretion and insulin resistance are the two main mechanisms leading to type 2 diabetes mellitus. Both abnormalities can be detected long before the onset of overt diabetes, at the pre-diabetic stage (impaired fasting glucose, IFG, impaired glucose tolerance, IGT, and combination of IFG and IGT), and are partially genetically determined. Since the prevalence and incidence of type 2 diabetes are rapidly increasing, it is important to get new information on the pathophysiology and genetics of this disease. However, previous studies on the pathophysiology of prediabetes and diabetes have often yielded inconclusive results, most probably due to the small sample size of many studies. Recent studies have identified 20 genetic loci convincingly associated with type 2 diabetes, but the mechanisms whereby these risk genes exert their effects have remained largely unknown. The aims of this study were to describe the pathophysiology of the prediabetic state, and to determine gene variants regulating insulin secretion, proinsulin conversion, and insulin sensitivity in two large studies of carefully phenotyped non-diabetic European subjects.

Impairment of peripheral insulin sensitivity was observed already at a relatively low fasting (FPG) and 2-hour glucose (2hPG) levels within the normoglycemic range. In contrast, the impairment of insulin secretion progressed substantially only in the diabetic range of FPG and 2hPG. Compensatory insulin secretion was entirely missing when FPG increased from the normal range to the IFG range, but it was present within the normal and IGT range of 2hPG. Peripheral insulin resistance was a predominant feature of isolated IGT, whereas impaired insulin secretion characterized isolated IFG. These findings suggest that type 2 diabetes develops through at least two distinct pathways: via elevation of FPG, where insulin secretion plays a crucial role, and via elevation of 2hPG, where insulin resistance seems to dominate. We also found that out of 18 type 2 diabetes-related loci, eight loci (*TCF7L2, SLC30A8, HHEX, CDKN2B, CDKAL1, MTNR1B, KCNJ11,* and *IGF2BP2*) were associated with impaired early-phase insulin release. In carriers of ≥11 risk alleles at these loci, insulin secretion was 32% lower than that of carriers of ≤3 risk alleles. Moreover, *CDKAL1* and *HHEX* SNPs were also associated with impaired first phase insulin release. Effects of *TCF7L2, SLC30A8, HHEX*, and *CDKAL1* on insulin secretion could be explained, at least in part, by impaired conversion of proinsulin to insulin. *HHEX, KCNJ11* and *TSPAN8* could also affect peripheral insulin sensitivity. In summary, our studies contribute new knowledge of the pathophysiology and genetics of type 2 diabetes.

National Library of Medicine Classification: WD 200.5.G6 , WK 810

Medical Subject Headings: Diabetes Mellitus, Type 2; Finland/epidemiology; Glucose Intolerance/genetics; Glucose Metabolism Disorders/ physiopathology; Glucose Tolerance Test; Insulin/secretion; Insulin Resistance; Polymorphism, Single Nucleotide; Proinsulin

To Anulka

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Alena Stančáková

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals I-IV in the text.

I **Stančáková A***, Javorský M*, Kuulasmaa T, Haffner SM, Kuusisto J, Laakso M. Changes in insulin sensitivity and insulin release in relation to glycemia and glucose tolerance in 6,414 Finnish men. *Diabetes* 58:1212-1221, 2009

II **Stančáková A**, Pihlajamäki J, Kuusisto J, Stefan N, Fritsche A, Häring H, Andreozzi F, Succurro E, Sesti G, Boesgaard TW, Hansen T, Pedersen O, Jansson PA, Hammarstedt A, Smith U, Laakso M; EUGENE2 Consortium. Single-nucleotide polymorphism rs7754840 of *CDKAL1* is associated with impaired insulin secretion in nondiabetic offspring of type 2 diabetic subjects and in a large sample of men with normal glucose tolerance. *J Clin Endocrinol Metab* 93:1924-1930, 2008

III Staiger H*, **Stančáková A***, Zilinskaite J, Vänttinen M, Hansen T, Marini MA, Hammarstedt A, Jansson PA, Sesti G, Smith U, Pedersen O, Laakso M, Stefan N, Fritsche A, Häring HU. A candidate type 2 diabetes polymorphism near the *HHEX* locus affects acute glucose-stimulated insulin release in European populations: results from the EUGENE2 study. *Diabetes* 57:514-517, 2008

IV **Stančáková A**, Kuulasmaa T, Paananen J, Jackson AU, Bonnycastle LL, Collins FS, Boehnke M, Kuusisto J, Laakso M. Association of 18 confirmed susceptibility loci for type 2 diabetes with indices of insulin release, proinsulin conversion, and insulin sensitivity in 5,327 nondiabetic Finnish men. *Diabetes* 58:2129-2136, 2009

*equal contribution

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Contents

ABBREVIATIONS

2hPG	2-hour plasma glucose during an OGTT
ADA	American Diabetes Association
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADP	Adenosine diphosphate
Akt	Protein kinase B
ALX4	Aristaless-like homeobox 4
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
CAMK1D	Calcium/calmodulin-dependent protein kinase I delta
CDC123	Cell division cycle 123 homolog
CDK	Cyclin-dependent kinase
CDK5RAP1	Cyclin-dependent kinase 5 regulatory subunit–associated protein 1
CDKAL1	Cyclin-dependent kinase 5 regulatory subunit associated protein 1–like 1
CDKN2	Cyclin-dependent kinase inhibitor 2
CEU	Population sample of Utah residents with ancestry from northern and western Europe
CIR	Corrected insulin response to an oral glucose load
DGI	Diabetes Genetic Initiative
DIAGRAM	Diabetes Genetics Replication And Meta-analysis
EUGENE2	European network on Functional Genomics of Type 2 Diabetes
EXT2	Exostoses (multiple) 2
FFAs	Free fatty acids
FPG	Fasting plasma glucose
FTO	Fat mass and obesity associated
FUSION	Finland - United States Investigation of Non- Insulin-Dependent Diabetes
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Facilitated glucose transporter
GWA	Genome-wide association
HapMap	Haplotype map of the human genome
HHEX	Hematopoietically expressed homeobox
HNF1B	Hepatocyte nuclear factor 1 homeobox B
HOMA-B	Homeostasis model assessment of insulin secretion
HOMA-IR	Homeostasis model assessment of insulin resistance
IAPP	Islets amyloid polypeptide
IDE	Insulin degrading enzyme
IFG	Impaired fasting glucose
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGF-II	Insulin-like growth factor 2
IGI	Insulinogenic index
IGT	Impaired glucose tolerance
IIFG	Isolated impaired fasting glucose
IIGT	Isolated impaired glucose tolerance
IMP2	Insulin-like growth factor 2 mRNA-binding protein
IR	Insulin receptor
IRS	Insulin receptor substrate
ISI	Insulin sensitivity index
IVGTT	Intravenous glucose-tolerance test
JAZF1	JAZF zinc finger 1
K_{ATP}	ATP-sensitive potassium channel
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
KIF11	Kinesin family member 11
LD	Linkage disequilibrium
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
LOC387761	Hypothetical gene
MAGIC	The Meta-Analyses of Glucose and Insulin-related traits Consortium

METSIM	Metabolic Syndrom In Men
MODY	Maturity-onset diabetes of the young
mRNA	Messenger ribonucleic acid
MTNR1B	Melatonin receptor 1B
NewDM	Newly diagnosed type 2 diabetes mellitus
NFG	Normal fasting glucose
NGT	Normal glucose tolerance
NOTCH2	Notch homolog 2 [Drosophila]
NR2C2	Nuclear receptor subfamily 2, group C, member 2
OGTT	Oral glucose-tolerance test
OR	Odds ratio
PDK1	Phopshoinositide-dependent protein kinase-1
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PPARG	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator-activated receptor response element
RNA	Ribonucleic acid
S_I	Insulin sensitivity index derived from an IVGTT
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element-binding protein
TCF7L2	Transcription factor 7-like 2
THADA	Thyroid adenoma associated
TNFa	Tumor necrosis factor alfa
TSPAN8	Tetraspanin 8
UCP2	Uncoupling protein 2
WFS1	Wolfram syndrome 1
WHO	World Health Organisation
Wnt	Wingless-type MMTV integration site family
WTCCC	Wellcome Trust Case Control Consortium
ZnT8	Zinc transporter 8

1. Introduction

Type 2 diabetes mellitus is a common metabolic disorder characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism. The two major pathophysiological defects in type 2 diabetes are impaired insulin secretion and insulin action (1). These metabolic abnormalities lead to long-term damage of various organs, causing their dysfunction and failure. Therefore, diabetes substantially increases the morbidity and mortality of affected individuals. Diabetes-related microvascular complications are responsible for the majority of new cases of blindness, kidney failure, and nontraumatic amputations. Furthermore, macrovascular complications such as stroke and cardiovascular disease-related deaths are 2–4 times more frequent in adults with diabetes than in the general population (2). These complications of type 2 diabetes are among the leading causes of mortality worldwide, and cause a significant decrease in the life-expectancy of diabetic patients.

The prevalence and incidence of type 2 diabetes is constantly increasing in almost all countries. The World Health Organization (WHO) estimates that approximately 180 million individuals are affected worldwide, and this number is expected to be doubled by the year 2030 (3). Moreover, the age of onset of type 2 diabetes is decreasing, and type 2 diabetes is increasingly observed in children (4). This increase in the incidence and prevalence is mainly due to adverse environmental factors of modern society, such as diets rich in carbohydrates and fat, and physical inactivity, although aging of the population and genetic factors also play important roles.

A considerable number of studies have been carried out to investigate the mechanisms leading to diabetes, and the genetic background of this disease. In spite of undisputable progress achieved over the recent years, our knowledge is far from complete. Moreover, findings across different studies are often inconsistent or even conflicting. One of the important reasons for these inconsistencies is the insufficient power of most of the studies due to a small sample size. Therefore, there is a need for large and thoroughly phenotyped cohorts, using accurate methods to estimate the metabolic parameters.

The aim of this study was to determine the characteristics of the pathophysiology of prediabetes, and to assess the possible effects of type 2

diabetes risk loci on insulin secretion and insulin sensitivity, based on two independent large cohorts of carefully phenotyped individuals.

2. Review of the literature

2.1 DEFINITION OF TYPE 2 DIABETES

Diagnosis of type 2 diabetes is based on fasting plasma glucose (FPG) concentration and/or 2-hour plasma glucose (2hPG) concentration during an oral glucose tolerance test (OGTT). The diagnostic cut-off points for diabetes are FPG 7.0 mmol/l, and 2hPG 11.1 mmol/l (Table 1).

2.2 DEFINITION AND CHARACTERISTICS OF PRE-DIABETIC STATES

Type 2 diabetes is preceded by a pre-diabetic state, characterized by mild elevation of fasting and/or postprandial glucose levels. This asymptomatic phase may last for years, and about one third of individuals with pre-diabetes finally develop type 2 diabetes (5). The pre-diabetic state is defined by an OGTT, and includes impaired fasting glucose (IFG, characterized by an elevated FPG), impaired glucose tolerance (IGT, characterized by an elevated 2hPG) or a combination of these (6). The diagnostic criteria for IFG and IGT are shown in Table 1. Two definitions of IFG have been presented: according to the American Diabetes Association (ADA) and WHO in 1997 (7), IFG is defined by FPG ≥6.1 and <7.0 mmol/l. In 2003, the ADA lowered this threshold to 5.6 mmol/l (6) in order to achieve a reasonable balance between sensitivity and specificity for diabetes prediction.

Table 1. Diagnostic criteria of glucose tolerance categories according to the WHO (7) and ADA (6) criteria.

	FPG			**2hPG**
	WHO	ADA		
NFG, NGT	<6.1	<5.6		<7.8
IIFG	≥6.1 & <7.0	≥5.6 & <7.0	and	<7.8
IIGT	<6.1	<5.6	and	≥7.8 & <11.1
IFG+IGT	≥6.1 & <7.0	≥5.6 & <7.0	and	≥7.8 & <11.1
Diabetes	≥7.0	≥7.0	and/or	≥11.1

FPG = fasting plasma glucose, 2hPG = 2-hour plasma glucose during an OGTT, NFG = normal fasting glucose, NGT = normal glucose tolerance, IIFG = isolated impaired fasting glucose, IIGT = isolated impaired glucose tolerance.

Both IFG and IGT predict similarly incident diabetes (8-10). In a large meta-analysis (11), the absolute annual incidence of diabetes in individuals with IFG or IGT varied from 5 to 10%, and was higher for individuals with a combination of IFG and IGT. IFG and IGT not only predict the development of type 2 diabetes, but are also associated with an increased risk of cardiovascular disease. This association is well established especially for IGT. In a large longitudinal Whitehall study (N=18,403), subjects with IGT had approximately double the risk of cardiovascular mortality compared with those with normal glucose tolerance (12). Several other studies have reported similar results (13, 14). Although conflicting reports have been published on the association between IFG and cardiovascular risk (15-18), results of the Framingham Heart Study showed that IFG was associated with increased risk of coronary heart disease in women, but not in men (19). Furthermore, a meta-analysis of 20 studies examining the relationship between glucose and incident cardiovascular events showed that progressive relationship exists already below the diabetic threshold for glucose levels (20).

In spite of similarities between IFG and IGT in predicting the risk of diabetes, they represent different metabolic states, and identify two distinct populations with only partial overlap. In most populations, IGT is more prevalent than IFG, increases with aging, is more common in women (18, 21), and is more strongly associated with cardiovascular disease than is IFG (5). These differences suggest that IFG and IGT are likely to have different pathophysiology.

2.3 PATHOPHYSIOLOGY OF TYPE 2 DIABETES AND PRE-DIABETES

Impaired insulin secretion and insulin resistance are the two main metabolic disturbances in the pathogenesis of type 2 diabetes. Both abnormalities often coexist in the same individuals (22). There is a strong link between insulin secretion and insulin sensitivity, and changes in one of these two produce adaptation in the other (23). There is no consensus on which one of the two abnormalities is the primary defect in the development of type 2 diabetes.

However, most researchers accept that type 2 diabetes develops when the pancreas is unable to secrete more insulin to compensate for existing insulin resistance. This is in accordance with an observation that insulin resistance is present early in the natural history of type 2 diabetes, whereas marked beta-cell dysfunction is a rather late event (24). Both impaired insulin secretion and insulin resistance are influenced by genetic and environmental factors.

2.3.1 Impaired insulin secretion

Insulin release from the beta-cells of the pancreatic islets in response to changes in blood glucose concentration is a complex phenomenon. Briefly, the process is initiated by the transport of glucose into the beta-cells through diffusion facilitated by GLUT2 transporters. In the beta-cell, glucose is metabolised to generate ATP, the central energy molecule, and the ATP/ADP ratio increases. This induces the closure of cell-surface ATP-sensitive K^+ channels, and leads to the depolarization of the cell-membrane. Next, transmembrane voltage-dependent Ca^{2+} channels are opened due to depolarisation, facilitating the influx of extracellular Ca^{2+} into the beta-cell. Finally, a rise in free cytosolic Ca^{2+} triggers the exocytosis of insulin (25) (Figure 1).

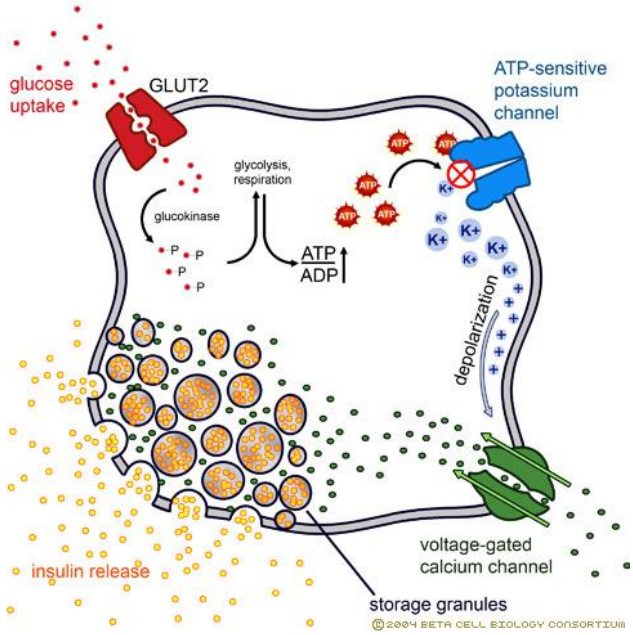

Figure 1: Insulin secretion by the beta-cell of the pancreatic islets (26).

Insulin is released form the pancreatic beta-cells in a biphasic manner in response to a rapid increase in blood glucose concentration. The first phase, which is a short-lasting (few minutes) increase in insulin secretion, is followed by a more slowly evolving second phase, which lasts as long as the glucose level is elevated. On the other hand, a slow increase in plasma glucose level induces a gradually larger secretion without the first phase (27).

In type 2 diabetic patients, the first phase of insulin release is substantially lower than in healthy subjects, and often absent. The second phase is also lower than in non-diabetic controls (28). Impairment of both first and second phase insulin release occurs early in the natural history of diabetes (29), and blunted first-phase insulin release can be demonstrated even in normoglycemic first-degree relatives of type 2 diabetic patients (30). Furthermore, beta-cell function deteriorates over the years following the diagnosis of type 2 diabetes (31).

There are several potential causes of beta-cell dysfunction in type 2 diabetes (32), e.g. reversible metabolic abnormalities (glucotoxicity and lipotoxicity), hormonal changes (inadequate incretin secretion and action), reduction of beta-cell mass due to apoptosis, and genetic abnormalities.

Chronic hyperglycemia has been shown to induce beta-cell dysfunction and apoptosis in animal models (33) and also in humans (34) (glucotoxicity). The mechanisms proposed include mitochondrial dysfunction with the production of reactive oxygen species, endoplasmic reticulum stress, and increased levels of intracellular calcium.

Elevation of free fatty acids (FFAs) has been shown to promote proapoptotic effects on beta-cells (35), possibly as a result of endoplasmic reticulum stress (36) (lipotoxicity). Moreover, high levels of FFAs can also contribute to beta-cell dysfunction through the intracellular accumulation of triglycerides as a response to the activation of the sterol regulatory element-binding proteins (SREBP) (37), or by increased expression of uncoupling protein 2 (UCP2), which regulates cellular ATP production (38). The deleterious effects of FFAs are observed predominantly in the presence of high glucose.

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are important gut hormones, called incretins, which are released after food intake, and they increase insulin release. In addition, GLP-1 acts as an inhibitor of secretion of glucagon, a protein secreted by pancreatic alpha-cells which contributes to hyperglycemia by stimulating hepatic glucose

production. Furthermore, both proteins have been shown to increase beta-cell proliferation and decrease beta-cell apoptosis (39). Attenuated release of GLP-1 has been found in patients with type 2 diabetes or IGT after the ingestion of a mixed meal (40).

Apoptosis of beta-cells is 3- to 10-fold more frequent in diabetic subjects than in weight-matched non-diabetic controls. Moreover, a 40% deficit in relative beta-cell volume has also been found in subjects with IFG (41), indicating that the loss of beta-cells is an early process in the pathogenesis of type 2 diabetes. The main mechanisms leading to increased beta-cell apoptosis include glucotoxicity, lipotoxicity, and deposits of islets amyloid polypeptide (IAPP). IAPP, which is co-secreted with insulin from beta-cells, exerts several physiological functions. Although IAPP is normally maintained in the form of soluble monomers, cytotoxic oligomers inducing apoptosis of beta-cells can also be formed. There are several possible mechanisms leading to the formation of oligomers (42), including insulin resistance which disproportionately increases the expression of IAPP compared with insulin expression (43, 44).

Gene variants may affect insulin secretion in type 2 diabetes through their effects on glucose-stimulated insulin release, incretin sensitivity or incretin secretion, proinsulin conversion, beta-cell proliferation, apoptosis etc.

2.3.1.1 Impaired proinsulin conversion as a marker of beta-cell dysfunction

The insulin gene is translated into proinsulin, a precursor of insulin. During insulin maturation, which takes place in the endoplasmic reticulum and Golgi apparatus of the beta-cells, proinsulin is cleaved by protein convertases 1 and 2 and carboxypeptidase E into mature insulin molecule and C-peptide (Figure 2). Normally, only <10% of synthesised proinsulin escapes this process and gets into the circulation. Therefore, the increased proinsulin/insulin ratio reflects the efficiency of proinsulin conversion.

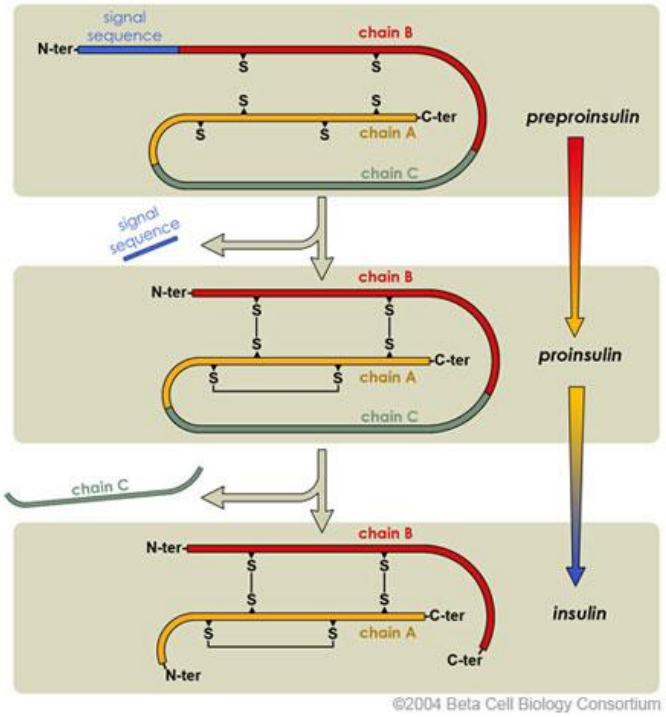

Figure 2: Scheme of insulin processing (26).

Individuals with type 2 diabetes typically exhibit an elevated proinsulin-to-insulin ratio (45), which has been shown to correlate with decreased acute insulin response to glucose (46). Moreover, hyperproinsulinemia has also been detected in individuals with pre-diabetes (47, 48), suggesting that impaired conversion of proinsulin to insulin is an early event in the development of type 2 diabetes.

2.3.2 Insulin resistance

Insulin exerts its biological functions by interacting with membrane-spanning insulin receptor (IR). Binding of insulin to IR elicits autophosphorylation of the IR, leading to binding of various scaffold proteins such as insulin receptor substrate (IRS) proteins. Phosphorylation of IRS proteins leads to their association with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and its activation. The subsequent steps, which involve PI3K-mediated actions of phopshoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB or Akt), lead to the facilitation of the translocation of glucose

transporter-4 (GLUT4)-containing vesicles to the cell surface (49). Finally, GLUT4 transports glucose into the cell. Other effects of insulin, such as its effects on glycogen synthesis, protein synthesis, lipogenesis, and supression of hepatic gluconeogenesis, are also mediated by PKB (50) (Figure 3).

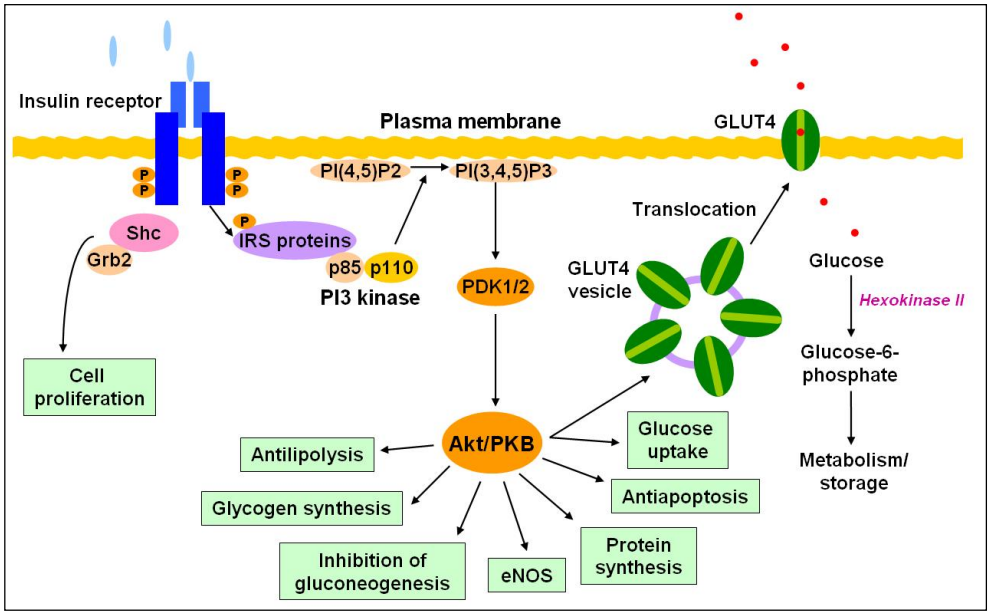

Figure 3: Scheme of insulin action [modified from (51, 52)].

The main insulin-sensitive tissues are skeletal muscle (accounting for 60-70% of whole-body glucose uptake), liver (~30%) and adipose tissue (~10%). Insulin resistance can be defined as the inability of these tissues to respond properly to normal circulating concentrations of insulin. To maintain euglycemia, the pancreas compensates by secreting increased amounts of insulin. However, elevated insulin levels can compensate for poor insulin action only for a limited time. After a period of compensated insulin resistance, pre-diabetes or diabetes usually develops, especially in coexistence with impaired beta-cell function. An early appearance of insulin resistance in the natural history of diabetes was demonstrated by Vaag et al., who found that young healthy offspring of diabetic parents commonly exhibit insulin resistance and impaired skeletal muscle insulin signalling many decades before the onset of overt type 2 diabetes (53).

Although the mechanisms resulting in insulin resistance are largely unknown, multiple abnormalities in the insulin signalling pathway have been

identified. The most important sites are IRS (-1 and -2), PI3K and PKB. Mutations in the *IRS1* gene in humans are associated with insulin resistance (54), and IRS-2 knockout mice show insulin resistance in muscle, fat and liver, and develop diabetes resulting from beta-cell failure (55). Reduced activation of the PI3-kinase/Akt signalling pathway can lead to reduced glucose transport and utilisation in the skeletal muscle and adipocytes (32). Mice lacking the PKBβ isoform are insulin-resistant and develop a phenotype closely resembling type 2 diabetes in humans (56, 57). Consistent with this finding, mutations in PKBβ have been identified in a family of severely insulin resistant patients (58).

Several environmental and lifestyle factors also affect insulin sensitivity, such as nutritional factors in utero, diet, physical activity, smoking, drugs, and particularly obesity. Obesity, and especially visceral adiposity, is strongly associated with insulin resistance and type 2 diabetes.

2.3.2.1 Adipose tissue and insulin sensitivity

Adipose tissue can modulate whole body glucose metabolism by regulating the levels of circulating FFAs, and also by secreting adipokines, thereby acting as an endocrine organ (50).

Insulin-resistant visceral adipose tissue is resistant to the antilipolytic effects of insulin and releases excessive amounts of FFAs. Metabolic overload of the liver and muscle with FFAs causes mitochondrial dysfunction with impaired FFA oxidation. In the liver, impaired FFA oxidation leads to redirection of FFAs into lipid species localized in ER and cytoplasm, which promotes the development of hepatic steatosis and hepatic insulin resistance. In skeletal muscle, metabolic overload and physical inactivity lead to incomplete oxidation of FFAs, and lipid-derived intermediates accumulate in mitochondria, contributing to both mitochondrial stress and insulin resistance (49). Moreover, elevated levels of plasma FFAs can increase insulin resistance also by affecting the insulin signalling cascade, particularly abolishing the insulin activation of IRS-1-associated PI3K-activity (59).

Adipokines secreted by the adipose tissue affect insulin sensitivity in either a positive (adiponectin, leptin, interleukin-10, etc.) or a negative way (TNFα, resistin, interleukin-6, retinol binding protein 4, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1 etc.). The best understood are the mechanisms whereby TNFα and adiponectin affect insulin sensitivity.

TNFα is the main factor that triggers the secretion of FFAs from the adipose tissue into the circulation (60). Furthermore, it mediates the repression of many genes responsible for glucose and FFA uptake and storage. The enhanced release of FFAs and cytokines as a result of TNFα action impairs insulin signalling in insulin responsive tissue, especially in skeletal muscle. TNFα has also been shown to downregulate the genes encoding adiponectin, GLUT4, IRS-1 etc.

Adiponectin improves insulin sensitivity by various mechanisms, resulting in a decrease in plasma FFA and glucose levels. In the liver, adiponectin induces FFA oxidation, decreases lipid synthesis, decreases uptake of FFA and represses gluconeogenesis. In skeletal muscle, adiponectin increases glucose and FFA oxidation. Adiponectin also suppresses the secretion of TNFα (61).

2.3.2.2 Liver and insulin sensitivity

Although the liver accounts for only ~30% of the whole-body glucose metabolism, hepatic insulin resistance plays an important role in the pathogenesis of type 2 diabetes. In the insulin-resistant liver, insulin does not inhibit sufficiently two key enzymes of gluconeogenesis (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase catalytic subunit). Consequently, this leads to increased hepatic glucose production in the fasting state and to fasting hyperglycemia.

2.3.2.3 Brain and insulin sensitivity

Recent observations have shown that the brain is also an important insulin-sensitive organ, even though glucose uptake by the brain is insulin-independent. Experimental studies in mice have shown that neuronal insulin signalling is required for intact control of body fat mass and glucose homeostasis (62-65). Impaired neuronal signalling by insulin (and also by leptin and nutrient-related signals) leads to hyperphagia, weight gain, and hepatic insulin resistance. One proposed mechanism of neuronal insulin resistance is the disruption of the IRS-PI3K signalling pathway, which mediates the neuronal actions of both insulin and leptin (66).

2.3.3 Hepatic glucose production

Increased hepatic glucose production resulting from unsuppressed gluconeogenesis and enhanced glycogenolysis in hepatocytes is an important mechanism contributing to hyperglycemia in type 2 diabetes. Hepatic glucose production is regulated by insulin and glucagon, which have opposite effects. Insulin suppresses both gluconeogenesis and glycogenolysis, and increased glucose hepatic output is therefore a consequence of insulin resistance in the liver. In contrast, glucagon stimulates gluconeogenesis and glycogenolysis, and increased glucagon secretion or enhanced hepatic glucagon sensitivity can contribute to the dysregulation of glucose production (67).

2.3.4 Insulin resistance and impaired insulin secretion in pre-diabetes

Epidemiological studies have shown that IFG and IGT represent two distinct subgroups of abnormal glucose tolerance (5, 68-70), which differ in their age and sex distribution (71, 72) and associated cardiovascular risk (73). Therefore, IFG and IGT are likely to have different pathophysiology. Many investigators have studied the role of insulin resistance and impaired insulin secretion in the pathogenesis of IFG and IGT. However, these studies have yielded contradictory results (69-88).

Most of the studies using the clamp method to assess insulin sensitivity in different categories of glucose tolerance have shown impairment of peripheral insulin sensitivity in IGT, whereas subjects with IFG have been shown to have normal or impaired (similarly as in IGT) peripheral insulin sensitivity (74-79). When insulin resistance was assessed using HOMA-IR (describing mainly hepatic insulin resistance), several studies (77, 83, 86, 88) reported increased insulin resistance in subjects with IFG, whereas others did not (74, 84, 85).

Studies on insulin secretion disturbances in IFG and IGT have reported conflicting results. While some studies applying the intravenous glucose tolerance test (IVGTT) or clamp methods to assess insulin secretion have reported impaired first-phase insulin secretion only in IFG but not IGT (74, 75, 78, 81), other studies have reported impaired first- and also second-phase insulin secretion in isolated IGT (76, 82). Studies assessing insulin secretion on the basis of fasting glucose and insulin levels or OGTT have reported even more controversial results, reporting impaired insulin secretion in IFG (85, 87), in IGT (83, 88), or in both (84, 86). Inconsistencies across these studies could be explained by differences in study populations, study designs and methods to

assess insulin sensitivity and insulin secretion, and most importantly by a small sample size.

The categorization of glucose tolerance is based on arbitrary cut-off points of glucose levels, and therefore different subgroups cannot fully account for changes in beta-cell function and insulin action when glycemia increases. Only a few studies have examined insulin secretion and/or insulin sensitivity as a function of glucose concentrations (78, 89-93). These studies have been, however, relatively small and most of them have been conducted in non-European populations.

2.4 GENETICS OF TYPE 2 DIABETES

2.4.1 Heritability of type 2 diabetes

It is generally agreed that type 2 diabetes has a strong genetic component. There are several lines of evidence supporting this view (94). For one thing, the prevalence of type 2 diabetes differs across different ethnic groups. For example, the prevalence of type 2 diabetes in Europeans is ~2%, whereas in Pima Indians in Arizona it reaches 50% (95). Moreover, type 2 diabetes often exhibits familial aggregation. Siblings of type 2 diabetic probands have nearly a 4-fold increased risk for type 2 diabetes compared with the general population (96). Furthermore, in twin studies there is a high concordance of type 2 diabetes in monozygotic twins (concordance rates 0.29-1.00), whereas in dizygotic twins the concordance is about 50% lower than in monozygotic twins (0.10-0.43) (97-100). Finally, there is a strong heritability of intermediate phenotypes, since both insulin sensitivity and insulin secretion have an important genetic component (heritability estimates 0.50-0.58 for insulin secretion, and 0.26-0.37 for insulin sensitivity) (99, 101).

The genetic contribution to type 2 diabetes arises from genetic variations in several genes, each conferring a small increase in the risk (94). These gene variations do not cause diabetes but increase its risk by interacting with other diabetes-susceptibility genes, the metabolic environment of the body (e.g. glucotoxicity and lipotoxicity) and life-style factors (e.g. sedentary life, excess calories, smoking, stress and chronic inflammation) (102). The most studied genetic variations determining the individual predisposition to type 2 diabetes (as well as to other complex diseases) are single nucleotide polymorphism

(SNPs), which cover ~90% of the sequence variation within the human genome (103).

2.4.2 Strategies in the search for the genes for type 2 diabetes

2.4.2.1 Candidate gene approach and linkage studies

The candidate gene approach focuses on the search for an association between type 2 diabetes and sequence variants in or near biologically defined candidate genes which have been chosen based on their known pathophysiological function. The importance of these variants is tested by comparing their frequency in diabetic and control individuals, or by testing their association with continuous outcomes. This approach cannot be used to identify novel genes and pathways (94).

The linkage strategy assumes that a disease-predisposing allele will pass from generation to generation with the variants at tightly linked loci. Linkage studies directly examine the transmission across the generations of both disease phenotype and marker alleles, trying to identify the causal loci. First, the susceptibility locus is localized to a chromosomal region. The specific gene and sequence variants are then identified within this region based on their physiologic impact. Alternatively, a dense map of markers is tested in cases and controls across the region of linkage. No prior knowledge about the gene or gene effects is necessary. This approach is very labour-intensive, since the chromosomal regions identified often encompass up to hundreds of genes.

Both approaches have led to the identification of a plethora of potential candidate genes for type 2 diabetes. However, most of the reported associations could not be replicated in other populations. However, at least three true type 2 diabetes candidates have been discovered by these approaches: *PPARG* and *KCNJ11* identified by the candidate gene approach, and *TCF7L2* identified by a linkage study. The low reproducibility of linkage and genetic association studies is due mostly to the insufficient power of these studies, over-interpretation of results, and incomplete knowledge of the etiopathogenesis of type 2 diabetes. Therefore, large-scale, biology-agnostic studies are necessary for further progress.

2.4.2.2 Genome-wide association (GWA) studies

The GWA strategy makes it possible to investigate genetic variation across the entire human genome and to identify genetic associations with continuous

traits or the presence or absence of the disease. This approach became feasible thanks to the sequencing of the human genome and the development of high-throughput genotyping technologies. An important step towards effective GWA studies was the completion of Stages I and II of the International HapMap Project (104, 105), which provided a set of informative single nucleotide polymorphisms (SNPs) to tag variation throughout the genome (106). Two main platforms for genome-wide SNP genotyping are available, Affymetrix and Illumina, containing up to ~1 000 000 SNPs selected from all three HapMap phases, the 1,000 Genomes Project, and published studies to tag common variations, taking linkage disequilibrium (LD) patterns into account. These SNP chips can capture > 90% of the common genetic variation reported in the HapMap (107).

The first GWA scans were published in 2007 (108-112), identifying six novel diabetes-susceptibility genes: *SLC30A8*, *HHEX-IDE*, *CDKN2A/2B*, *IGF2BP2*, *CDKAL1* and *FTO*. The first scan (108) replicated the previously known association between *TCF7L2* and type 2 diabetes, and discovered SNPs in *SLC30A8*, *HHEX-IDE, EXT2-ALX4* and *LOC387761* as novel type 2 diabetes loci. Three other GWA scans (WTCCC, DGI and FUSION) (109-111) confirmed the known *PPARG*, *KCNJ11* and *TCF7L2* loci as well as *SLC30A8* and *HHEX-IDE,* and identified *CDKN2A/2B*, *IGF2BP2*, *CDKAL1* as novel diabetes-susceptibility loci. Simultaneously, deCODE investigators reported *SLC30A8*, *HHEX-IDE*, and *CDKAL1* signals (112). The associations of *EXT2-ALX4* and *LOC387761* found in the first scan were not confirmed in these four subsequent scans. Furthermore, SNPs in *FTO* were found to be associated with obesity and therefore contribute indirectly to the risk of type 2 diabetes (113).

In 2008, a meta-analysis (DIAGRAM) including four previous GWA scans (WTCCC, DGI, FUSION and deCODE) identified six additional diabetes-susceptibility genes: *JAZF1*, *CDC123/CAMK1D*, *TSPAN8/LGR5*, *THADA*, *ADAMTS9* and *NOTCH2* (114). At the end of 2008, two GWA scans conducted in Japanese samples identified SNPs in *KCNQ1* robustly associated with type 2 diabetes (115,116). Most recently, a meta-analysis of multiple GWAS (MAGIC, The Meta-Analyses of Glucose and Insulin-related Traits Consortium) confirmed the *MTNR1B* locus as a candidate for type 2 diabetes, with major effects on fasting glucose and insulin secretion (117). A total of 20 confirmed type 2 SNPs with their effect sizes from the original GWA scans are presented in Figure 4.

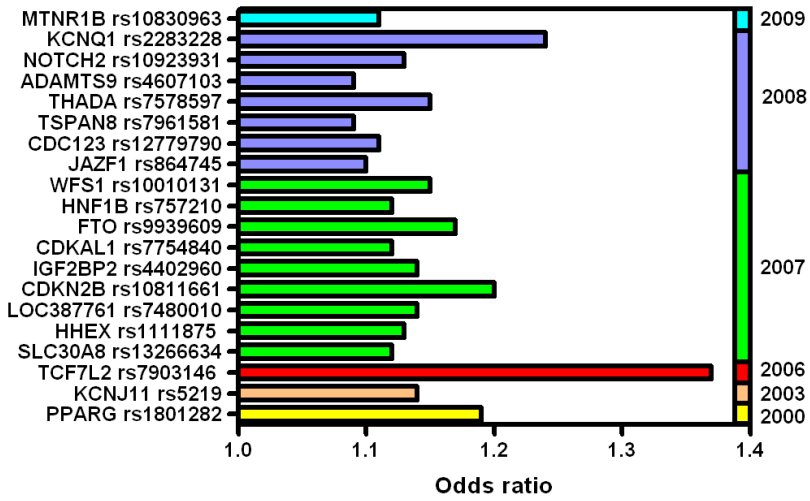

Figure 4. Confirmed type 2 diabetes-related SNPs with their effect sizes (odds ratios) in chronological order.

2.4.3 Type 2 diabetes loci identified by candidate gene approach or linkage studies

PPARG encodes for the peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear hormone receptor superfamily which regulates the transcription of various genes after binding to their PPAR response elements (PPREs). PPARγ regulates mainly the expression of adipose-specific genes, and acts as an important regulator of adipocyte differentiation. PPARγ also contributes to the regulation of FFA metabolism by stimulating uptake, storage, and oxidation of FFAs in adipocytes (118), thereby decreasing their plasma levels. These actions, together with effects of PPARγ activation on the concentration of circulating pro-inflammatory proteins and adiponectin, can modulate insulin sensitivity. PPARγ also acts as a modulator of intracellular insulin-signalling events. Moreover, it may act as a tumor suppressor gene, inhibiting the growth of several cell types and inducing apoptosis.

A missense mutation Pro12Ala (rs1801282) in exon B of *PPARG2* was identified in 1997 (119). The association of the common Pro allele with an increased risk of type 2 diabetes was first reported in 1998 (120) and was replicated in several studies including a meta-analysis (121) and GWA studies (109-111). Although the effect size is small (carriers of Pro/Pro genotype have

1.25-fold increase in the risk of type 2 diabetes), the high frequency of the Pro allele (~85% in Europeans) leads to a considerable population-attributable risk of type 2 diabetes (25%) (121).

The association of the Pro12Ala variant with diabetes is explained, at least in part, by its effect on whole-body insulin sensitivity. Since exon B harbouring the Pro12Ala polymorphism is only present in the PPARγ2 isoform, which is expressed in adipose tissue, it is probable that the Pro12Ala polymorphism exerts its insulin-sensitizing effect directly in the adipose tissue. Insulin-resistant adipose tissue releases FFAs into the circulation, which may further impair insulin sensitivity in skeletal muscle and the liver. The effect of *PPARG2* on insulin sensitivity is supported by the fact that PPARγ is the specific molecular target of thiazolidinediones, the insulin-sesitizing antidiabetic drugs (122).

KCNJ11 (Potassium inwardly-rectifying channel, subfamily J, member 11) encodes for the Kir6.2 subunit of the ATP-sensitive potassium channel (K_{ATP}). K_{ATP} channels regulate the flux of K^+ ions across the cell membranes, and their importance in insulin secretion was established 25 years ago (123). Rare activating mutations of *KCNJ11* cause permanent neonatal diabetes mellitus with a severe defect in insulin secretion (124), due to permanent opening of K_{ATP} channels in the plasma membrane of beta-cells (125). On the other hand, common polymorphisms in *KCNJ11* predispose to type 2 diabetes.

A missense mutation E23K (rs5219, Glu23Lys), was first shown to be associated with type 2 diabetes in 2003 (126). Several subsequent studies confirmed the original findings (127, 128), including GWAS (109-111). Although the effect size is small (odds ratio (OR) ~1.2), the high frequency of the risk allele (~50%) leads to a sizeable population-attributable risk. The mechanism is thought to be a beta-cell dysfunction due to a small increase in K_{ATP} channel activity (129).

TCF7L2 (transcription factor 7-like 2) encodes for the high mobility group box-containing transcription factor that serves as a nuclear receptor for β-catenin. It mediates the wingless-type MMTV integration site family (WNT) signalling pathway, a key developmental and growth regulatory mechanism of the cell. Wnt signalling has also been shown to regulate pancreatic beta-cell proliferation (130), and heterodimerization of *TCF7L2* with β-catenin induces transcription of several genes implicated in glucose metabolism, such as proglucagon (the prohormone of glucagon), glucagon-like peptide 1 (GLP-1) and GLP-2 (131).

In 2006, Grant and colleagues showed a significant association between a microsatelite marker (DG10S478), located in intron 3 of *TCF7L2*, and the risk of type 2 diabetes in an Icelandic cohort (132). Since then, the association of rs7903146, which is in strong linkage disequilibrium with DG10S478, with type 2 diabetes has been replicated in several studies (133-140). Moreover, in GWAS rs7903146 appeared as the strongest signal for diabetes risk reported to date, with OR ~1.4 (108-112). The mechanism behind this association is likely to be impaired insulin secretion (135).

WFS1 (Wolfram syndrome 1) encodes a ubiquitously expressed transmembrane glycoprotein, wolframin, which maintains calcium homeostasis of the endoplasmic reticulum. Mutations in this gene cause Wolfram syndrome (DIDMOAD), which includes diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (141). Several intronic SNPs in *WFS1* were found to be associated with the risk of type 2 diabetes (142). The mechanism explaining this association could be impaired insulin secretion, since wolframin deficiency in *Wfs1* knockout mice leads to beta-cell loss, probably due to endoplasmic reticulum stress leading to beta-cell apoptosis (143), and consequently to insufficient insulin secretion (144).

HNF1B (Hepatocyte nuclear factor 1 homeobox B) encodes a transcription factor expressed in the liver, pancreatic islets, kidney, and uterus (145). Mutations in *HNF1B* are a rare cause of maturity-onset diabetes of the young (MODY), accounting for approximately 1% of MODY cases, but are a relatively common cause of non-diabetic renal disease, particularly cystic renal disease (145). Common variants in *HNF1B* were associated with the risk of type 2 diabetes and also with prostate cancer in a GWA study (146).

2.4.4 Type 2 diabetes loci identified by GWA studies

2.4.4.1 CDKAL1 and HHEX

CDKAL1 (cyclin-dependent kinase 5 regulatory subunit associated protein 1–like 1) encodes a protein of unknown function which shares protein domain similarity with CDK5 regulatory subunit–associated protein 1 (CDK5RAP1). CDK5RAP1 is a neuronal protein that specifically inhibits the activation of cyclin-dependent kinase 5 (CDK5) (147). CDK5 is a small serine/threonine protein kinase recognized as an essential molecule in the brain, but it also displays several extraneuronal effects (148), and is thought to play a role in the

pathophysiology of beta-cell dysfunction and predisposition to type 2 diabetes (149). *CDKAL1* is also expressed in human pancreatic islets (109). Therefore, it is likely that *CDKAL1* and CDK5-mediated pathways in beta-cells are related.

GWA studies have identified several SNPs (rs7756992, rs7754840, rs10946398, rs4712523 and rs9465871) in intron 5 of *CDKAL1* associated with type 2 diabetes (109-112). The strongest association signals were observed for two SNPs (rs7754840 and rs10946398), which are in complete linkage disequilibrium (r^2 = 1.0 according to HapMap CEU). Moreover, the type 2 diabetes risk alleles of rs7756992 and rs7754840 were also associated with impaired insulin secretion (110, 112). Therefore, impaired insulin secretion represents a plausible mechanism of increased diabetes risk associated with *CDKAL1* variants.

HHEX (hematopoietically expressed homeobox) encodes for a member of the homeobox family of transcription factors, which are involved in developmental processes. *HHEX* is also involved in the Wnt signaling pathway, which is fundamental for cell growth and development (150, 151). *HHEX* is expressed in the embryonic ventral-lateral foregut, which gives rise to the ventral pancreas and the liver (152), and is highly expressed in both tissues (109). Knockout of this gene was shown to impair proliferation of endodermal epithelial cells, positioning of ventral foregut endoderm cells relative to the mesoderm, and budding and morphogenesis of the ventral pancreas, provoking lethality during mid-gestation (152).

SNP rs11117875, located within 13kb from the *HHEX* gene, has been shown to be associated with type 2 diabetes in GWAS (108-112). Regarding the role of *HHEX* in pancreas development and its high expression in beta-cells, it is likely that this SNP confers a diabetes risk by affecting beta-cell function. However, a potential contribution of another two genes within the locus tagged by rs11117875 (*IDE* encoding insulin degrading enzyme, and *KIF11* encoding kinesin family member 11) to the association with type 2 diabetes cannot be excluded.

2.4.4.2 Other loci

SLC30A8 (solute carrier family 30 (zinc transporter), member 8) encodes for a pancreatic beta-cell-expressed zinc transporter, ZnT8. It is located in the secretory granules, where it provides zinc for insulin maturation and storage (153). Previous studies have demonstrated a relationship between perturbations in zinc metabolism and carbohydrate metabolism (154). An

association between the rs13266634 (Arg325Trp) polymorphism in *SLC30A8* and type 2 diabetes has been shown in several GWA studies (108-112). Subsequent studies have reported an association of this SNP with pancreatic beta-cell dysfunction (155-160). Moreover, ZnT8 knockout mice were found to have impaired glucose tolerance with reduced insulin secretion (161).

CDKN2B (cyclin-dependent kinase inhibitor 2B) encodes for a cyclin-dependent kinase inhibitor, which forms a complex with cyclin-dependent kinase 4 (CDK4) or CDK6, and prevents their activation. It functions as a cell growth regulator that controls cell cycle G1 progression. This gene lies adjacent to the tumor suppressor gene *CDKN2A* in a region that is frequently mutated and deleted in a wide variety of tumours (162). Both proteins encoded by *CDKN2B* and *CDKN2A* are expressed in pancreatic islets (109-111) and play a role in the regulation of pancreatic beta-cell replication (163-165). A polymorphism rs10811661 located 125kb from *CDKN2B* and *CDKN2A* has been associated with type 2 diabetes in three genome-wide association studies (109-111) and in several subsequent studies (166-170).

IGF2BP2 (insulin-like growth factor 2 mRNA binding protein 2) encodes for the protein IMP2, a member of the insulin-like growth factor 2 (IGF-II) mRNA-binding protein (IMP) family. It regulates IGF2 translation, and is implicated in RNA localization and stability (171). IMP proteins (IMP1-3) are expressed in developing embryos and are essential for normal embryonic growth and development. Only IMP2 is expressed in adult organs, such as the gut, brain, muscle, and pancreatic islets (171). An association of SNPs in intron 2 of *IGF2BP2* with type 2 diabetes was inferred from a series of GWA studies (109-111) and several subsequent replication studies in different populations (157, 159, 160, 166, 169, 170, 172, 173). The reported association with the *IGF2BP2* locus based on all studies (OR 1.14) represents a 3% difference in allele frequency between the case and control groups in over 34,000 subjects (174).

FTO (fat mass and obesity associated) is a member of the non-heme dioxygenase superfamily, which encodes for a 2-oxoglutarate-dependent nucleic acid demethylase. *Fto* mRNA is abundant mainly in the brain, particularly in the hypothalamic nuclei governing energy balance, and it has been shown to change its expression according to the feeding/starvation state in mice (175). A cluster of common SNPs in intron 1 of *FTO* was found to be significantly associated with type 2 diabetes (113). However, the association was completely abolished after adjustment for BMI (body mass index),

suggesting that *FTO* confers diabetes risk through its association with obesity. Subsequent studies confirmed *FTO* as an "obesity candidate" (176-180). Moreover, *FTO* may influence cerebrocortical insulin sensitivity, since rs8050136 in *FTO* has been associated with impaired insulin-stimulated cerebrocortical activity (181).

JAZF1 (JAZF zinc finger 1) encodes for a nuclear protein with three C2H2-type zinc fingers, and functions as a transcriptional repressor of the transcription factor *NR2C2* (nuclear receptor subfamily 2, group C, member 2) gene (182). *NR2C2* is widely expressed, and *Nr2c2*$^{-/-}$ knockout mice display growth retardation and hypoglycemia due to reduced gluconeogenesis (183, 184). *JAZF1* is expressed in the pancreas (182), and it is speculated that a gain-of-function variant in this gene may lead to postnatal growth restriction which also affects pancreatic beta-cell mass and function. The meta-analysis of 3 GWA studies identified rs864745 in intron 1 of *JAZF1* as being associated with type 2 diabetes (114). Another SNP in *JAZF1* was found to be associated with prostate cancer in a GWA study (185).

CDC123 (cell division cycle 123 homolog [*S. cerevisiae*]) and *CAMK1D* (calcium/calmodulin-dependent protein kinase I delta) are the closest genes to rs12779790, associated with type 2 diabetes in GWA meta-analysis (114). This SNP is located ~90 kb from *CDC123* and ~63.5 kb from *CAMK1D. CDC123* encodes for a protein involved in cell cycle regulation and nutritional control of gene transcription (184). *CAMK1D* regulates granulocyte function (186). The mechanisms whereby these genes increase the risk of type 2 diabetes are not known.

TSPAN8 (tetraspanin 8) encodes for a member of the transmembrane 4 superfamily. Tetraspanin 8 is a widely expressed cell surface glycoprotein known to form complexes with integrins to regulate cell motility in cancer cell lines (187). Since 6-integrin binding to laminin has been shown to negatively affect pancreatic beta-cell mass maintenance (188), it is possible that variation in *TSPAN8* biologically influences pancreatic beta-cell function. *TSPAN8* is also expressed in carcinomas of the colon, liver, and pancreas. SNP rs7961581, shown to be associated with type 2 diabetes in GWA meta-analysis (114), resides 110 kb upstream of *TSPAN8.*

THADA (thyroid adenoma associated) is a widely expressed gene with unknown function, although there is evidence suggesting that it may be involved in the death receptor pathway and apoptosis (189). It was found as a

target gene of specific chromosomal rearrangements observed in thyroid benign tumors (189). A nonsynonymous SNP rs7578597 in exon 24 of *TSPAN8* was shown to be associated with type 2 diabetes in a GWA meta-analysis (114).

ADAMTS9 (ADAM metallopeptidase with thrombospondin type 1 motif, 9) encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. ADAMTS9 is a secreted metalloprotease expressed in many tissues including skeletal muscle and the pancreas (114). *ADAMTS9* is located on chromosome 3p14.3-p14.2, an area known to be lost in hereditary renal tumors. SNP rs4607103, found to be associated with type 2 diabetes in GWA meta-analysis (114), resides ~38 kb upstream of *ADAMTS9*.

NOTCH2 (Notch homolog 2 [Drosophila]) encodes for a member of the Notch family, playing a role in a variety of developmental processes by controlling cell fate decisions. In mice, Notch2 is expressed in embryonic ductal cells of branching pancreatic buds during pancreatic organogenesis, the likely source of endocrine and exocrine stem cells (190). SNP rs10923931, found to be associated with type 2 diabetes in GWA meta-analysis (114), resides within intron 5 of *NOTCH2*.

KCNQ1 (potassium voltage-gated channel, KQT-like subfamily, member 1) encodes for the pore-forming subunit of the voltage-gated potassium channel. It is mainly expressed in the heart, where it is required for the repolarization phase of the action potential in cardiac muscle (191), and in other tissues including the brain, adipose tissue, liver, pancreas and pancreatic islets (192-194). Mutations in *KCNQ1* are associated with cardiac arrhythmias (192, 195, 196). The contribution of *KCNQ1* to the molecular pathogenesis of type 2 diabetes is not clear, although it might play a role in beta-cell function (116, 197). SNPs in *KCNQ1* were robustly associated with type 2 diabetes in two GWA studies conducted in Japanese populations (115, 116).

MTNR1B (melatonin receptor 1B) encodes for one of two subtypes of a receptor for melatonin. Melatonin is a hormone predominantly secreted by the pineal gland, playing an important role in the regulation of circadian and seasonal rhythms of several biological functions. There is evidence that melatonin has inhibitory effects on insulin secretion (198), and might be involved in circadian lowering of nocturnal insulin levels (199). Melatonin receptors are members of the G protein-coupled receptor family. They are mainly expressed in the brain (suprachiasmatic nucleus, hippocampus), but

they have also been found in human and rodent pancreatic islets, MTNR1A predominating in glucagon producing alfa-cells, and MTNR1B in insulin-producing beta-cells (reviewed in 200). Two SNPs, rs1387153 and rs10830963, were reported to be associated with type 2 diabetes, elevated fasting plasma glucose, and impaired insulin secretion measured by HOMA-B (117, 201). SNP rs10830963 is located within the single intron of *MTNR1B*.

LOC387761 (a hypothetical gene). Two SNPs (rs7480010 and rs9300039) near this gene have been associated with type 2 diabetes in two GWA studies (108, 111). The function of the protein product of *LOC387761* is not known.

3. Aims of the study

The main aim of this study was to describe the pathophysiology of the prediabetic state, and to assess the possible effects of type 2 diabetes risk loci on insulin secretion, proinsulin conversion, and insulin sensitivity.

The specific aims of the study were:

1. To describe the changes in insulin secretion and insulin sensitivity across the entire range of fasting and 2-hour plasma glucose from normal glucose tolerance to type 2 diabetes in a large population-based study

2. To investigate the association of the *HHEX* locus with insulin secretion and insulin sensitivity in non-diabetic offspring of type 2 diabetic patients

3. To investigate the association of the *CDKAL1* locus with insulin secretion and insulin sensitivity in non-diabetic offspring of type 2 diabetic patients

4. To investigate the association of 18 type 2 diabetes risk loci with insulin secretion, proinsulin conversion, and insulin sensitivity in a large population-based study.

4. Subjects and methods

4.1 SUBJECTS

The METSIM study *(Studies I, IV)*
A total of 6,414 men from the ongoing population-based cross-sectional METSIM Study (Metabolic Syndrome in Men Study) were included in the study. The subjects, aged from 45 to 70 years, were randomly selected from the population register of the town of Kuopio, in eastern Finland (population 95 000). Every participant had a one-day outpatient visit to the Clinical Research Unit at the University of Kuopio, including an interview on the history of previous diseases and current drug treatment, and an evaluation of glucose tolerance and cardiovascular risk factors. Fasting blood samples were drawn after 12 hours of fasting followed by an OGTT. The study was approved by the Ethics Committee of the University of Kuopio and Kuopio University Hospital, and carried out in accordance with the Helsinki Declaration.

The EUGENE 2 Study *(Studies II, III)*
The subjects in this study were healthy non-diabetic offspring of patients with type 2 diabetes. One of the parents (a proband) had to have type 2 diabetes, and the spouse a normal glucose tolerance in an OGTT or a lack of history of type 2 diabetes in first-degree relatives. The probands (N=536) were randomly selected among type 2 diabetic subjects living in the regions of five study centers in Europe constituting the EUGENE2 consortium: the Lundberg Laboratory for Diabetes Research (Gothenburg, Sweden), the Polyclinic Mater Domini of the University Magna Graecia (Catanzaro, Italy), the Steno Diabetes Center (Copenhagen, Denmark), the Kuopio University Hospital (Kuopio, Finland), and the Tübingen University Hospital (Tübingen, Germany). Type 2 diabetes among the probands was defined according to the WHO criteria (7). The offspring (children of a diabetic proband and his/her spouse) were invited to the study. Altogether 846 offspring from the five European clinical centres were included in the study, as follows: Catanzaro, Italy (N=110), Copenhagen, Denmark (N=270), Gothenburg, Sweden (N=100), Kuopio, Finland (N=217) and Tübingen, Germany (N=149). A standard medical history was obtained from all participants, and they underwent physical examination, routine blood tests, and OGTT. A subgroup of 758 subjects underwent an IVGTT on a separate occasion, followed by a hyperinsulinemic-euglycemic clamp in four centres (N

= 575). The study protocol was approved by appropriate Institutional Review Boards. All study subjects gave informed consent.

Table 2: Baseline characteristics of EUGENE2 and METSIM study populations.

	EUGENE2 *(Studies II, III)*	METSIM	
		All *(Study I)*	Non-diabetic *(Study IV)*
Men / women	368/478	6414/-	5327/-
Age (years)	40.0 ± 10.2	57.7 ± 6.8	58.4 ± 6.5
Body mass index (kg/m^2)	26.7 ± 5.0	27.0 ± 3.9	26.8 ± 3.8
Fasting plasma glucose (mmol/l)	5.1 ± 0.5	5.8 ± 0.8	5.7 ± 0.5
2-hour plasma glucose (mmol/l)	6.3 ± 1.5	6.5 ± 2.4	6.1 ± 1.7
Fasting plasma insulin (pmol/l)	51 ± 57	52 ± 38	49 ± 34
2-hour plasma insulin (pmol/l)	328 ± 294	331 ± 341	313 ± 315

4.2 METHODS

Clinical measurements *(Studies I-IV)*
Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. BMI was calculated as weight (kg) divided by height (m) squared. Waist (at the midpoint between the lateral iliac crest and lowest rib) and hip circumference (at the level of the trochanter major) were measured to the nearest 0.5 cm.

Oral glucose tolerance test *(Studies I-IV)*
A 2-h OGTT (75 g of glucose) was performed, and samples for plasma glucose and insulin were drawn at 0, 30 and 120 min in the METSIM study, and at 0, 30, 60, 90, and 120 min in the EUGENE2 study. Glucose tolerance was evaluated according to the WHO criteria (7) in *Studies II, III*, and according to the ADA criteria (6) in *Studies I, IV*.

IVGTT and the euglycemic hyperinsulinemic clamp *(Studies II-III)*
IVGTT was performed to determine the first-phase insulin secretion capacity after an overnight fast. A bolus of glucose (300 mg/kg in a 50% solution) was injected within 30 seconds into the antecubital vein. Samples for the measurement of plasma glucose and insulin (arterialized venous blood) were drawn at –5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 minutes. At 60 min after the glucose bolus, the euglycemic hyperinsulinemic clamp was started to evaluate insulin sensitivity (insulin infusion of 240 pmol/m^2/min) (202). Glucose was

clamped at 5.0 mmol/l for the next 120 minutes by the infusion of 20% glucose at various rates according to glucose measurements performed at 5-minute intervals. The mean amount of glucose infused during the last hour was used to calculate the rates of whole-body glucose uptake (presented as M-value).

Laboratory determinations

In the METSIM study *(Studies I, IV)*, plasma glucose was measured by enzymatic hexokinase photometric assay (Konelab Systems Reagents, Thermo Fischer Scientific, Vantaa, Finland), insulin by immunoassay (ADVIA Centaur Insulin IRI, no. 02230141, Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), and proinsulin by immunoassay (Human Proinsulin Ria kit, Linco Research, St. Charles, MO, USA). Proinsulin data were available for 2,697 subjects.

In the EUGENE2 study *(Studies II, III)*, plasma glucose was measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus, Yellow Springs Instrument Co., Inc, Ohio, USA) in all centers. Since plasma insulin levels were measured by different methods (except for the Gothenburg center, which had their insulin measured in Tubingen), the assay applied in Tubingen (microparticle enzyme immunoassay; Abbott Laboratories, Tokyo, Japan) was selected as a reference assay.

Genotyping *(Studies I-IV)*

DNA was isolated from whole blood using commercial DNA isolation kits. In the EUGENE2 study, *CDKAL1* rs7754840 and *HHEX* rs1111875 were genotyped. In the METSIM study, a total of 19 confirmed type 2 diabetes-related SNPs were genotyped *(PPARG* rs1801282, *KCNJ11* rs5219, *TCF7L2* rs7903146, *SLC30A8* rs13266634, *HHEX* rs1111875, *LOC387761* rs7480010, *CDKN2B* rs10811661, *IGF2BP2* rs4402960, *CDKAL1* rs7754840, *HNF1B* rs757210, *WFS1* rs10010131, *JAZF1* rs864745, *CDC123* rs12779790, *TSPAN8* rs7961581, *THADA* rs7578597, *ADAMTS9* rs4607103, *NOTCH2* rs10923931, *KCNQ1* rs2283228). Genotyping of all SNPs except *MTNR1B* rs10830963 was performed with the TaqMan Allelic Discrimination Assay (Applied Biosystems). *MTNR1B* rs10830963 was genotyped by Sequenom iPlex gold SBE (Sequenom). In the EUGENE2 study, the genotyping success rate was 99.7%, and the error rate was 0% among 3.3% of DNA samples regenotyped. In the METSIM study, the TaqMan genotyping call rate was 100%, with an error rate of 0% among 4.5% of DNA samples genotyped in duplicate, and the Sequenom iPlex call rate for *MTNR1B* rs10830963 was 96.8%, with an error rate of 0% among 4.2% of DNA samples genotyped in duplicate. All SNPs were consistent with Hardy-

Weinberg equilibrium (P>0.05) except for *HNF1B* rs757210 (P<0.0001). This SNP was therefore omitted from all statistical analyses.

Calculations *(Studies I-IV)*

The trapezoidal method was used to calculate the glucose, insulin, and proinsulin area under the curve (AUC) during the OGTT *(Studies I-IV)*, and insulin and glucose AUC during the first (0-10 min), second (10-60 min) and entire (0-60 min) phase of IVGTT *(Studies II-III)*. Surrogate indices of insulin sensitivity and insulin secretion (including Matsuda ISI, HOMA-IR, HOMA-B, insulinogenic index, corrected insulin response) were calculated according to published formulas (80, 90, 203-206). The index of early-phase insulin release ($InsAUC_{0-30}/GluAUC_{0-30}$) during an OGTT was calculated as the total insulin area under the curve divided by the total glucose area under the curve during the first 30 min of an OGTT *(Studies I, IV)*. Four indices of proinsulin conversion were calculated *(Study IV)*: proinsulin/insulin ratio in the fasting state ($Proins_0/Ins_0$), an index of proinsulin conversion to insulin during the first 30 min ($ProinsAUC_{0-30}/InsAUC_{0-30}$), 30 to 120 min ($ProinsAUC_{30-120}/InsAUC_{30-120}$) and 0 to 120 min ($ProinsAUC_{0-120}/InsAUC_{0-120}$) of an OGTT. Clamp-derived insulin sensitivity (M-value) was calculated as the glucose infusion rate necessary to maintain euglycemia during the last 60 min (steady state) of the clamp (in μmol/kg/min), or as an insulin sensitivity index (S_I) derived from an IVGTT (207) *(Studies II, III)*.

Statistical analysis

Study I: Data were presented as means ± SD, median (25th, 75th percentile) for continuous variables or as count (percentage) for categorical variables. Variables with a non-normal skewed distribution were logarithmically transformed before analysis. Continuous variables were compared across the categories of glucose tolerance by the analysis of variance (ANOVA) or after adjustment for covariates using the general linear model. Pair-wise comparisons between the groups were performed by Bonferroni post-hoc tests (with P-value adjustment for multiple testing for each variable). Categorical variables were examined by the χ^2 test. Spearman's rank correlation was used to compare the surrogate indices with the reference measures. P-value <0.05 was considered statistically significant.

Studies II, III: The results for continuous variables were given as means ± SD. Odds ratios were presented with the 95% confidence intervals. Variables with skewed distribution were logarithmically transformed for statistical analyses.

The differences between the groups were assessed by the ANOVA for continuous variables and by the χ^2 test for non-continuous variables. The linear mixed model and logistic regression analysis were applied to adjust for confounding factors. For mixed model analysis we included center and pedigree as random factors, genotype and gender as fixed factors, and continuous variables adjusted for as covariates. Power calculations were performed using G*power software available at http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/. *P*-value <0.05 was considered statistically significant.

Study IV: Effects of SNPs on continuous traits were presented as effect sizes [B (SE)] per copy of the type 2 diabetes risk allele, estimated and adjusted for covariates by linear regression, using untransformed dependent variables. *P* values were calculated using logarithmically transformed variables (all except for age) due to their skewed distribution. The effect of genetic risk score on $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ was analyzed by linear regression adjusted for age, BMI and Matsuda ISI due to the significant association of genetic risk score with these covariates. Hardy-Weinberg equilibrium was tested by the χ^2 test. $P<0.05$ was considered nominally significant, $P<6.9\text{x}10^{-4}$ calculated using Bonferroni correction for multiple comparisons was considered statistically significant, given 72 independent tests for 18 SNPs and 4 outcomes measured [obesity (BMI), insulin release ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$), insulin sensitivity (Matsuda ISI), and proinsulin conversion ($ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$)]. The power of the sample was estimated using the Bioconductor's GeneticsDesign package version 1.1 (http://www.bioconductor.org/packages/2.3/bioc/html/GeneticsDesign.html).

All analyses except for the power calculations were conducted with the SPSS v.14 programs (SPSS, Chicago, IL, USA).

5. Results

5.1 CHANGES IN INSULIN SENSITIVITY AND INSULIN RELEASE IN RELATION TO GLYCEMIA AND GLUCOSE TOLERANCE IN THE METSIM STUDY *(Study I)*

Baseline characteristics. Out of 6,414 subjects included (Table 2), 2,168 (34%) had NGT, 2,859 (45%) had IIFG, 217 (3%) had IIGT, 701 (11%) had a combination of IFG and IGT, and 469 (7%) had newly diagnosed type 2 diabetes (NewDM). A total of 492 subjects with previously diagnosed diabetes were excluded from statistical analyses.

Insulin sensitivity according to fasting and 2-hour plasma glucose concentration. Categories of FPG (steps by 0.5 mmol/l) and 2hPG (steps by 1.0 mmol/l) were generated, and categories with FPG < 5.0 mmol/l and 2hPG < 5.0 mmol/l were set as the reference categories. We observed a considerable decrease (by 17%) in age- and BMI-adjusted peripheral insulin sensitivity (Matsuda ISI) within the normal range of FPG, compared with the reference category. Insulin sensitivity further decreased to -50% within the range of IFG, and decreased to -67% in the diabetic range of FPG (Figure 5A). A substantial decrease in insulin sensitivity (-37%) was also observed within the normal range of 2hPG. Insulin sensitivity further decreased to -51% within the IGT range, and to -57% within the diabetic range of 2hPG (Figure 5B).

Insulin release according to fasting and 2-hour plasma glucose concentration. Age- and BMI-adjusted early-phase insulin release ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$) decreased only slightly (-4%) within the normal range of FPG. It further decreased within the range of IFG and diabetes to -25% and -70%, respectively (Figure 5C). The early-phase insulin release decreased by -6% within the normal range of 2hPG, and further decreased to -23% and -50% within the range of IGT and diabetes, respectively (Figure 5D). Age- and BMI-adjusted total insulin release ($InsAUC_{0\text{-}120}/GluAUC_{0\text{-}120}$) decreased to -13% within the range of IFG, and to -70% within the diabetic range of FPG (Figure 5E). Total insulin release increased by 14% with higher 2hPG up to 9.9 mmol/l, and then decreased to -45% within the diabetic range of 2hPG (Figure 5F). The largest decreases in both early-phase (-32% to -50%) and total (-17% to -45%) insulin release were observed within the range of FPG from 7.0 to 7.9 mmol/l (Figure 5C and 5E).

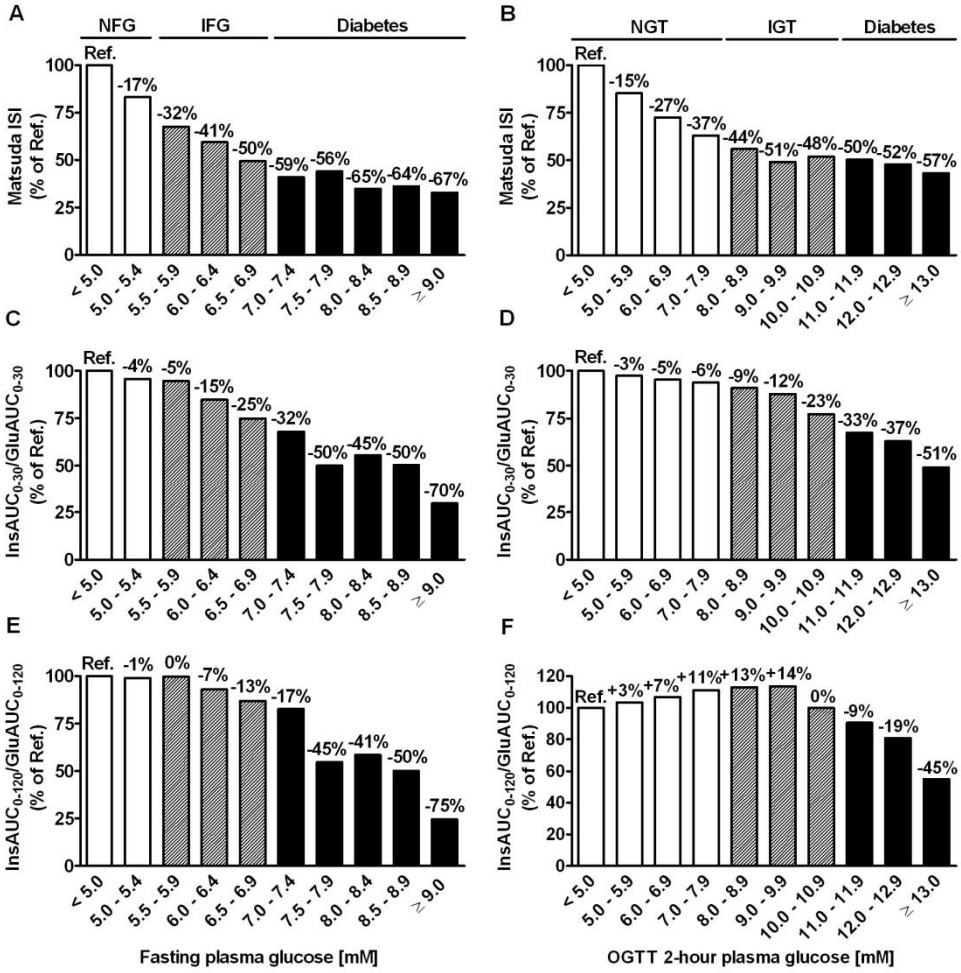

Figure 5. Insulin sensitivity (Matsuda ISI, **A,B**)**,** early-phase insulin release ($InsAUC_{0-30}/GluAUC_{0-30}$, **C,D**) and total insulin release during OGTT ($InsAUC_{0-120}/GluAUC_{0-120}$, **E,F**) across the categories of fasting (FPG) and 2-hour plasma glucose (2hPG). Bars display the value of insulin sensitivity or insulin release relative to the reference category (fasting plasma glucose < 5.0 mmol/l, 2-h plasma glucose < 5.0 mmol/l). Calculations were based on geometric means, adjusted for age and BMI with the general linear model. Cut-off values for different categories of FPG in mg/dl: 90.1 (5.0 mmol/l), 99.1 (5.5 mmol/l), 108.1 (6.0 mmol), 117.1 (6.5 mmol/l), 126.1 (7.0 mmol/l), 135.1 (7.5 mmol/l), 144.1 (8.0 mmol/l), 153.2 (8.5 mmol/l), 162.2 (9.0 mmol/l). Cut-off values for different categories of 2hPG in mg/dl: 90.1 (5.0 mmol), 108.1 (6.0 mmol), 126.1 (7.0 mmol/l), 144.1 (8.0 mmol/l), 162.2 (9.0 mmol/l), 180.2 (10.0 mmol/l), 198.2 (11.0 mmol/l), 216.2 (12.0 mmol/l), 234.2 (13.0 mmol/l).

Disposition index. The early-phase DI_{30} and total DI_{120} decreased with higher FPG within the normal range by -21% and -18%, respectively. Within the IFG range, the reduction in DI_{30} and DI_{120} reached -63% and -57%. As a function of 2hPG, DI_{30} and DI_{120} decreased to -41% and -30% in the normal range, and further decreased to -60% and -48% in the IGT range.

Compensatory insulin secretion. Compensatory insulin secretion was not observed in spite of a significant decrease in insulin sensitivity within the normal range of FPG, but in contrast the early-phase insulin release started to fall. However, compensatory total insulin secretion started already at low 2hPG levels and insulin release increased up to 10 mmol/l, and then started to fall. A decrease in DI indices was substantial already in the normal ranges of FPG and 2hPG.

Insulin sensitivity and insulin release according to glucose levels in non-obese and obese individuals. No significant interaction between BMI and glucose levels in determining insulin sensitivity or insulin release was found.

Insulin sensitivity and insulin release in categories of glucose tolerance. Age- and BMI-adjusted peripheral insulin sensitivity (Matsuda ISI) was significantly lower by 26% in IIFG, by 30% in IIGT, by 42% in IFG+IGT, and by 46% in NewDM, compared with NGT (Figure 6A). Matsuda ISI was significantly lower in individuals with IIGT than in individuals with IIFG (P=0.0016). A significantly greater decrease in IIFG than in IIGT (-31% vs. -16%; P=0.0028) was found when insulin sensitivity was assessed with 1/HOMA-IR. 1/HOMA-IR was reduced by 39% in the IFG+IGT group, and by 45% in NewDM (Figure 6B). Categories of glucose tolerance status differed significantly also with respect to other indices of insulin sensitivity.

Compared with NGT, the age- and BMI-adjusted early-phase insulin release ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$) was significantly lower by 8% in IIFG, not changed in IIGT, lower by 16% in IFG+IGT and by 43% in NewDM (Figure 6C). The difference between IIFG and IIGT was not statistically significant (-8% vs. 0%, P=1.0). The total insulin release ($InsAUC_{0\text{-}120}/GluAUC_{0\text{-}120}$) was significantly lower in IIFG (-6%) and in NewDM (-29%), whereas no significant changes were observed in IIGT or in IFG+IGT compared with NGT (Figure 6D). Individuals with IIFG had significantly lower total insulin release than individuals with IIGT (-6% vs. +16%, P=0.001).

Disposition index. The early-phase DI_{30} was lower in IIGT than in IIFG (-36% vs. -29% compared with NGT, P=0.0003). In the IFG+IGT group, DI_{30} was 53%

lower, and in NewDM 68% lower than in NGT (Figure 6E). In contrast, the total DI_{120} was lower to the same extent in IIFG and IIGT (by 27%). DI_{120} was 44% lower in the IFG+IGT group and 62% lower in NewDM than in NGT (Figure 6F).

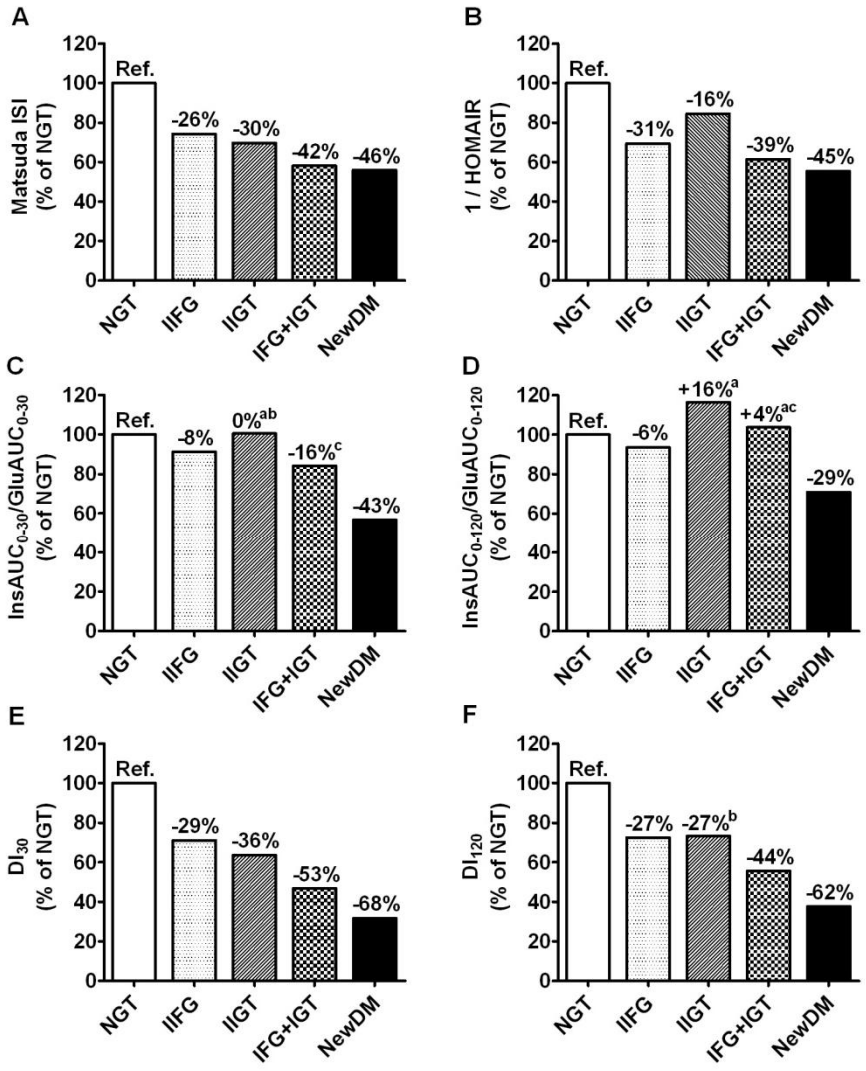

Figure 6. Insulin sensitivity (Matsuda ISI, **A**; 1/HOMAIR, **B**), early-phase ($InsAUC_{0-30}/GluAUC_{0-30}$, **C**) and total insulin release ($InsAUC_{0-120}/GluAUC_{0-120}$, **D**), disposition index for early insulin release (DI_{30} = Matsuda ISI x $InsAUC_{0-30}/GluAUC_{0-30}$, **E**), and disposition index for total insulin release (DI_{120} = Matsuda ISI x $InsAUC_{0-120}/GluAUC_{0-120}$), **F**) in different categories of glucose tolerance. Bars show the percentage of each

index relative to NGT (reference, 100%). Calculations were based on geometric means, adjusted for age and BMI (ANCOVA). All pairwise comparisons were statistically significant (P<0.05, Bonferroni posthoc test) except for those marked: [a]P>0.05 vs. NGT, [b]P>0.05 vs. IIFG, [c]P>0.05 vs. IIGT, [d]P>0.05 vs. IFG+IGT.

5.2 ASSOCIATION OF *CDKAL1* RS7754840 POLYMORPHISM WITH IMPAIRED INSULIN SECRETION IN THE EUGENE2 AND METSIM STUDIES *(Study II)*

The EUGENE2 study

Baseline characteristics. Altogether, 846 subjects from the EUGENE2 study were included in the study (women 56.5%) (Table 2). Of these, 17% had abnormal glucose tolerance (IFG and/or IGT). The frequency of the minor C allele of rs7754840 was 0.33 (96 homozygous and 373 heterozygous carriers of the C allele among 846 subjects). The genotype distribution followed the Hardy-Weinberg equilibrium (P=0.911).

OGTT data. The glucose and insulin responses during an OGTT according to genotypes of rs7754840 are shown in Figure 7. The type 2 diabetes risk C allele was significantly associated with higher glucose levels at 30 min (P=0.034) and 60 min (P=0.005), as well as with higher glucose AUC (P=0.016). With respect to insulin response, the C allele was significantly associated with lower insulin levels at 30 min (P=0.011). Insulin levels at 0, 60, 90 and 120 min, and insulin AUC also tended to be lower in CC homozygotes. A significant difference in the insulinogenic index (IGI, P=0.001) and corrected insulin response to an oral glucose load (CIR, P<0.001) was observed between the genotypes. IGI was 53% lower and CIR 26% lower in the CC homozygotes compared with the GG homozygotes. No significant difference in the HOMA-beta index was observed.

Insulin sensitivity. No significant difference was observed between the genotypes in the M-value (Figure 8A) or HOMA-IR index, but there was a significant difference in S_I values in the Copenhagen center (P=0.006). There was a significant negative correlation between the M-value and HOMA-IR (r=-0.479; P<0.001), as well as between S_I and HOMA-IR (r=-0.650; P<0.001), therefore we adjusted our results for HOMA-IR, available from all study centres.

Insulin release. Figure 8 (B, C) shows the first-phase (0-10 min) and second-phase (10-60 min) glucose and insulin responses in an IVGTT. Under the additive model the C allele was associated significantly with higher glucose AUC over basal glucose during the second-phase of the IVGTT (*P*=0.003), resulting in a 15% difference between the GG and CC genotypes. No significant effect on first-phase glucose response was observed. Furthermore, the C allele was significantly associated with lower first-phase insulin AUC over basal insulin levels (P=0.002). The first-phase insulin release was 11% lower in the GC heterozygotes than in the GG homozygotes, and 13% lower in the CC homozygotes thank in the GC heterozygotes, suggesting an additive effect of the C allele on the first-phase insulin release. No significant effect of rs7754840 on the second-phase insulin release was observed. We also found a significant association between the disposition index (M-value x first phase insulin release) and the *CDKAL1* genotype under the recessive model (*P*=0.028). Under the additive model this association was no longer significant.

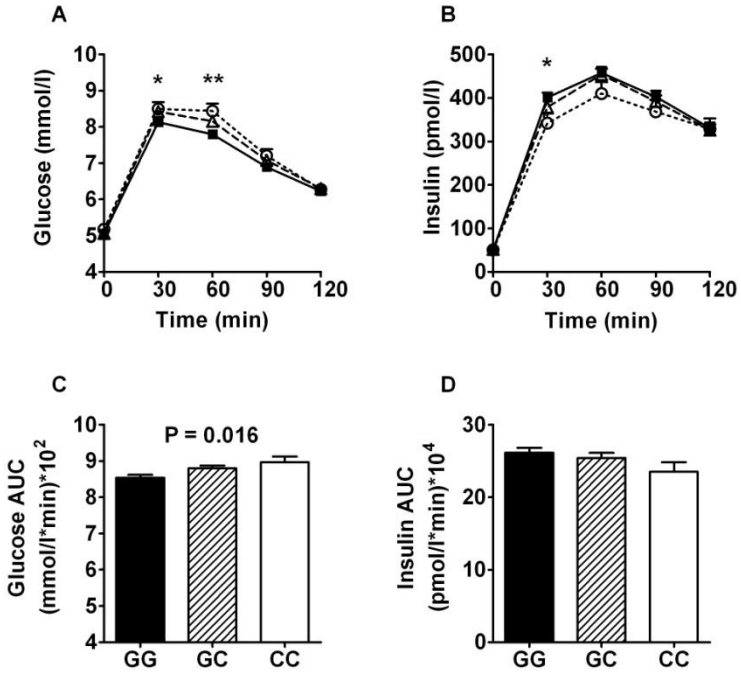

Figure 7. Plasma glucose (A) and insulin (B) levels during an OGTT, and glucose (C) and insulin (D) levels under the curve (AUC) during an OGTT according to SNP rs7754840 in all subjects. P-values are adjusted for age, BMI, gender, family and center,

and are calculated over the three genotype groups (ANOVA). *P<0.05, **P<0.01. In A and B, black squares and the solid line indicate GG, white triangles and the dashed line indicate GC, and white circles and the dotted line indicate CC. In C and D, the filled bars indicate GG, striated bars indicate GC and open bars indicate CC. Insulin levels are log-transformed in statistical analyses. Data are given as adjusted means ± SE.

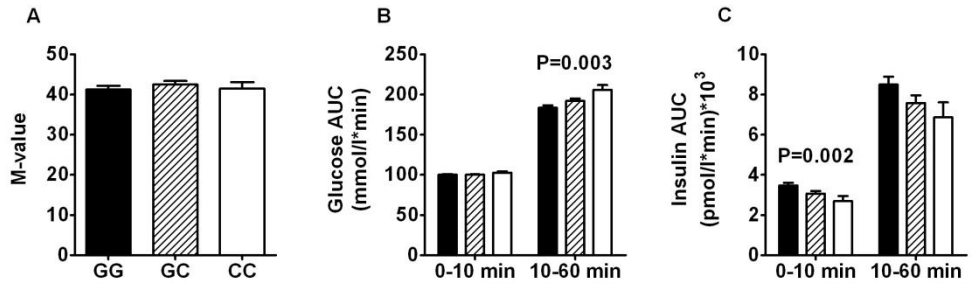

Figure 8. Insulin sensitivity measured by clamp (A), the first-phase and second-phase glucose levels under the curve over basal glucose (B) and first-phase and second-phase insulin levels under the curve over basal insulin (C) during the IVGTT according to SNP rs7754840 in all subjects (Copenhagen was excluded from analyses of insulin sensitivity and second-phase insulin secretion). *P*-values are adjusted for age, BMI, gender, family and center, and are calculated over the three genotype groups (ANOVA). Insulin levels are log-transformed in statistical analyses. Filled bars indicate GG, striated indicate GC and open bars indicate CC. Data are given as adjusted means ± SE.

The METSIM study

Baseline characteristics. An independent sample of 3,900 middle-aged Finnish men from the ongoing population-based study was studied. Of 3,367 non-diabetic subjects (mean age 59.0±5.8 years; BMI 26.9±3.8 kg/m^2), 2,405 (71.4%) had NGT, 632 (18.8%) had IFG and 330 (9.8%) had IGT. To examine the association of rs7754840 with type 2 diabetes, 533 diabetic subjects were compared with subjects having NGT.

Association of rs7754840 with type 2 diabetes. We observed a significant association of rs7754840 with type 2 diabetes under the recessive model [OR 1.346 (1.044; 1.120); P=0.022] indicating a 1.3-fold higher risk in the CC homozygotes than in carriers of the G allele. Under the additive model, the effect was signifficant only when comparing the GG and CC homozygotes [OR

1.422 (1.072; 1.882); *P*=0.014] and remained significant also after adjustement for age and BMI.

OGTT data. In subjects with NGT, a significant assocation of the C allele with lower insulin AUC was observed (*P*<0.001). After the adjustment for age, BMI and the HOMA-IR index the effect remained significant ($P_{adjusted}$=0.041). Furthermore, we observed an association of the C allele with significantly lower values of IGI (Figure 9), being 9% lower in GC heterozygotes than in the GG homozygotes and 2% lower in the CC homozygotes than in GC heterozygotes (*P*<0.001; $P_{adjusted}$=0.012). Analyses of all non-diabetic subjects provided very similar results.

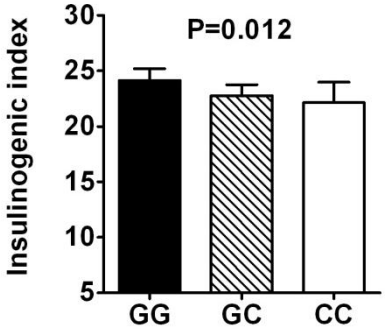

Figure 9. Insulinogenic index values according to SNP rs7754840 in subjects with normal glucose tolerance from the replication sample of Finnish middle-aged men. *P*-value is adjusted for age, BMI and HOMA-IR, and is calculated over the three genotype groups (ANOVA). Insulinogenic index and HOMA-IR values are log-transformed in statistical analysis. Data are given as adjusted means ± SE.

5.3 ASSOCIATION OF A POLYMORPHISM NEAR THE *HHEX* LOCUS WITH IMPAIRED ACUTE GLUCOSE-STIMULATED INSULIN RELEASE IN THE EUGENE2 STUDY *(Study III)*

Baseline characteristics. Altogether, 844 subjects from the EUGENE2 study were included in the study (women 57%, age 40±10 year, BMI 26.6±4.9 kg/m^2). Of these, 691 (82%) had NGT, and 153 (18%) had IFG and/or IGT. The frequencies of the minor alleles of rs1111875 and rs7923837 were 0.39 and 0.34, respectively. The genotype distributions followed the Hardy-Weinberg equilibrium (P>0.05). Both SNPs were in strong, but not complete, linkage disequilibrium (r^2 = 0.779). The two SNPs were not associated with anthropometric data such as age, weight, height, BMI, or waist and hip circumference.

OGTT data. Neither rs1111875 nor rs7923837 were associated with fasting glucose and insulin levels, 2-hour glucose levels, insulin levels at 30 min of an OGTT, or the AUCs of the glucose and insulin during an OGTT before and after adjustment for centre, family relationship, gender, age, and BMI (Table 3). However, additional adjustment for HOMA-IR and glucose levels at 30 min revealed a trend towards lower insulin levels at 30 min in the carriers of the type 2 diabetes risk A allele of rs7923837 (P=0.066).

Insulin sensitivity. Neither rs1111875 nor rs7923837 were associated with the M-value or HOMA-IR, although association of rs1111875 with HOMA-IR became slightly significant after adjustment for centre, family relationship, gender, age and BMI (Table 3).

Insulin release. *HHEX* rs7923837 was significantly associated with first-phase insulin release (both unadjusted, P=0.013, and adjusted for centre, family relationship, gender, age, BMI, and HOMA-IR, P=0.014) (Table 3). In contrast, *HHEX* rs1111875 was not associated with either first-phase or second-phase insulin release during the IVGTT (Table 3). To assess whether the association of rs7923837 with first-phase insulin release is already detectable before the alteration of glucose tolerance, we analysed 691 subjects with NGT. In this subgroup, the effect of rs7923837 on insulin release was no longer significant, which could be due to reduced sample size

Table 3. Associations of *HHEX* SNPs rs1111875 and rs7923837 with metabolic parameters (N = 844).

SNP (MAF)	*HHEX* rs1111875 (0.39)						*HHEX* rs7923837 (0.34)					
Genotype	CC	CT	TT	P_1	P_2	P_3	GG	GA	AA	P_1	P_2	P_3
N	303	418	123	-	-	-	356	395	91	-	-	-
Fasting glucose (mM)	5.1 ± 0.55	5.09 ± 0.54	5.01 ± 0.44	0.3	0.11	-	5.11 ± 0.56	5.08 ± 0.52	5.01 ± 0.43	0.4	0.3	-
Glucose 120 min OGTT (mM)	6.28 ± 1.58	6.25 ± 1.53	6.2 ± 1.42	1	0.8	-	6.27 ± 1.56	6.27 ± 1.54	6.15 ± 1.40	0.9	0.8	-
AUC glucose OGTT (mM·min)	872 ± 175	862 ± 181	863 ± 151	0.7	0.9	-	870 ± 177	863 ± 178	862 ± 152	0.9	0.9	-
Fasting insulin (pM)	48.3 ± 32.5	54.2 ± 73.8	44.4 ± 29.9	0.22	0.06	-	48.1 ± 31.7	54.4 ± 75.9	44.8 ± 27.5	0.5	0.2	-
Insulin 30 min OGTT (pM)	360 ± 225	403 ± 282	385 ± 234	0.11	0.15	-	362 ± 220	407 ± 288	382 ± 240	0.13	0.4	-
AUC insulin OGTT (pM·min)	247˙575 ± 154˙834	263˙378 ± 188˙720	246˙755 ± 135˙307	0.4	0.7	-	244˙007 ± 147˙702	268˙633 ± 194˙318	242˙962 ± 133˙361	0.4	0.7	-
AUC insulin 0-10 min IVGTT (pM·min)*	3˙580 ± 2˙842	4˙013 ± 3˙232	3˙616 ± 2˙359	0.17	0.19	0.23	3˙433 ± 2˙434	4˙173 ± 3˙514	3˙647 ± 2˙209	0.013	0.046	0.014
AUC insulin 0-10 min IVGTT over basal insulin (pM·min)*	3˙062 ± 2˙502	3˙471 ± 2˙749	3˙198 ± 2˙206	0.15	0.2	0.21	2˙956 ± 2˙282	3˙602 ± 2˙919	3˙220 ± 2˙061	0.016	0.049	0.025
AUC insulin 10-60 min IVGTT (pM·min)*	11˙164 ± 11˙340	11˙450 ± 10˙699	9˙576 ± 7˙098	0.3	0.4	1	10˙559 ± 9˙987	11˙877 ± 11˙649	9˙652 ± 6˙086	0.19	0.7	0.4
AUC insulin 10-60 min IVGTT over basal insulin (pM·min)*	8˙601 ± 9˙316	8˙737 ± 8˙177	7˙449 ± 6˙222	0.3	0.5	0.8	8˙205 ± 8˙810	9˙006 ± 8˙526	7˙491 ± 5˙196	0.14	0.5	0.22
HOMA-IR (mM·µU/ml)	11.1 ± 8.2	12.8 ± 20.6	10 ± 7.0	0.17	0.04	-	11.1 ± 7.9	12.9 ± 21.1	10.1 ± 6.5	0.4	0.17	-
M-value (µmol/kg/min)**	42.7 ± 16.5	41.1 ± 16.6	41 ± 16.4	0.5	0.6	-	42.8 ± 17.2	41.2 ± 16.5	40 ± 14.2	0.4	0.5	-

Data are presented as means ± SD. P_1 values are unadjusted, P_2 values are adjusted for centre, family relationship, gender, age, and BMI, and P_3 values are adjusted for centre, family relationship, gender, age, BMI, and HOMA-IR. *IVGTT data were available from 758 subjects, and ** clamp data from 575 subjects.

5.4 ASSOCIATION OF 18 CONFIRMED SUSCEPTIBILITY LOCI FOR TYPE 2 DIABETES WITH INDICES OF INSULIN RELEASE, PROINSULIN CONVERSION, AND INSULIN SENSITIVITY IN THE METSIM STUDY *(Study IV)*

Baseline characteristics. A total of 5,327 nondiabetic men (Table 2) were included in the study. Of these, 3,594 (68%) subjects had NGT, 884 (17%) had isolated IFG, 503 (9%) had isolated IGT, and 346 (6%) had both IFG and IGT. Subjects with type 2 diabetes (*N*=898) were excluded from the analyses.

Insulin sensitivity. None of the 18 SNPs had a significant effect on Matsuda ISI in the primary analyses carried out under the additive model adjusted for age. Two SNPs, *HHEX* rs1111875 and *KCNJ11* rs5219, were nominally associated with Matsuda ISI, with effect sizes ranging from +2% to +4% per risk allele (*P*=0.010 and 0.005) (Table 4). Adjustment for BMI did not have a major impact on these associations, but revealed another nominal association between *TSPAN8* rs7961581 and Matsuda ISI (*P*=0.008, effect size -2% per risk allele). However, both *KCNJ11* rs5219 and *HHEX* rs1111875 were also associated with $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$. Adjustment for $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ abolished the effect of *KCNJ11* rs5219 (*P*=0.906), but strengthened the effect of *HHEX* rs1111875 on Matsuda ISI ($P=3.6\times10^{-5}$).

Insulin release. Altogether, eight SNPs (in or near *KCNJ11*, *TCF7L2*, *SLC30A8*, *HHEX*, *CDKN2B*, *IGF2BP2*, *CDKAL1*, and *MTNR1B*) were nominally or significantly associated with $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$. The largest effects on $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ (from -6% to -9% per risk allele) were observed for *TCF7L2* rs7903146, *HHEX* rs1111875, *CDKAL1* rs7754840, and *MTNR1B* rs10830963, and were statistically significant in both primary analyses and analyses adjusted for age, BMI and Matsuda ISI (Table 4). Effect sizes of the SNPs in/near *KCNJ11*, *SLC30A8*, *CDKN2B*, and *IGF2BP2* were <-5% per risk allele. Adjustment of the effects of these SNPs for BMI and Matsuda ISI in addition to age attenuated the initially significant effect of *KCNJ11* rs5219 (*P*=0.024), strengthened the associations of *SLC30A8* rs13266634 and *CDKN2B* rs10811661 to a significant level ($P=3.2\times10^{-4}$, and 1.7×10^{-4}), and did not change the nominal association of *IGF2BP2* rs4402960 with $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ (*P*=0.004) (Table 4).

Table 4. Associations of 18 SNPs with early-phase insulin release ($InsAUC_{0-30}/GluAUC_{0-30}$), proinsulin conversion ($ProinsAUC_{0-30}/InsAUC_{0-30}$), insulin sensitivity (Matsuda ISI), and disposition index (DI= $InsAUC_{0-30}/GluAUC_{0-30}$ x Matsuda ISI) in non-diabetic subjects.

Gene	Alleles	$InsAUC_{0-30}$ / $GluAUC_{0-30}$			$ProinsAUC_{0-30}$ / $InsAUC_{0-30}$			Matsuda ISI			Disposition index		
SNP	MAF (%)	Effect size B (SE)	P	P*	Effect size B (SE)	P	P*	Effect size B (SE)	P	P†	Effect size B (SE)	P	P†
PPARG rs1801282	C/G 15.5	0.63 (0.57)	0.316	0.664	0.14 (0.45)	0.991	0.560	-0.11 (0.11)	0.364	0.054	-0.30 (1.99)	0.958	0.810
KCNJ11 rs5219	G/A 47.7	-1.14 (0.41)	3.8E-04	0.025	0.49 (0.32)	0.115	0.531	0.25 (0.08)	0.005	0.008	-1.32 (1.40)	0.362	0.231
TCF7L2 rs7903146	C/T 17.7	-1.78 (0.53)	3.9E-05	9.8E-07	0.75 (0.42)	0.002	6.0E-04	0.12 (0.11)	0.228	0.920	-6.51 (1.87)	8.3E-05	3.4E-06
SLC30A8 rs13266634	C/T 39.1	-0.83 (0.41)	0.013	3.2E-04	0.73 (0.33)	1.9E-05	1.2E-05	-0.00 (0.08)	0.871	0.679	-4.19 (1.46)	0.001	4.2E-04
HHEX rs1111875	C/T 46.9	-2.73 (0.40)	3.2E-12	1.4E-14	0.80 (0.32)	9.7E-06	6.5E-06	0.17 (0.08)	0.010	0.017	-8.89 (1.42)	2.5E-09	1.2E-10
LOC387761 rs7480010	A/G 17.5	-0.51 (0.54)	0.540	0.290	-0.33 (0.44)	0.829	0.194	0.17 (0.11)	0.087	0.345	3.57 (1.91)	0.094	0.189
CDKN2B rs10811661	A/G 14.5	-1.15 (0.58)	0.021	1.7E-04	0.31 (0.47)	0.285	0.211	-0.03 (0.12)	0.847	0.413	-6.30 (1.99)	4.3E-04	0.001
IGF2BP2 rs4402960	C/A 32.1	-1.34 (0.43)	0.004	0.004	0.14 (0.34)	0.263	0.368	0.08 (0.09)	0.182	0.440	-4.22 (1.53)	0.038	0.014
CDKAL1 rs7754840	G/C 37.0	-1.68 (0.42)	3.4E-05	2.2E-06	0.32 (0.34)	3.1E-04	0.001	0.12 (0.08)	0.181	0.176	-5.25 (1.48)	1.6E-04	6.4E-05
WFS1 rs10010131	G/A 45.0	-0.56 (0.41)	0.048	0.397	0.01 (0.33)	0.402	0.081	0.14 (0.08)	0.055	0.100	0.23 (1.44)	0.986	0.808
JAZF1 rs864745	A/G 48.5	0.15 (0.41)	0.551	0.554	-0.25 (0.32)	0.792	0.968	-0.10 (0.08)	0.198	0.067	-1.31 (1.43)	0.301	0.241
CDC123 rs12779790	A/G 21.5	-0.82 (0.49)	0.059	0.062	-0.07 (0.39)	0.486	0.598	0.07 (0.10)	0.369	0.433	-2.36 (1.73)	0.196	0.043
TSPAN8 rs7961581	A/G 19.4	0.23 (0.51)	0.525	0.891	-0.29 (0.41)	0.120	0.310	-0.15 (0.10)	0.343	0.008	-0.75 (1.80)	0.635	0.308
THADA rs7578597	A/G 5.0	-2.09 (0.93)	0.263	0.232	-1.24 (0.73)	0.425	0.267	0.04 (0.18)	0.659	0.373	-3.51 (3.27)	0.355	0.410
ADAMTS9 rs4607103	G/A 26.1	-0.66 (0.47)	0.335	0.221	-0.04 (0.37)	0.087	0.069	-0.04 (0.09)	0.809	0.587	-2.13 (1.65)	0.308	0.332
NOTCH2 rs10923931	C/A 13.8	-0.56 (0.59)	0.228	0.668	-0.95 (0.47)	0.360	0.080	0.16 (0.12)	0.054	0.060	1.21 (2.09)	0.244	0.300
KCNQ1 rs2283228	A/C 6.2	-1.03 (0.84)	0.161	0.093	0.31 (0.66)	0.176	0.353	0.10 (0.17)	0.701	0.284	-3.29 (2.96)	0.162	0.221
MTNR1B rs10830963	C/G 36.0	-2.02 (0.42)	1.4E-07	1.0E-13	-0.21 (0.33)	0.301	0.189	0.03 (0.08)	0.577	0.436	-9.65 (1.47)	6.7E-11	3.8E-13

Effect size shown is B-coefficient (SE) per copy of the type 2 diabetes risk allele, and was calculated using untransformed variables adjusted for age by linear regression. *P* values were calculated using log-transformed variables (due to their skewed distribution) by linear regression. *P* values are adjusted for age; P^* values are adjusted for age, BMI, and Matsuda ISI; $P^\dagger$ values are adjusted for age and BMI. In the entire cohort, the means±SE of the examined parameters and the number of subjects with

available data were as follows: $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ 30.4±0.29 pmol/mmol (N=5298), $ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$ 12.5±0.23 (N=2697), Matsuda ISI 7.03±0.06 [mg/dl, mU/l] (N=5295), and DI 163.7±1.02 (N=5295). *P* values significant after correction for multiple testing ($P<6.9x10^{-4}$) are in bold. Risk alleles are underlined. Results for the additive model are presented.

Table 5. Associations of 4 SNPs with proinsulin/insulin ratio at fasting state ($Proins_0/Ins_0$), during 0 to 30 min ($ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$), 30 to 120 min ($ProinsAUC_{30\text{-}120}/InsAUC_{30\text{-}120}$) and 0 to 120 min ($ProinsAUC_{0\text{-}120}/InsAUC_{0\text{-}120}$) of an OGTT in non-diabetic subjects

Gene	Alleles	$Proins_0$ / Ins_0			$ProinsAUC_{0\text{-}30}$ / $InsAUC_{0\text{-}30}$			$ProinsAUC_{30\text{-}120}/InsAUC_{30\text{-}120}$			$ProinsAUC_{0\text{-}120}/InsAUC_{0\text{-}120}$		
SNP	MAF (%)	Effect size B (SE)	P	P*	Effect size B (SE)	P	P*	Effect size B (SE)	P	P*	Effect size B (SE)	P	P*
TCF7L2 rs7903146	C/<u>T</u> 17.7	1.20 (1.22)	0.042	0.021	0.75 (0.42)	0.002	6.0E-04	0.55 (0.44)	0.005	1.1E-03	0.57 (0.43)	0.004	0.001
SLC30A8 rs13266634	<u>C</u>/T 39.1	1.59 (0.96)	0.006	0.003	0.73 (0.33)	1.9E-05	1.2E-05	0.64 (0.35)	1.1E-04	4.2E-05	0.64 (0.34)	8.2E-05	2.8E-05
HHEX rs1111875	<u>C</u>/T 46.9	0.74 (0.94)	0.365	0.622	0.80 (0.32)	9.7E-06	6.5E-06	0.69 (0.34)	0.002	0.002	0.71 (0.33)	0.001	6.6E-04
CDKAL1 rs7754840	G/<u>C</u> 37.0	-0.39 (0.98)	0.313	0.775	0.32 (0.34)	3.1E-04	0.001	0.36 (0.35)	0.003	0.009	0.35 (0.35)	0.002	0.005

Effect size shown is B-coefficient (SE) per copy of the type 2 diabetes risk allele, and was calculated using untransformed variables adjusted for age by linear regression. *P* values were calculated using log-transformed variables (due to their skewed distribution) by linear regression. *P* values are adjusted for age, P^* values are adjusted for age, BMI and Matsuda ISI. In the entire cohort, the means±SE of the examined parameters and the number of subjects with available data were as follows: $Proins_0/Ins_0$ 36.3±0.67 (N=2712), $ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$ 12.5±0.23 (N=2697), $ProinsAUC_{30\text{-}120}/InsAUC_{30\text{-}120}$ 14.1±0.24 (N=2693), $ProinsAUC_{0\text{-}120}/InsAUC_{0\text{-}120}$ 13.8±0.24 (N=2692). *P* values significant after correction for multiple testing ($P<6.9x10^{-4}$) are in bold. Risk alleles are underlined. Results for the additive model are presented.

Proinsulin conversion. Four SNPs (in/near *HHEX*, *SLC30A8*, *TCF7L2*, and *CDKAL1*) were associated with $ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$, with effect sizes ranging from +3% to +6% per risk allele (Tables 4 and 5). For *HHEX* rs1111875 and *SLC30A8* rs13266634 the effects were significant regardless of the adjustments used (adjusted for age: $P=9.7x10^{-6}$ and $1.9x10^{-5}$; adjusted for age, BMI and Matsuda ISI: $P=6.5x10^{-6}$ and $1.2x10^{-5}$). In contrast, adjustment for BMI and Matsuda ISI attenuated the significant effect of *CDKAL1* rs7754840 to a nominal level ($P=0.002$), and strengthened the nominal effect of *TCF7L2*

rs7903146 to a significant level (P=6.0x10^{-4}). Similar results, although slightly attenuated, were obtained when alternative indices of proinsulin conversion based on proinsulin and insulin AUCs during 0-120 min or 30-120 min of an OGTT were used (ProinsAUC$_{0\text{-}120}$/InsAUC$_{0\text{-}120}$ and ProinsAUC$_{30\text{-}120}$/InsAUC$_{30\text{-}120}$, Table 5). *SLC30A8* rs13266634 and *TCF7L2* rs7903146 were also nominally associated with the fasting proinsulin/insulin ratio (Proins$_0$/Ins$_0$, Table 5). Overall, these results were consistent with associations of *TCF7L2*, *SLC30A8*, *HHEX* and *CDKAL1* with insulin release, since the risk alleles associated with lower insulin release were also associated with higher proinsulin/insulin ratio.

Combined effect of risk alleles on insulin release. To evaluate the combined effect of multiple type 2 diabetes risk alleles (denoted as the risk allele throughout the text) on InsAUC$_{0\text{-}30}$/GluAUC$_{0\text{-}30}$ we calculated a genetic risk score as the sum of weighted risk alleles (47) at SNPs significantly or nominally associated with InsAUC$_{0\text{-}30}$/GluAUC$_{0\text{-}30}$ in initial analyses (*KCNJ11*, *TCF7L2*, *SLC30A8*, *HHEX*, *CDKN2B*, *IGF2BP2*, *CDKAL1* and *MTNR1B*). For each subject, the number of risk alleles (0, 1, or 2) per SNP was weighted for their effect sizes (shown in Table 4; average effect size per allele among 8 SNPs was 1.58, which was considered as one weighted risk allele), and the sum of weighted alleles for each subject was rounded to the closest integer. Subjects with ≤3 and ≥11 weighted risk alleles were pooled to obtain larger numbers. InsAUC$_{0\text{-}30}$/GluAUC$_{0\text{-}30}$ gradually decreased with an increasing number of risk alleles (relative effect size -4% per allele, P=9.3x10^{-44} adjusted for age, BMI, and Matsuda ISI). Subjects with ≥11 weighted risk alleles (N=190) had 32% lower InsAUC$_{0\text{-}30}$/GluAUC$_{0\text{-}30}$ than subjects with ≤3 weighted risk alleles (N=163) (Figure 10). We also performed similar analysis using non-weighted risk alleles. The difference in InsAUC$_{0\text{-}30}$/GluAUC$_{0\text{-}30}$ between subjects with ≤3 and ≥11 risk alleles was -37% (relative effect size -4% per risk allele, P=3.8x10^{-28}).

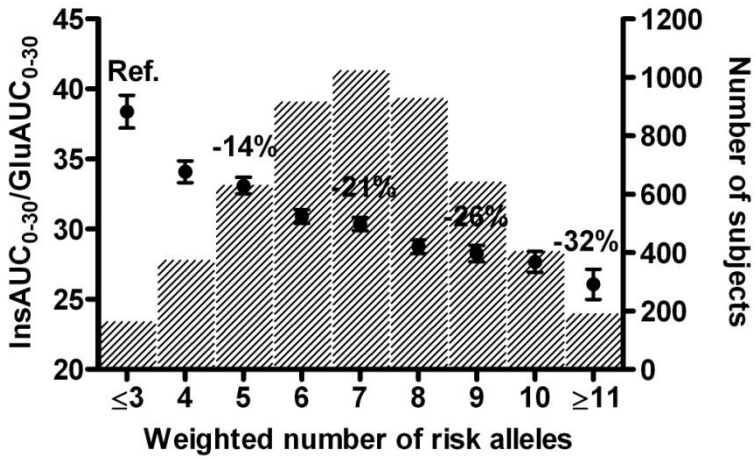

Figure 10. Early-phase insulin release ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$) according to the number of risk alleles in 8 insulin secretion-related SNPs (*KCNJ11* rs5219, *TCF7L2* rs7903146, *SLC30A8* rs13266634, *HHEX* rs1111875, *CDKN2B* rs10811661, *IGF2BP2* rs4402960, *CDKAL1* rs7754840 and *MTNR1B* rs10830963). For each subject, the number of type 2 diabetes risk alleles (0, 1, 2) per SNP was weighted for their effect sizes (shown in Table 4; average effect size per risk allele among 8 SNPs was 1.58, which was considered as one weighted risk allele). The effect of the number of the risk alleles on $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ was significant ($P=9.3\times10^{-44}$, adjusted for age, BMI and Matsuda ISI). Data are shown as means±SE (adjusted for age, BMI and Matsuda ISI). Bars show numbers of subjects in each category.

6. Discussion

6.1 REPRESENTATIVENESS OF THE STUDY SUBJECTS AND EVALUATION OF THE METHODS

This work was based on the results from two cohorts (EUGENE2 and METSIM), both of them exceptional for their large size and detailed phenotyping of the participants.

Studies I and IV were performed in >6000 (>5000 non-diabetic) Finnish middle-aged men from the ongoing population-based METSIM study. The large sample size of this study and careful phenotyping of the participants allowed us to investigate both the pathophysiology of the prediabetic state, and the genetics of insulin secretion and insulin sensitivity. Inconclusive results of previous studies on the pathophysiology of prediabetes indicate that large population-based studies are needed to obtain reliable results. In the METSIM study, the entire spectrum of glucose tolerance status from NGT to diabetes allowed us to examine in detail the changes in insulin secretion and insulin sensitivity with increasing glycemia. Large cohorts with sufficient statistical power are also necessary for genetic-association studies, since the effects of type 2 diabetes risk variants on (pre)diabetic phenotypes are modest. The limitation of the METSIM study is that it includes only Finnish men, and therefore the validity of the results for women, or for other populations is uncertain. Previous studies have not reported sex differences in insulin secretion, but in some studies women have been more insulin sensitive than men (208-210). On the other hand, there is no indication that the association of type 2 diabetes risk variants with parameters of glucose metabolism is dependent on gender (68-71). Due to the large size of the METSIM Study we could not use the most accurate methods to evaluate insulin sensitivity (clamp) and insulin secretion (IVGTT or hyperglycemic clamp) or hepatic insulin sensitivity (tracer techniques). However, we validated our OGTT-derived indices of insulin secretion and insulin sensitivity against the gold-standard measures in the Kuopio sample from the EUGENE2 study. Finally, in spite of the large sample size we did not have sufficient power (>80%) to detect small effects (<6% per allele) of the examined SNPs on Matsuda ISI and $InsAUC_{0-30}$/$GluAUC_{0-30}$, which may explain the negative findings for 9 of 18 SNPs in *Study IV*.

The EUGENE2 study (*Studies II, III*) included 846 young and healthy non-diabetic offspring of patients with type 2 diabetes. Offspring of type 2 diabetic patients are known to have increased risk of type 2 diabetes, and display abnormalities in insulin secretion and/or insulin action long before the onset of type 2 diabetes. Therefore, such a population is ideal for association studies of gene variants and early disturbances in insulin secretion and insulin action. Insulin secretion and insulin sensitivity in the EUGENE2 study were measured by IVGTT and euglycemic hyperinsulinemic clamp, considered as "gold standard" methods providing the most accurate results. The limitation of the EUGENE2 study is that it included five different European populations, and therefore genetic differences between the populations might influence the results. However, similar results have been also found in other European populations.

6.2 PATHOPHYSIOLOGY OF THE PREDIABETIC STATE: INSULIN SENSITIVITY AND INSULIN SECRETION IN RELATION TO HYPERGLYCEMIA *(Study I)*

Subjects with prediabetes (IFG and/or IGT) are at increased risk of developing type 2 diabetes (211-213) and cardiovascular disease (214). Previous studies have suggested that IFG and IGT, defined by fasting and 2-hour glucose levels, might have different etiologies, metabolic profile and prognostic importance. However, no large-scale population-based studies on the relationship of hyperglycemia with insulin secretion and insulin sensitivity have been conducted.

Hyperglycemia as a continuous trait

We observed that insulin sensitivity (Matsuda ISI) decreased substantially already at relatively low glucose levels within the normal range of FPG and 2hPG. Insulin sensitivity further decreased through the IFG/IGT range, and reached its minimum within the diabetic range of FPG and 2hPG. In contrast, early-phase insulin release ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$) decreased only slightly within the normal range of FPG and 2hPG, but declined substantially through the diabetic range of FPG and 2hPG. Compensatory insulin secretion was entirely missing when FPG increased from the normal range to the IFG range, but was observed within the normal and IGT range of 2hPG.

Previous studies addressing this question have been considerably smaller in size (78, 89-92), but some of them have shown similar trends in changes of insulin secretion and insulin sensitivity with increasing glycemia (89, 90, 92). In one study (89), a decrease in insulin sensitivity (measured by clamp) with higher 2hPG levels within the NGT group was associated with higher BMI, suggesting that the observed effect is caused by obesity. In contrast, we found that insulin sensitivity decreased comparably in both non-obese and obese individuals within the non-diabetic range of 2hPG, suggesting that obesity does not affect insulin sensitivity related to hyperglycemia. Changes in early-phase and total insulin secretion with increasing glycemia were also independent of obesity in our study. A previous study demonstrated that the dynamic aspects of beta-cell response to glucose were unaltered in morbidly obese non-diabetic subjects (215).

Hyperglycemia categorized to IFG and IGT

We demonstrated that peripheral insulin resistance (Matsuda ISI) was a predominant feature of IIGT, whereas impairment in early and total insulin release during an OGTT characterized IIFG. This finding provides support for the notion that IFG and IGT result from distinct metabolic abnormalities.

Results of previous studies have been inconsistent with respect to differences in insulin sensitivity between IIFG and IIGT. Lower peripheral insulin sensitivity in subjects with IIGT compared with subjects with IIFG has been reported in some studies using the clamp method or IVGTT (74, 75, 77, 79), but similar impairment in insulin action has also been found in both IIFG and IIGT (78, 81). In two studies the decrease in insulin sensitivity in IIGT compared with IIFG was related to obesity (76, 82). In our study, the decrease in insulin sensitivity was significantly greater in IIGT than in IIFG. The decrease was quite similar in non-obese and obese subjects with IIGT, indicating that the reduction in peripheral insulin sensitivity in IIGT was not explained by obesity.

Conflicting findings have also been published on 1/HOMA-IR, reflecting mainly hepatic (but also peripheral) insulin sensitivity (76, 77, 79, 83-88). In our study 1/HOMA-IR was more reduced in IIFG than in IIGT. However, reliable results on hepatic insulin sensitivity can be obtained only by using the tracer techniques (78, 216).

Examination of insulin secretion revealed that individuals with IIFG had impairment in both early-phase and total glucose-stimulated insulin

release, whereas individuals with IIGT had increased total insulin release. Some previous studies assessing insulin secretion by IVGTT or clamp have similarly reported impaired insulin release in individuals with IIFG (74, 75, 78, 79, 81), although others have found impaired insulin release in individuals with IGT (76, 80). Studies based on OGTT measurements have reported impaired early-phase insulin secretion (the insulinogenic index) in individuals with IGT only (86, 88), or in individuals with IGT or IFG (77, 83, 87). The inconsistencies in the findings from previous studies could be due to different study designs, different methods, and most importantly a small sample size.

Our findings are supported by those of a recent large prospective study investigating the natural history of prediabetes. This study has demonstrated that individuals who progressed within 5 years from NGT to IIFG displayed stationary beta-cell failure and progressive hepatic insulin resistance, whereas those who progressed from NGT to IIGT had low insulin sensitivity with inadequate compensatory insulin secretory response (217).

Summary

Changes in insulin sensitivity and insulin secretion in response to increasing glucose levels (regarded as a continuous trait or as categories of glucose tolerance) in our study suggest that there are two major pathways for the development of type 2 diabetes. One pathway leads to diabetes via the elevation of FPG (IFG), most probably due to an insulin secretion defect, and another via postprandial hyperglycemia (IGT), most probably due to insulin resistance. This hypothesis is supported by our finding that subjects with postprandial hyperglycemia within the non-diabetic range (or IGT) displayed a compensatory increase in total (but not early-phase) insulin secretion, whereas in subjects with fasting hyperglycemia (or IFG) this compensatory hypeinsulinemia was entirely missing. Moreover, insulin release (early-phase and total) linearly decreased with increasing FPG levels, indicating a defect in insulin secretion. Prospective follow-up studies including subjects with IIFG and IIGT are needed, however, to confirm this hypothesis.

6.3 TYPE 2 DIABETES SUSCEPTIBILITY LOCI *(Studies II-IV)*

Although recent advances in the genetics of type 2 diabetes have led to the identification of 20 diabetes risk SNPs, little is known about their function and the mechanisms whereby they increase the risk of diabetes. We investigated a total of 18 diabetes-risk SNPs in two populations for their associations with insulin secretion, insulin resistance, and proinsulin conversion.

6.3.1 Loci associated with insulin secretion

Insulin secretion has an important genetic component, as suggested by twin studies reporting heritability estimates >50% (99,218). We found that out of 18 diabetes risk loci, eight loci (*TCF7L2, SLC30A8, HHEX, CDKN2B, CDKAL1, MTNR1B, KCNJ11,* and *IGF2BP2*) were associated with impaired insulin release. This finding confirms the importance of the genes regulating insulin secretion in determining the genetic susceptibility to type 2 diabetes.

6.3.1.1 CDKAL1

CDKAL1 was identified by GWAS as a susceptibility gene for type 2 diabetes (109-112). We replicated the association of *CDKAL1* rs7754840 with type 2 diabetes in the initial sample of 3900 Finnish men from the METSIM study. Furthermore, we demonstrated in two studies that the type 2 diabetes risk allele of rs7754840 was significantly associated with impaired early-phase insulin secretion measured either directly by an IVGTT (EUGENE2 study) or estimated from an OGTT (METSIM Study), but not with insulin sensitivity (measured by clamp or OGTT). The association remained significant after adjustment for covariates including BMI and insulin sensitivity.

An association of *CDKAL1* variants with insulin release during an OGTT was first found in two GWA studies (110, 112), and replicated in several subsequent studies (159, 160, 172, 219, 220). However, the OGTT applied in the initial studies does not allow the accurate estimation of either the first- and second-phase insulin secretion, or insulin sensitivity. We showed that the effect of rs7754840 on insulin secretion was mainly due to impaired first-phase insulin release. Several prospective studies have indicated that impaired first-phase insulin secretion is an independent predictor for the progression from NGT or IGT to type 2 diabetes (221, 222).

The mechanisms underlying the association of rs7754840 with impaired insulin secretion are not clear. Considering the similarity of the *CDKAL1*

protein product and CDK5RAP1 (CDK5-inhibitor) in the brain, it is possible that *CDKAL1* is involved in CDK5-mediated regulation of beta-cell function. Inhibition of CDK5 activity seems to have a positive impact on insulin gene expression and secretion during glucotoxic conditions (223). However, further studies are needed to fully elucidate the function of *CDKAL1* in CDK5-mediated pathways in pancreatic beta-cells.

6.3.1.2 HHEX

Two SNPs near the *HHEX* gene, rs1111875 and rs7923837 (both in strong LD), have been associated with increased risk of type 2 diabetes in GWAS (108-111). Moreover, rs1111875 has been shown in initial studies to affect the acute insulin response during an OGTT (166) and β-cell glucose sensitivity (219). We found an association of the type 2 diabetes risk allele in rs7923837 with impaired first-phase insulin release during IVGTT in the EUGENE2 study. Furthermore, we confirmed the association of rs1111875 with early-phase insulin secretion during an OGTT in the METSIM study, and this SNP had the largest effect size (-6.7% per diabetes risk allele) among 18 type 2 diabetes susceptibility SNPs tested. These associations remained significant after adjustment for covariates and correction for multiple testing. In agreement with our observations, several studies have also reported an association of *HHEX* with impaired insulin secretion measured by OGTT or IVGTT (155, 160, 166, 219, 220, 224).

In spite of the growing evidence that *HHEX* affects insulin secretion, the mechanisms behind this association remain unclear. HHEX is a transcription factor highly expressed in pancreatic islets, necessary for embryonic formation of the ventral pancreas (152). Therefore, the defect in glucose-stimulated insulin release associated with rs1111875 could arise from mild alterations in the embryonic organogenesis of the ventral pancreas. It is noteworthy that the signal tagged by rs1111875 is a region of extended linkage disequilibrium that includes *IDE* (insulin degrading enzyme) and *KIF11* (kinesin-interacting factor 11) genes, which could also be potential candidates.

6.3.1.3 Other loci

In addition to *CDKAL1* and *HHEX*, *TCF7L2, SLC30A8, CDKN2B, MTNR1B, KCNJ11,* and *IGF2BP2* loci were also associated with impaired early-phase insulin release in the METSIM study, although only associations of *TCF7L2,*

SLC30A8, CDKN2B, and *MTNR1B* remained significant after correction for multiple testing.

TCF7L2 is the most important candidate gene for type 2 diabetes to date, confirmed in many populations. Since its discovery, several studies have reported associations of *TCF7L2* variants (especially rs7903146) with impaired insulin response to glucose during an OGTT or IVGTT (135, 160, 225-227). Our results are in agreement with those of these reports. *TCF7L2* is known to play a crucial role in the WNT signalling pathway, which is required for beta-cell growth, differentiation and function. Moreover, it is also important for the regulation of GLP-1 expression and secretion in intestinal L cells. Schäfer et al. showed that variations in *TCF7L2* are associated with impaired GLP-1-induced insulin secretion (228), so it is likely that *TCF7L2* variants affect beta-cell function both directly and indirectly through impaired GLP-1 secretion or signalling.

MTNR1B is the most recent candidate gene for type 2 diabetes, found to be strongly associated with the risk of type 2 diabetes, higher FPG levels, and lower basal insulin secretion measured by HOMA-B in a GWA meta-analysis (MAGIC) (117). Subsequent reports found an association of type 2 diabetes risk allele in rs10830963 with decreased early insulin secretion during an OGTT and IVGTT (201, 229). These results are in agreement with our finding. The relationship between the *MTNR1B* variant and insulin secretion seems to be biologically credible, since *MTNR1B* is expressed in the beta cells, and is thought to mediate an inhibitory effect of melatonin on insulin secretion (200). Moreover, melatonin receptors are overexpressed in the islets of patients with type 2 diabetes (230).

The effects of the SNPs in or near *KCNJ11*, *SLC30A8*, *CDKN2B*, and *IGF2BP2* on insulin secretion in the METSIM Study were more modest in size (<5% reduction per risk allele). Previous studies assessing the effects of these loci on the measures of insulin secretion have been inconclusive (112, 127, 155, 160, 172, 220, 224, 231), most probably due to insufficient power to detect modest effects of these SNPs. The mechanisms of action of these SNPs are also mostly a matter of speculation (*CDKN2B*, *SLC30A8*) or are unknown (*IGF2BP2*). *CDKN2B*, similarly as *CDKAL1*, plays a role in the regulation of the cell cycle, suggesting a possible link between cell cycle regulation and beta-cell function. On the other hand, the effect of *KCNJ11* rs5219 (Glu23Lys) on insulin secretion is biologically plausible, since *KCNJ11* encodes the Kir6.2 subunit of the K_{ATP} channel, which is necessary for insulin secretion. Experimental studies

suggest that rs5219 causes an insulin secretion defect through a small increase in K_{ATP} channel activity (232, 233).

A few studies have reported associations of variants in *WFS1* (234), *TSPAN8* (235), *JAZF1* (235), *CDC123* (235), *LOC387761* (231), and *KCNQ1* (116) loci with insulin secretion, but our study failed to confirm such associations.

6.3.1.4 Combined effect of 8 SNPs on insulin secretion

When type 2 diabetes risk alleles in the 8 insulin secretion-related SNPs (*TCF7L2, SLC30A8, HHEX, CDKN2B, CDKAL1, MTNR1B, KCNJ11,* and *IGF2BP2*) were combined, we observed a gradual decrease in early-phase insulin secretion during an OGTT with an increasing number of risk alleles, reaching -32% in subjects with ≥11 compared with subjects with ≤3 risk alleles. An observation similar to our results was reported in a study by Pascoe et al. (225), where carriers of 9 or more risk alleles in 7 genes exhibited 21.8% lower insulin secretion (assessed by the insulinogenic index), and 26.6% lower glucose sensitivity of beta-cells, than carriers of 4 or less risk alleles.

6.3.2 Loci associated with proinsulin conversion

A total of four insulin secretion-related loci (*TCF7L2, SLC30A8, HHEX,* and *CDKAL1*) were also associated with indices of proinsulin conversion in the METSIM Study, suggesting that these loci may affect insulin secretion, at least partially, through impaired proinsulin conversion.

An association of *TCF7L2* rs7903146 with proinsulin levels (236, 237) or the proinsulin/insulin ratio (238, 239) has been previously reported. Although the mechanisms behind this association are not clear, impaired glucagon-like peptide 1 signaling seems to be involved (228). Moreover, binding sites for *TCF7L2* have been found in the promoters of genes encoding proprotein convertase 1 and 2 (237), supporting this mechanism.

The association of *SLC30A8* rs13266634 and *CDKAL1* rs7754840 with the proinsulin/insulin AUC ratio during an OGTT was also shown in a recent study (160). Since *SLC30A8* encodes the zinc transporter ZnT8, which plays an important role in the storage and maturation of insulin in the granules of the beta-cells (240), there is a possibility that genetic variants affecting the function of ZnT8 could impair proinsulin processing. However, currently it is not known whether rs1326634 (Arg325Trp) SNP affects the functional properties of ZnT8.

Our finding of the association between the *HHEX* variant and impaired proinsulin conversion has not previously been reported, and the mechanism behind this association is not known. On the other hand, one study has reported an association between *MTNR1B* rs10830963 and the proinsulin/insulin ratio (201), which we could not confirm in our study. It is noteworthy that the increased proinsulin/insulin ratio does not necessarily represent a specific defect in proinsulin processing, as proinsulin concentrations rise under most conditions of stressed beta-cells. Therefore, these findings require further investigation.

6.3.3 Loci associated with insulin sensitivity

It is well accepted that insulin resistance is mainly modulated by lifestyle factors, such as a lack of physical activity and diet rich in carbohydrates and saturated fatty acids, leading to obesity. However, twin studies have shown that the genetic component of insulin resistance is also significant, although less than that of insulin secretion. The estimated heritability of insulin resistance is ~40% (241).

Out of 18 type 2 diabetes risk loci, *KCNJ11*, *HHEX*, and *TSPAN8* were nominally associated with peripheral insulin resistance (Matsuda ISI) in the METSIM Study. The associations did not persist after the correction for multiple testing. Surprisingly, we did not confirm an association of *PPARG* with Matsuda ISI, the only diabetes risk gene known to affect insulin sensitivity to date, although a trend for the association was present. Most of the previous studies reporting the association between *PPARG2* rs1801282 (Pro12Ala) and insulin resistance have applied HOMA-IR, which reflects mainly insulin resistance in the liver (120, 242-248). *PPARG* isoform 2, bearing the Pro12Ala polymorphism, is expressed prominently in adipose tissue and to a lower extent in the liver (249), but not in skeletal muscle. Therefore it is possible that Pro12Ala affects insulin sensitivity predominantly in adipose tissue and the liver, whereas Matsuda ISI representing mostly muscle insulin sensitivity might not reflect sufficiently insulin sensitivity in these tissues. Interestingly, mice with deleted PPARγ in adipose tissue display insulin resistance in adipose tissue and the liver, but not in muscle (250), which is consistent with our hypothesis.

Type 2 diabetes risk alleles of *KCNJ11* rs5219 and *HHEX* rs1111875 were, surprisingly, nominally associated with greater sensitivity in our study. Although a similar observation has not been reported for *HHEX*, a recent

study has shown that homozygous carriers of type 2 diabetes risk allele of rs5219 of *KCNJ11* had, besides an insulin secretion defect, a ~40% increase in liver insulin sensitivity measured by clamp and tracer infusion (251). Therefore, increased insulin sensitivity in non-diabetic carriers of the risk alleles might reflect a compensation for impaired insulin secretion.

The association of *TSPAN8* rs7961581 with Matsuda ISI became nominally significant only after adjustment for obesity (BMI). Only one study has reported a similar nominal association of rs7961581 with Matsuda ISI and HOMA-IR (252). *TSPAN8* encodes for a widely expressed cell surface glycoprotein tetraspanin 8, and its role in the pathogenesis of diabetes is unclear.

A few studies have also found an association between *TCF7L2* variants and insulin sensitivity (140, 253, 254). However, we could not confirm such an association.

6.3.4 Loci with unknown function

We did not find any associations with indices of insulin secretion, insulin sensitivity and proinsulin conversion for 8 of 18 loci (*LOC387761*, *WFS1*, *JAZF1*, *CDC123*, *THADA*, *ADAMTS9*, *NOTCH2*, and *KCNQ1)*. This might be due to the insufficient power of the METSIM Study to detect potentially very modest effects of these SNPs on the measured parameters, indicating that even larger studies are needed to clarify their mode of action. There is also a possibility that mechanisms other than impaired insulin secretion, proinsulin conversion, and peripheral insulin resistance (such as tissue-specific insulin sensitivity of the liver, adipocytes, and brain, glucagon and incretin secretion, etc.) explain their associations with the diabetes risk.

6.4 CONCLUDING REMARKS

Investigation of the pathophysiology and genetics of type 2 diabetes has attracted considerable interest for many years. However, most progress in both fields has been made only recently, due to the availability of larger, well-powered study populations, and more precise and/or efficient phenotyping methods.

Although both impaired insulin secretion and insulin resistance play indisputably important roles in the development of prediabetes and type 2

diabetes, recent studies (including this work) suggest that insulin secretion is probably more important than insulin resistance. According to our findings, a reduction of insulin sensitivity by as much as 50% did not lead to diabetic hyperglycemia, whereas the reduction of early-phase secretion by >25% (and a much smaller reduction in total insulin secretion) increased fasting and 2-hour glucose levels into the diabetic range. Nevertheless, type 2 diabetes could be developed *via* at least two distinct pathways: one leading to diabetes through an increase in FPG, where an insulin secretion defect plays a crucial role, and another leading to diabetes through an increase in 2hPG, where insulin resistance is likely to play a dominant role. This hypothesis could be tested in a longitudinal large population-based study.

Results from the genetic studies of type 2 diabetes (including this work) also confirm the importance of insulin secretion in the pathogenesis of type 2 diabetes, since most of the confirmed type 2 diabetes risk loci were shown to affect insulin secretion. On the other hand, with the exception of *PPARG2,* no convincing candidate gene for insulin resistance was found. This lack of "insulin-resistance genes" might indicate that environmental and lifestyle factors rather than genetic variations are central in determining a common type of insulin resistance.

In spite of the considerable progress in studies of the genetics of type 2 diabetes, gaps in our knowledge remain. For example, the diabetes-susceptibility SNPs discovered to date explain only 5–10% of the variation of genetic risk of type 2 diabetes (255), and therefore many more variants are expected to be discovered. Studies on the copy number variation and rare mutations especially could bring new information. Moreover, many of the SNPs identified so far are located far (even hundreds of kilobases) from the known genes and do not necessarily represent the causal variants, but only a signal from a genomic region associated with type 2 diabetes. Therefore, further investigation of these loci (including fine-mapping and functional studies) is necessary to clarify their roles in the pathophysiology of type 2 diabetes.

In conclusion, the findings of our studies contribute new knowledge on the pathophysiology and genetics of type 2 diabetes. Future progress in this field could provide information enabling better prediction of an individual's risk of type 2 diabetes and individually tailored lifestyle modification programs and pharmacological therapy to prevent or treat type 2 diabetes.

7. Summary

The main findings of Studies I-IV were:

Study I: The impairment of peripheral insulin sensitivity starts at relatively low fasting and 2-hour glucose levels, already within the normoglycemic range. In contrast, the impairment of insulin secretion progresses substantially only in the diabetic range of fasting and 2-hour glucose levels. Peripheral insulin resistance is the predominant feature of IIGT, whereas impaired insulin secretion characterizes IIFG.

Study II: CDKAL1 rs7754840 was associated with type 2 diabetes in Finnish men, and with impaired first-phase insulin release in young non-diabetic offspring of type 2 diabetic patients.

Study III: HHEX rs7923837 was associated with impaired first-phase insulin release in young non-diabetic offspring of type 2 diabetic patients.

Study IV: From a total of 18 type 2 diabetes-related loci, eight loci were significantly (*TCF7L2, SLC30A8, HHEX, CDKN2B, CDKAL1* and *MTNR1B*) or nominally (*KCNJ11*, and *IGF2BP2*) associated with impaired early-phase insulin release during an OGTT. The effects of *TCF7L2, SLC30A8, HHEX*, and *CDKAL1* on insulin secretion could be explained, at least in part, by impaired conversion of proinsulin to insulin. *HHEX, KCNJ11* and *TSPAN8* were nominally associated with the Matsuda index of peripheral insulin sensitivity.

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