DISSERTATIONS IN
HEALTH
SCIENCES

Jagadish Vangipurapu

Liver Insulin Resistance and Hyperglycemia

Genetic Determinants and Association with Cardiovascular Risk Factors

Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF
EASTERN FINLAND

JAGADISH VANGIPURAPU

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on Friday, the 24th of August 2012, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
121

Department of Medicine, Institute of Clinical Medicine
School of Medicine, Faculty of Health Sciences
University of Eastern Finland
Kuopio University Hospital
Kuopio
2012

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Distributor:
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P.O.Box 1627
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ISBN: 978-952-61-0843-8 (print)
ISBN: 978-952-61-0844-5 (PDF)
ISSN: 1798-5706 (print)
ISSN: 1798-5714 (PDF)
ISSNL: 1798-5706

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Vangipurapu, Jagadish
Liver insulin resistance and hyperglycemia: Genetic determinants and association with cardiovascular risk factors. 104 p.
University of Eastern Finland, Faculty of Health Sciences, 2012
Publications of the University of Eastern Finland. Dissertations in Health Sciences 121.

ISBN: 978-952-61-0843-8 (print)
ISBN: 978-952-61-0844-5 (PDF)
ISSN: 1798-5706 (print)
ISSN: 1798-5714 (PDF)
ISSNL: 1798-5706

ABSTRACT

The prevalence of type 2 diabetes is increasing alarmingly worldwide and is attributable to an increase in the prevalence of obesity and a sedentary lifestyle. The two major pathophysiological features of type 2 diabetes are impaired insulin secretion and insulin resistance. Impaired insulin secretion is mainly due to pancreatic beta-cell dysfunction or/and decreased beta-cell mass. Insulin resistance is observed predominantly in skeletal muscle, liver and adipose tissue. Insulin resistance in these tissues is the underlying cause of several metabolic disturbances, and plays a critical role not only in the pathogenesis of type 2 diabetes, but also in the development of cardiovascular disease (CVD). Genome-wide association studies have identified several gene loci associated with type 2 diabetes and hyperglycemia. Most of these loci are known to be associated with insulin secretion and only a few with insulin resistance. The main objective of this study was to investigate the role of insulin resistance in insulin sensitive tissues, especially in the liver, in non-diabetic participants.

A novel surrogate index for hepatic insulin resistance, Liver IR index, was developed. Unlike other markers that include only insulin and glucose measurements, Liver IR index takes into consideration several metabolic traits related to hepatic insulin resistance, e.g. BMI, fat mass and high-density lipoprotein cholesterol levels. This index strongly correlates with the direct measure of hepatic insulin resistance based on the clamp technique with tracers. It was found that type 2 diabetes risk loci, rs1111875 of *HHEX* and rs5219 of *KCNJ11*, were significantly associated with increased hepatic insulin sensitivity. The combined effect of five type 2 diabetes risk loci on liver insulin resistance was <2%. Therefore, the effect of increased insulin sensitivity of *HHEX* and *KCNJ11* loci on liver insulin sensitivity seems to be minor and might be compensatory for their effects on the impairment of insulin secretion. The Pro12Pro genotype of *PPARG* was associated with decreased adipose tissue insulin sensitivity. It was also noted that insulin resistance, particularly in the liver, plays a major role in the adverse changes in the CVD risk factors, mainly those related to dyslipidemia (total triglycerides, apolipoprotein B

and total cholesterol levels) and low-grade inflammation. Finally, several single nucleotide polymorphisms associated with fasting hyperglycemia, indicating that these loci mainly regulate insulin release (basal: *G6PC2*, *MTNR1B*, and *DGKB*; glucose-stimulated: *MTNR1B*, *TCF7L2*, *CRY2*, *FADS1*, *GCK* and *SLC30A8*) and/or proinsulin processing (*MADD*, *TCF7L2*, *SLC30A8* and *GCKR*). Only one locus (*GCKR*) was associated with insulin sensitivity.

National Library of Medical Classification: WG 120, WI 702, WK 810, WK 820, WK 880

Medical Subject Headings: Cardiovascular Diseases; Diabetes Mellitus, Type 2; Risk Factors; Insulin Resistance; Liver; Adipose Tissue; Hyperglycemia; Blood Glucose; Genes; Genetic Loci; Dyslipidemias; Inflammation; Polymorphism, Single Nucleotide

Acknowledgements

This study was performed in the Department of Internal Medicine, University of Eastern Finland and the Kuopio University Hospital.

With due respect, I would like to express my deepest gratitude towards my principal supervisor, Prof. Markku Laakso. His guidance and suggestions have been extremely significant in shaping the current study. His expertise in the field of human genetics and medicine is unassailable. His excellent planning skills, undeterred focus and zeal are an inspiration. I express my heartfelt thanks towards him for providing me this opportunity to pursue my doctoral studies under his supervision.

I sincerely extend my thanks to Prof. Johanna Kuusisto for being my second supervisor and providing me her support and advice during my studies.

I wish to thank Prof. Giorgio Sesti and Prof. Norbert Stefan for reviewing my thesis and providing encouraging comments. Additionally, I would also thank Dr. Ewen Mac Donald for his suggestions in improving the thesis linguistically.

I am thankful to Dr. Alena Stancakova and acknowledge her support for helping me develop the basic skills and knowledge in statistics and genetics of type 2 diabetes. Her professional know-how and problem-solving skills are admirable. I am also grateful to all other co-authors and collaborators involved in the study for their helpful advice.

I further express my sincere thanks to my close colleagues and friends – Mr. Nagendra Yaluri, who provided his friendly support from my early days in Kuopio, Mr. Shalem Raju Modi, in whom I found a long-lasting companion, and Mr. Niyas K Saleem, with whom I shared several nice coffee-breaks. Thanks to all of you for the interesting discussions and all your support during this time.

It is my pleasure to especially thank Mr. Teemu Kuulasmaa, who was always ready to help me with any kind of computer related issues. I am thankful to Mr. Jussi Paananen for his helpful suggestions and tips in fulfilling my bioinformatics related tasks.

I further would like to acknowledge my new colleagues and friends – Dr. Henna Cederberg, your arrival to the department has filled up new energy; Mr. Yuvaraj Mahendran – sharing office with you has been a pleasure; Mr. Maykel Lopez

Rodriguez and Ms. Fatemeh Rostami – short conversations with you have been exciting. I additionally would like to thank Dr. Martin Javorsky and Dr. Jianjun Wang, the former researchers in our laboratory.

I wish to express my gratitude towards all the staff of the Department of Internal Medicine. More importantly, I will be indebted to Ms. Seija Laitinen and Ms. Leena Uschanoff, for always being eager to take us in and around Kuopio and exchanging cultural aspects. A special thanks to Ms. Raija Raisanen, Ms. Katja-Kostian Kokko and Ms. Eeva Oittinen for handling all administrative issues which were always hassle-free. I am sure that I missed out several important friends and well-wishers here in Kuopio and also back at home, but I wish to thank each one of you individually for your support.

With utmost respect and love, I dedicate this thesis to my parents and I would like to heartily thank them for their continuous encouragement. I am indebted to all my family members, particularly my brother and sister for their support in helping me pursue my studies, so far away from home. I would personally like to thank my wife, for extending her unconditional love and I greatly appreciate the support she provided in all possible ways during this process of completing my thesis.

I acknowledge the Graduate School - Doctoral Program in Molecular Medicine (DPMM), UEF and North Savo Regional Foundation for providing me the necessary funding without which the study would have been impossible.

Jagadish Vangipurapu

Kuopio, 2012

List of original publications

I **Vangipurapu J***, Stančáková A*, Kuulasmaa T, Paananen J, Kuusisto J; EGIR-RISC Study Group, Ferrannini E, Laakso M. A novel surrogate index for hepatic insulin resistance. *Diabetologia 54:540-543, 2011*

II **Vangipurapu J***, Stančáková A*, Pihlajamäki J, Kuulasmaa TM, Kuulasmaa T, Paananen J, Kuusisto J, Ferrannini E, Laakso M. Association of indices of liver and adipocyte insulin resistance with 19 confirmed susceptibility loci for type 2 diabetes in 6,733 non-diabetic Finnish men. *Diabetologia 54:563-571, 2011*

III **Vangipurapu J**, Stančáková A, Kuulasmaa T, Soininen P, Kangas AJ, Ala-Korpela M, Kuusisto J, Laakso M. Association between liver insulin resistance and cardiovascular risk factors. *J Inter Med. Apr 5, 2012* [*Epub ahead of print*]

IV Stančáková A, **Vangipurapu J**, Jackson AU, Bonnycastle LL, Morken MA, Paananen J, Collins FS, Boehnke M, Kuusisto J, Laakso M. Association of 16 SNPs Increasing Fasting Glucose Level with Indices of Insulin Release, Proinsulin Conversion, and Insulin Sensitivity in 8,750 Non-diabetic Finnish Men. *Manuscript*

*equal contribution

Some unpublished results are also presented

Contents

Abbreviations

ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8
ADAMTS9	A disintegrin and metalloproteinase with thrombospondin motifs 9
ADCY5	Adenylate cyclase 5
ADP	Adenosine diphosphate
ADRA2A	Adrenergic alpha-2A receptor
Akt	Protein kinase B
ALT	Alanine aminotransferase
ATP	Adenosine triphosphate
BCL11A	B-cell CLL/lymphoma 11A
BMI	Body mass index
C/EBP	CCAAT/enhancer-binding protein
C2CD4B	C2 calcium-dependent domain containing 4B
CAMK1D	Calcium/calmodulin-dependent protein kinase 1 D
CAPN10	Calpain 10
CDC123	Cell division cycle 123 homolog
CDKAL1	Cyclin-dependent kinase 5 regulatory sub-unit associated protein 1-like 1
CDKN2	Cyclin-dependent kinase inhibitor 2
CENTD2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1 (ARAP1)
CHCHD9	Coiled-coil-helix-coiled-coil-helix domain containing 2 pseudogene 9
CRP	C-reactive protein
CRY2	Cryptochrome 2
CVD	Cardiovascular disease
DGKB	Diacylglycerol kinase beta
DUSP9	Dual specificity phosphatase 9
EGIR	European group for study of insulin resistence
EGP	Endogenous glucose production
FADS	Fatty acid desaturase
Foxa2	Forkhead box A2
Foxo1	Forkhead box O1
FPI	Fasting plasma insulin
G6PC2	Glucose-6-phosphatase catalytic 2
GCK	Glucokinase
GCKR	Glucokinase regulator
GIPR	Gastric inhibitory polypeptide receptor
GLIS3	GLIS family zinc finger 3
SLC2A4	Glucose transporter 4
GRS	Genetic risk score

GWA	Genome-wide association
HDL	High-density lipoprotein
HHEX	Hematopoietically expressed homeobox
HMGA2	High mobility group AT-hook 2
HNF1A	Hepatocyte nuclear factor 1 homeobox A
HNF1B	Hepatocyte nuclear factor 1 homeobox B
HOMA-B	Homeostasis assessment of insulin secretion
HOMA-IR	Homeostasis assessment of insulin resistance
IDE	Insulin degrading enzyme
IDL	Intermediate-density lipoprotein
IFG	Impaired fasting glucose
IGF1	Insulin like growth factor 1
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGT	Impaired glucose tolerance
IL6	Interleukin 6
IR	Insulin resistance
IRS	Insulin receptor substrate
JAZF1	Juxtaposed with another zinc finger gene 1
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
KLF14	Kruppel-like factor 14
LDL	Low-density lipoprotein
MADD	MAPK activating death domain
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK	Mitogen-activated protein-kinase
METSIM	Metabolic Syndrome in Men
MODY	Maturity onset diabetes of the young
mRNA	messenger ribonucleic acid
MTNR1B	Melatonin receptor 1B
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-essential fatty acids
NGT	Normal glucose tolerance
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NOTCH2	Notch homolog 2
PDK1	Pyruvate dehydrogenase kinase, isozyme 1
PDX1	Pancreatic and duodenal homeobox 1
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate

PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PRC1	Protein regulator of cytokinesis 1
PROX1	Prospero homeobox 1
RBMS1	RNA binding motif, single stranded interacting protein 1
RISC	Relationship between insulin sensitivity and cardiovascular disease
SE	Standard error
SH2	SRC homology 2
SLC2A2	Solute carrier family 2 member 2
SLC30A8	Zinc-transporter 8
SNP	Single nucleotide polymorphism
SREBP1c	Sterol regulatory element binding protein-1c
TCF7L2	Transcription factor 7-like 2
THADA	Thyroid adenoma associated
TNF-α	Tumour necrosis factor-α
TP53INP1	Tumor protein p53 inducible nuclear protein 1
TSPAN8	Tetraspanin 8
WFS1	Wolfram syndrome 1
WHR	Waist hip ratio
VLDL	Very-low density lipoprotein
VPS13C	Vacuolar protein sorting 13 homolog C
ZBED3	Zinc finger, BED domain-containing 3 protein
ZFAND6	Zinc finger, AN1-type domain 6

1. Introduction

Diabetes mellitus is a chronic disease currently affecting an estimated 346 million people worldwide, as reported by the World Health Organisation (2011). This number is expected to grow up to 439 million adults by 2030 [1]. The prevalence of type 2 diabetes has risen alarmingly in the recent past owing to the trends towards increasing obesity and adoption of a sedentary lifestyle, and type 2 diabetes is now being diagnosed also in children and adolescents. As a consequence, the prevalence of microvascular and macrovascular complications is increasing, leading to severe outcomes such as blindness, kidney failure and cardiovascular disease (CVD).

Several non-modifiable risk factors such as age, gender, family history, race and ethnicity increase the risk of type 2 diabetes, along with various modifiable risk factors such as obesity, physical inactivity, diet, smoking, elevated blood pressure and dyslipidemia. Identical twin pairs show a higher concordance rate for diabetes than do dizygotic twins [2,3]. The first-degree relatives of diabetic individuals have up to four times greater lifetime risk of developing diabetes compared to the general population [4]. The lifetime risk of developing type 2 diabetes is 40% for individuals with one parent with type 2 diabetes, and 70% if both parents are affected [4]. The likelihood of developing type 2 diabetes is greater in certain ethnic groups. For example, the risk of diabetes in African-Americans, Hispanics, and Native Americans is approximately 2, 2.5, and 5 times greater, respectively, than in Caucasians [5].

Disturbances in normal glucose homeostasis cause impaired glucose tolerance that further progresses to overt diabetes. One of the major pathophysiological features leading to type 2 diabetes is impaired insulin secretion. Impaired insulin secretion is observed when the pancreatic beta-cells are not able to produce enough insulin to normalize glucose levels. The beta-cells initially respond to elevated glucose levels by producing higher amounts of insulin, resulting in hyperinsulinemia. Over time, the beta-cells lose this capability and thus, insulin levels decline. Impaired insulin secretion is observed early in the natural history of diabetes and has a strong genetic basis [6,7]. This impairment in insulin secretion is attributable to defective beta-cell function or/and decreased beta-cell mass.

Insulin resistance, the other major pathophysiological feature of type 2 diabetes, is observed prominently in skeletal muscle, liver and adipose tissue. The reduced sensitivity of these tissues to insulin is observed in individuals with type 2

diabetes. Insulin resistance in these tissues is the underlying cause of several metabolic disturbances, playing a critical role not only in the pathogenesis of type 2 diabetes, but also in other complex diseases, e.g. CVD. It associates with several abnormalities in the lipid and lipoprotein levels and blood pressure, resulting in an increased risk of CVD [8]. Thus, insulin resistance is considered as an independent risk factor for atherosclerosis and CVD [8,9]. CVD is the leading cause of mortality in individuals with type 2 diabetes.

Hyperglycemia is the primary consequence of insulin resistance in combination with impaired insulin secretion. Hyperglycemia, an elevation in fasting or postprandial glucose levels, is a prediabetic state that greatly increases the risk for future diabetes and CVD [10]. It is also considered as a major initiator of many diabetic microvascular complications such as retinopathy, neuropathy and nephropathy [11]. Hyperglycemia induces cellular dysfunction and damage through a variety of mechanisms such as oxidative stress leading to alterations in the expression and function of several genes and proteins [11].

Genetic association studies can identify gene variants that predispose to complex diseases such as type 2 diabetes and CVD. Over the past few years, several genome-wide association studies have resulted in the identification of novel risk loci or polymorphisms associated with type 2 diabetes and also with other metabolic traits, including hyperglycemia [12-20]. The majority of these risk loci are associated with impaired insulin secretion, and only a few with insulin resistance.

The current study aimed to examine tissue-specific insulin resistance, mainly in the liver, and the effect of type 2 diabetes loci on tissue-specific insulin resistance and its implication in CVD in a large cohort of non-diabetic individuals. Furthermore, the study investigated the association of loci regulating fasting hyperglycemia with indices of insulin secretion and insulin sensitivity.

2. Review of the literature

2.1 PATHOPHYSIOLOGY OF TYPE 2 DIABETES

The major metabolic disturbances leading to type 2 diabetes are insulin resistance and impaired insulin secretion. There is a dynamic balance between insulin sensitivity and insulin secretion [6]. Type 2 diabetes develops when this balance is

disrupted, i.e. when the increase in insulin secretion is unable to compensate for the prevailing insulin resistance.

2.1.1 Insulin secretion

The secretion of insulin from the pancreatic beta-cells is a complex process. The most important stimulus for insulin secretion, glucose, enters the cytoplasm of the beta-cell via a facilitated glucose transporter, SLC2A2. The presence of glucose inside the cell is recognized by a glucokinase that acts as a glucose sensor. Glucose is metabolized to ATP through a series of biochemical pathways. Elevation of the ATP/ADP ratio triggers the closure of the ATP-sensitive K^+ channels, leading to cell membrane depolarization which in turn results in the opening of voltage-dependent Ca^{2+} channels causing extracellular calcium to enter into the cell that acts as a trigger of insulin exocytosis [21].

Insulin release from the pancreatic beta-cells occurs in a biphasic pattern upon induction by glucose. The first phase is an acute transient phase. This is an initial burst of insulin that occurs within a few minutes after the elevation in glucose levels. The second phase of insulin release that follows is slower and more sustained. Studies based on isolated beta-cells have revealed that the first phase burst of insulin release arises from the readily releasable pool of insulin granules present at the plasma membrane, whereas the second phase insulin secretion occurs from newly synthesized insulin granules [21-24].

Impaired insulin secretion is the major pathophysiological mechanism leading to type 2 diabetes. In hyperglycemic conditions, the beta-cells secrete increasing amounts of insulin in attempts to overcome insulin resistance but with time, insulin secretion declines. In type 2 diabetes, the first phase of insulin release is often blunted or absent and the second phase is also diminished [7,25,26]. The defect in insulin secretion mainly occurs due to decreased beta-cell mass or impaired beta-cell signaling or genetic abnormalities [27-29]. A reduction of about 20-40% in the beta-cell mass can be measured in type 2 diabetes subjects. The beta-cell mass might be reduced due to impaired neogenesis or enhanced apoptosis. Approximately 60-70% of beta-cell function is already lost in individuals with impaired glucose tolerance [30]. A genetic basis of beta-cell dysfunction has been demonstrated in studies based on first degree relatives of type 2 diabetic subjects and in twin studies [31,32]. The two incretins, GLP-1 and GIP, play a major role in stimulating insulin secretion following oral glucose administration. This potentiating effect of the

incretins is reported to be defective in type 2 diabetic subjects and their first-degree relatives [33].

Insulin is a heterodimeric protein comprising of a 21 amino acid A-chain and a 30 amino acid B-chain linked by disulfide bridges. Preproinsulin, the early precursor of insulin, is larger and comprises of 110 amino acids. It includes a 24 amino acid signal sequence and a 35 amino acid connecting peptide linking the A and B chain as shown in Figure 1.

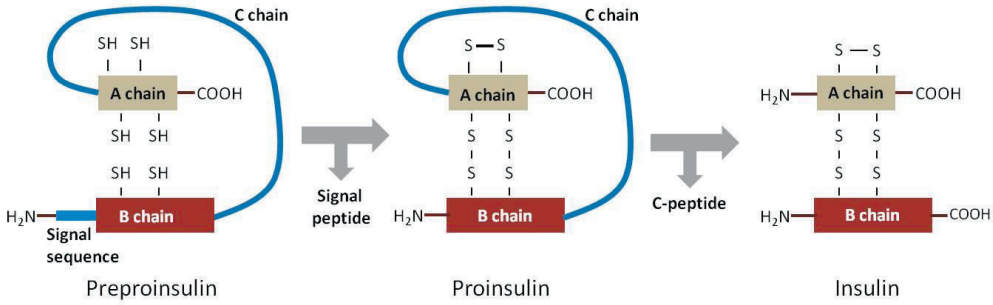

Figure 1. Precursors of insulin (adapted from [34])

Insulin is produced in the endoplasmic reticulum in its precursor form and packaged into secretory vesicles in the Golgi apparatus. The secretory granules are the major site of insulin processing. The main enzymatic machinery involves proprotein convertase 1/2 and carboxypeptidase E that convert preproinsulin into a mature heterodimeric insulin molecule and C-peptide. Only a small part (<10%) of the newly synthesized proinsulin escapes from this conversion process. Therefore, the relative proportion of proinsulin to insulin (PI/I ratio) provides an estimate for the efficiency of proinsulin processing [35].

Basal intact proinsulin levels have been shown to be higher not only in obese and non-obese subjects with type 2 diabetes but also in obese subjects without type 2 diabetes [36]. A high PI/I ratio is associated with elevated glucose levels, impaired insulin secretion, insulin resistance and increased risk of type 2 diabetes [37-40].

2.1.2 Insulin resistance

Ever since its initial identification by Himsworth (1936), who classified subjects as being either insulin sensitive or insulin insensitive [41], insulin resistance has been

recognized as a characteristic feature of a spectrum of metabolic disturbances including obesity, hypertension and type 2 diabetes. Insulin resistance is defined as a decreased responsiveness of target tissues to normal circulating levels of insulin. Studies based on euglycemic insulin clamp have provided direct quantitative evidence that the progression from normal to impaired glucose tolerance is associated with the development of severe insulin resistance.

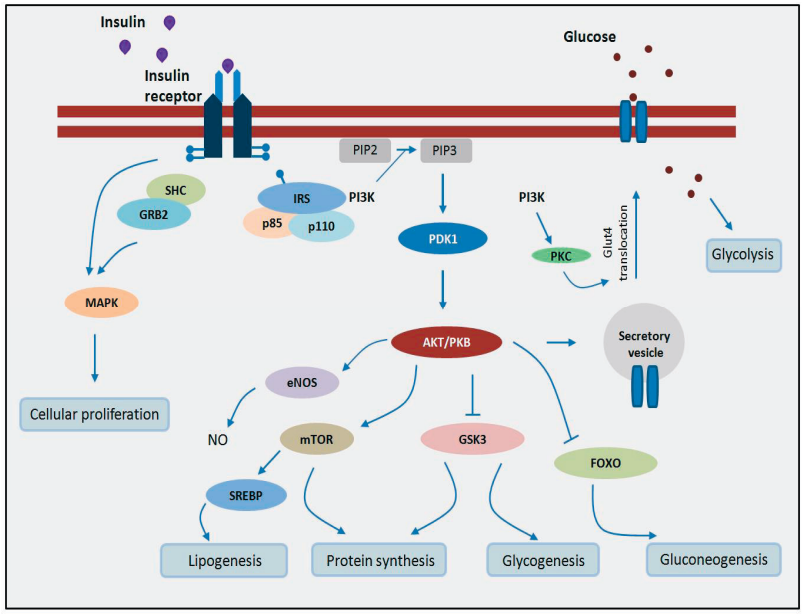

Figure 2. Insulin signaling pathway (modified from [42])

Insulin exerts its biological functions by binding to the insulin receptor, which initiates a cascade of signaling events [43], as depicted in Figure 2. The binding of insulin to the extracellular α-subunit of the insulin receptor stimulates autophosphorylation on multiple tyrosine residues in the cytoplasmic portion of the transmembrane β-subunit. This results in the activation of its intrinsic tyrosine kinase, which in turn catalyses the phosphorylation of intracellular substrates including the insulin receptor substrate (IRS). Once phosphorylated, IRS proteins bind and activate several Src homology 2 (SH2) domain proteins including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase is known to play a central role in insulin-stimulated intracellular translocation of GLUT-4 and glucose uptake in adipose tissue and skeletal muscle. Activated PI 3-kinase generates lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3). The increase

in the level of PIP3 leads to the activation of a protein kinase cascade, first stimulating the protein pyruvate dehydrogenase kinase, PDK1, which phosphorylates and activates serine/threonine kinase, Akt (also known as protein kinase B) that further interacts with several other downstream effectors.

Several defects in insulin signaling involving the IR/IRS/PI 3-kinase/PKC/Akt/SLC2A4 cascade have been detected in subjects with insulin resistance [43,44]. Thus, it is possible that decreased expression or increased degradation of these key components of insulin signaling might be responsible for the development of insulin resistance.

Inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been linked to insulin resistance [45]. A key mechanism by which cytokines such as TNF-α induce insulin resistance involves site-specific serine phosphorylation of IRS-1 [46]. Serine phosphorylation might induce the dissociation of IRS proteins from the insulin receptor or hinder access to tyrosine phosphorylation sites of IRS proteins or induce IRS protein degradation. The insulin signaling pathway also regulates Forkhead transcription factors, mainly Foxo1 and Foxa2. In insulin resistant conditions, the constitutive activation of Foxo1 and Foxa2 results in elevated gluconeogenesis, hyperglycemia and high levels of lipid oxidation [47].

Insulin resistance and endothelial dysfunction commonly occur together and both traits can be detected early in the pathogenesis of atherosclerosis [48]. Insulin exerts its vascular effects primarily by augmenting the availability of endothelium-derived nitric oxide (NO), a potent vasodilator. In experimental conditions mimicking the state of insulin resistance, endothelial-mediated vasodilation was found to be impaired, partly due to the inability of insulin to stimulate the activity of the key enzyme responsible for NO synthesis, nitric oxide synthase (NOS), in vascular endothelial cells [48-50].

Insulin resistance is traditionally defined only by disturbances in glucose metabolism. This glucocentric view, that states that hyperglycemia is the primary disease caused by a combination of insulin resistance and beta-cell loss, is challenged by a more lipocentric view. Hyperglycemia, insulin resistance and beta-cell loss could be secondary to lipotoxicity or ectopic lipid deposition at least to some extent. Insulin resistance is the major identifiable defect in subjects at risk for type 2 diabetes and might precede its development by several years [51-53]. Insulin

resistance is also important in the development of CVD. This process can include several stages as depicted in Figure 3.

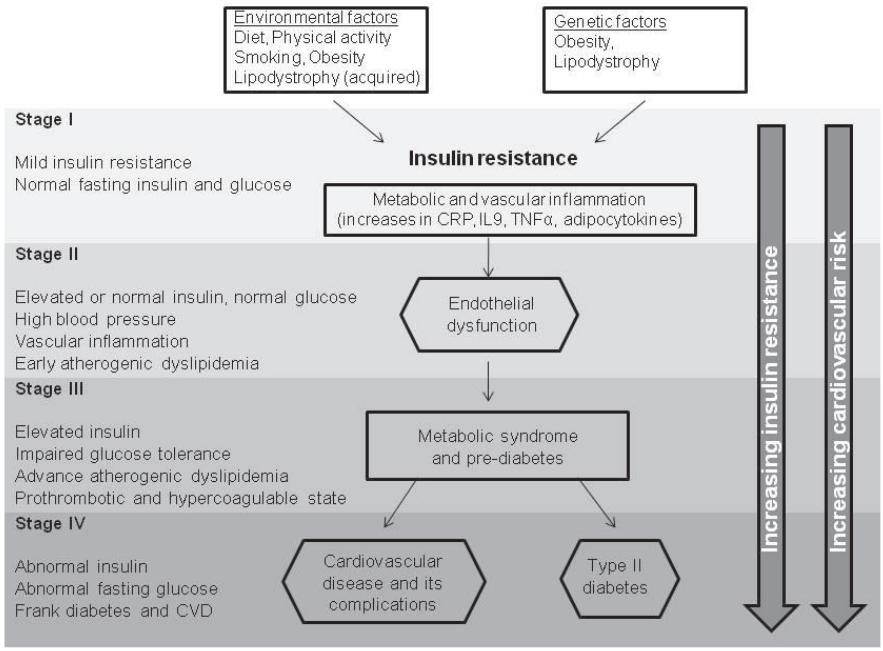

Figure 3. Different stages in the progression of insulin resistance to type 2 diabetes and cardiovascular disease (adapted from [54])

Other genetic or acquired factors that could lead to insulin resistance include mainly obesity and the presence of excess glucocorticoids as observed in Cushing's syndrome or in patients receiving other steroid therapies, or subjects with hormonal imbalances as in polycystic ovarian syndrome, and lipid accumulation in the liver.

2.2 TISSUE-SPECIFIC INSULIN RESISTANCE

Whole-body glucose homeostasis can be maintained when all the tissues are normally sensitive to insulin. Insulin resistance has tissue-specific effects that have been demonstrated in studies based on transgenic mice, e.g. insulin receptor knockout mice. Mice lacking the muscle insulin receptor alone are characterized by reduced insulin-stimulated muscle glucose uptake, but total-body glucose

homeostasis remains normal [55]. Adipose tissue insulin receptor knockout mice exhibited impaired adipose tissue glucose uptake but were protected against obesity, glucose intolerance, and dyslipidemia and manifested a prolonged lifespan. In contrast, knockout of the insulin receptor in the liver resulted in both fasting and postprandial hyperglycemia and the subsequent development of peripheral (muscle) insulin resistance [55].

In humans, skeletal muscle accounts for ~80% of the insulin-stimulated glucose uptake, liver ~15-20% and adipose tissue <5% [6]. However, in the insulin resistant state, all these tissues together contribute to the development of hyperglycemia as presented in Figure 4.

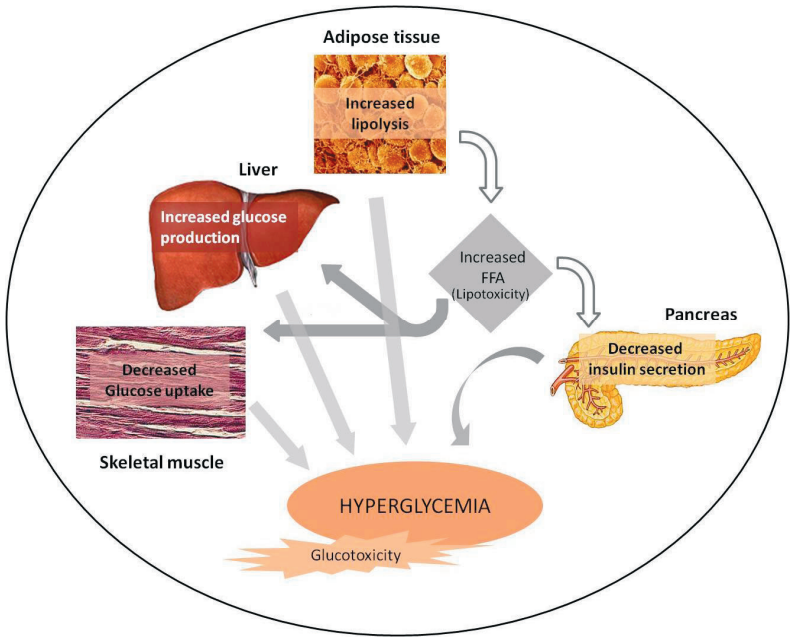

Figure 4. Major metabolic defects in tissues causing hyperglycemia (adapted from [56])

2.2.1 Skeletal muscle

Skeletal muscle tissue accounts for up to 80% of insulin-mediated glucose uptake in the post-prandial state [6]. Thus, insulin resistance in skeletal muscle contributes significantly to disturbances in glucose homeostasis, and is present even decades before beta-cell failure and overt hyperglycemia [57,58]. In agreement with this notion, even lean normal glucose-tolerant offspring of two parents with type 2 diabetes exhibit moderate to severe skeletal muscle insulin resistance [59-64].

Skeletal muscle stores 1-2% of its tissue mass as glycogen. Glycogen synthesis in the skeletal muscle is one of the primary pathways of glucose disposal. In subjects with type 2 diabetes, baseline glycogen concentrations are ~30% lower than in matched controls, indicating that the process of glycogen synthesis has been profoundly impaired [65]. During hyperinsulinemia, type 2 diabetic patients display a blunted elevation in intramyocellular glucose-6-phosphate concentrations in comparison with their age-weight matched control subjects [66], suggesting that glucose transport mediated by GLUT-4, and/or its phosphorylation, mediated by hexokinase, are the rate limiting steps in insulin-stimulated glucose disposal in skeletal muscle. Similar findings have been observed in lean, insulin-resistant offspring of type 2 diabetic patients [67]. A reduction of GLUT-4 mediated glucose transport, leading to lower insulin stimulated glycogen synthesis, is considered as the central feature in skeletal muscle insulin resistance [68].

Mitochondria are the major organelles involved in fatty acid oxidation and they are known to have a major role in energy metabolism. Normal glucose-tolerant subjects with both parents affected with diabetes, have reduced expression of key mitochondrial genes which regulate oxidative metabolism in skeletal muscle [69]. In contrast, mitochondria from normal glucose-tolerant subjects without any family history of diabetes respond to insulin by increasing ATP production by 90%, whereas mitochondria from insulin resistant offspring increase ATP production by only 5% [70-72].

In young and lean insulin resistant subjects, skeletal muscle insulin resistance leads to increased levels of plasma triglycerides and lower plasma levels of high-density lipoprotein cholesterol [73]. The energy derived from ingested carbohydrates is diverted away from muscle glycogen synthesis into increased hepatic de novo lipogenesis that might be partly due to the upregulation of the insulin-dependent sterol regulatory element binding protein-1c, SREBP1c, a master transcriptional regulator of lipogenic enzymes, thus leading to the development of atherogenic dyslipidemia [74,75].

2.2.2 Liver

The liver plays a major role in glucose homeostasis. It has a primary role in endogenous glucose production or gluconeogenesis during fasting, and storage of glucose as glycogen (glycogenesis) in the fed state, both processes being regulated by insulin. Insulin resistance in the liver results in impaired suppression of hepatic

glucose production, a trait that can be observed not only in type 2 diabetes but also in pre-diabetes [76-78].

The major contributors to endogenous glucose production (EGP) are gluconeogenesis and glycogenolysis. The dynamic equilibrium between glucose production and glucose utilization is regulated by insulin and glucagon. The liver is an important tissue for glucose homeostasis, and it is the predominant site for EGP with only a minor contribution made by the kidney (<20%). The liver is involved in directing glucose fluxes both during transition from fasting to the fed state and under pathological conditions such as diabetes mellitus. Subjects with type 2 diabetes exhibit increased endogenous glucose production, and synthesize only 25-45% of hepatic glycogen when compared with non-diabetic individuals [79]. Thus, defective regulation of glycogenolysis as well as gluconeogenesis occurs as a consequence of hepatic insulin resistance, as observed in obese non-diabetic and type 2 diabetic humans.

Insulin primarily suppresses EGP via a direct interaction with hepatic receptors or indirectly through its action on lipolysis in the fat cell. In the liver, it mediates its inhibitory effects on glucose production by inhibiting the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. In individuals with abnormal glucose tolerance, and especially in individuals with type 2 diabetes, the suppression of EGP is inadequate and contributes to both fasting and postprandial hyperglycemia [77,78].

Hyperglycemia is an important determinant of vascular complications of diabetes. Short-term fluctuations in glycemic control, acute hyperglycemia, and also chronic hyperglycemia play a key role in the pathogenesis of vascular complications associated with diabetes. Both are involved in the development of various microvascular and macrovascular complications [80]. Hyperglycemia is not only a consequence of, but also an important factor for the worsening of both insulin resistance and insulin deficiency as it results in glucose toxicity that further aggravates the pathophysiological state, mainly by increasing oxidative stress [81].

Studies that have employed isotope tracer techniques to assess visceral fat metabolism in vivo in obese subjects have shown that ~20% of non-essential fatty acids (NEFA) delivered into the liver are derived from lipolysis of visceral fat [82]. Thus, insulin resistance in the liver could be mainly due to visceral obesity and chronic inflammation associated with it. Obesity is characterized by the production of excess NEFA and inflammation is characterized by abnormal production of

cytokines and adipokines e.g., TNF-α, IL-6, leptin and resistin. Increased NEFA flux from adipose tissue to the liver also leads to dyslipidemia, mainly via increased production of very-low density lipoprotein (VLDL) particles [83].

2.2.3 Adipose tissue

Adipose tissue, mainly white adipose tissue, acts as a reservoir of energy that is stored in the form of fat. More importantly, adipose tissue is an endocrine organ that secretes several cytokines/adipokines and which modulates whole body glucose metabolism. The importance of fat tissue in glucose homeostasis has been demonstrated in fatless mouse models [84,85]. These mice are extremely insulin resistant, and introduction of fat restores their sensitivity to insulin.

The major effect of insulin on adipose tissue is to activate lipoprotein lipase that binds NEFAs and glycerol together to form triglycerides, the storage form of fat. Adipose tissue is extremely sensitive to insulin concentrations, inhibiting lipolysis at insulin concentrations that are much lower than those needed to inhibit hepatic glucose production or to stimulate muscle glucose uptake. However, in subjects with type 2 diabetes, lipolysis is resistant to insulin when compared to non-diabetic subjects [86].

It is widely accepted that adipose tissue plays a central role in the development of insulin resistance [87,88]. In insulin-resistant states, there is marked adipocyte resistance to the antilipolytic effect of insulin, resulting in the elevation of plasma NEFA levels. Fasting and postprandial NEFA levels have generally been found to be elevated in obesity and type 2 diabetes [89,90]. During nutritional excess, adipocytes become hypertrophic and develop a gene expression pattern that closely resembles that of macrophages [91], leading to the secretion of various adipokines. Thus, the overproduction of the proinflammatory cytokines such as IL-6, TNF-α and CRP and a deficiency of the anti-inflammatory cytokines like adiponectin exacerbates insulin resistance.

Recent studies have indicated that disturbances in the PPARγ and CCAAT/enhancer-binding protein (C/EBP) signaling play an important role in adipose tissue as they are essential for adequate adipogenesis, metabolic regulation, and adipose tissue insulin sensitivity [92].

2.3 IDENTIFICATION OF RISK LOCI FOR TYPE 2 DIABETES

2.3.1 Family based linkage analysis and candidate gene approach

Linkage analysis is most useful when the disease under investigation follows a monogenic form of inheritance with a large effect size. This technique is based on the use of genetic markers in a family pedigree coupled with phenotypic information to identify chromosomal regions showing a linkage with a specific trait or disease. Causative genetic variations of several monogenic forms of diabetes, e.g. maturity-onset diabetes of the young and neonatal diabetes, have been successfully identified using this approach [93].

Only two genes associated with type 2 diabetes have been reported to be identified by linkage analyses, *CAPN10* and *TCF7L2*, although the *CAPN10* locus could not be replicated in genome-wide association studies [94,95].

The candidate gene approach focuses on a specific candidate gene or a selected genetic region chosen based on a known biological function. This approach is biased in assuming that a specific locus causes a disease based on prior knowledge. Many candidate genes have been investigated based on this principle. However, only a few genes for type 2 diabetes, such as *PPARG* and *KCNJ11*, have been identified with this approach [96-98].

2.3.2 Genome-wide association studies

With the advent of genome-wide association (GWA) studies, owing to the completion of Human Genome Project and Hapmap Project [99], it became possible to test hundreds of thousands of SNPs simultaneously for their association with complex diseases. This method allowed the discovery of multiple gene variants each individually having only a small effect. Almost all disease categories have been addressed, including metabolic, neurodegenerative and cardiovascular diseases and several types of cancer. The first six GWA studies for type 2 diabetes performed in 2007, reported more than 10 new genetic loci that significantly modified the risk of type 2 diabetes [12-20]. In addition, *PPARG*, *KCNJ11* and *TCF7L2* were confirmed as risk loci for type 2 diabetes in these studies.

After a series of GWA studies that reported several new loci associated with type 2 diabetes, meta-analyses combining several GWA studies were performed [16]. These studies combined several GWA scans that were based on

large populations, and thus had substantial power to detect genetic variants with small effect sizes. This approach identified several new genetic loci for type 2 diabetes.

Additional GWA studies that reported association with continuous glycemic traits, such as fasting glucose, fasting insulin, HOMA-B and HOMA-IR led to the identification of new genetic variants [18]. After completion of deep sequencing of the human genome, it might be possible to identify additional risk loci for type 2 diabetes. The various loci associated with type 2 diabetes or other glycemic traits are listed in Table 1.

2.4 GENETIC LOCI ASSOCIATED WITH TYPE 2 DIABETES OR HYPERGLYCEMIA

The variants can be broadly classified based on their effects either on beta-cell function or insulin sensitivity or loci with unknown role in type 2 diabetes, and are briefly described below.

2.4.1 Variants regulating β-cell function

ADRA2A, encodes the adrenergic alpha-2A receptor, which is a member of the G-protein coupled receptor superfamily. This gene is located on chromosome 10q24-26 and interestingly, it contains no introns. ADRA2A inhibits lipolysis and thus promotes weight gain [101]. Since Adra2a mediates adrenergic suppression of insulin secretion, *Adra2a* knockout mice exhibit enhanced insulin secretion [102], and animals with beta-cell specific overexpression of *Adra2a* are glucose-intolerant [103]. A recent study revealed that the risk allele carriers of variant rs553668, exhibited overexpression of the receptor and as a consequence, they displayed reduced insulin secretion and had an increased risk of type 2 diabetes [104].

C2CD4B, encoding C2 calcium-dependent domain containing 4B, is located on chromosome 15q22.2. The biological function of this protein coding gene is yet unknown, but it might play a role in inflammation as its expression is upregulated by cytokines [105]. Recent studies have reported the association of a SNP rs11071657 with fasting hyperglycemia and a reduced glucose-stimulated insulin response [18,106].

Table 1. Genetic variants associated with type 2 diabetes, insulin secretion, insulin resistance and other glycemic traits (modified from *Grarup et al.* [100])

Gene	Lead SNP	Risk allele frequency	Type of study	GWA trait	Other reported association with physiologic phenotypes
Variants regulating β-cell function					
ADRA2A	rs10885122	0.90	MA	FG	Reduced insulin release during OGTT
C2CD4B	rs11071657	0.59		FG	Reduced insulin release during OGTT Increased fasting proinsulin
CDC123/ CAMK1D	rs12779790	0.23	MA MA	T2D	Reduced insulin release during OGTT Reduced arginine-stimulated and second-phase glucose-stimulated insulin release during hyperglycemic clamp
CDKAL1	rs10946398	0.31	GWA	T2D	Impaired conversion of proinsulin to insulin Reduced insulin release during OGTT Reduced insulin release after IV glucose stimulation Reduced birth weight
CDKN2A CDKN2B	rs10811661	0.79	GWA	T2D	Reduced insulin release during OGTT Reduced insulin release during IVGTT
CENTD2	rs1552224	0.88	MA	T2D	Lower HOMA-B
DGKB/ TMEM195	rs2191349	0.47	MA	FG, T2D	Reduced insulin release during OGTT
FADS1	rs174550	0.63	MA	FG	Reduced insulin release during OGTT
G6PC2	rs560887	0.70	MA	FG HOMA-B HbA1c	Increased insulin release during OGTT Increased insulin release during IVGTT Increased basal hepatic glucose production Decreased risk of T2D
GCK	rs1799884	0.20	MA	FG, T2D, HbA1c	Increased glucose levels during OGTT Reduced insulin release derived from OGTT
GIPR	rs10423928	0.18	MA	2 h-G	Reduced insulin release during OGTT Increased fasting proinsulin Impaired incretin effect
GLIS3	rs7034200	0.53	MA	FG	Reduced insulin release during OGTT
HHEX	rs1111875	0.56	GWA	T2D	Reduced insulin release during OGTT
IDE	rs5015480		GWA		Borderline significant reduced insulin release during IV glucose stimulation Reduced birth weight
IGF2BP2	rs4402960	0.29	GWA	T2D	Reduced insulin release during OGTT Reduced insulin release during IVGTT Reduced insulin release after IV tolbutamide stimulation
HNF1A	rs7957197	0.85	MA	T2D	
HNF1B	rs4430796	0.43	CS	T2D	
JAZF1	rs864745	0.52	MA	T2D	Reduced insulin release derived from OGTT
KCNJ11/ ABCC8	rs5219	0.50	CS	T2D	Reduced insulin release during OGTT Increased glucagon levels during hyperglycemic clamp
KCNQ1	rs2237895	0.61	GWA	T2D	Reduced insulin release during OGTT Reduced glucose-stimulated incretin secretion
MADD	rs7944584	0.69	MA	FG	Higher fasting proinsulin
MTNR1B	rs10830963	0.30		FG, T2D, HbA1c	Reduced insulin release during OGTT Reduced insulin released during IVGTT
PROX1	rs340874	0.50	MA	FG, T2D	Reduced insulin release during OGTT

SLC30A8	rs13266634	0.75	GWA	T2D, FG	Impaired conversion of proinsulin to insulin Reduced insulin release during OGTT Reduced insulin release during IVGTT
TCF7L2	rs7901695		LA	T2D, FG, 2 h-G	Impaired conversion of proinsulin to insulin
	rs7903146	0.25			Reduced insulin release during OGTT Reduced incretin effect Reduced glucagon levels
THADA	rs7578597	0.92	MA	T2D	Reduced GLP-1—and arginine-stimulated insulin release during hyperglycemic clamp
TSPAN8	rs7961581	0.23	MA	T2D	Reduced insulin release during OGTT
WFS1	rs10010131	0.27	CS	T2D	Reduced insulin release during OGTT Reduced GLP-1 induced insulin release during hyperglycemic clamps
Variants regulating insulin sensitivity					
ADAMTS9	rs4607103	0.81	MA	T2D	Reduced insulin-stimulated glucose uptake during hyperinsulinemic-euglycemic clamp Increased glucose-stimulated insulin release
DUSP9	rs5945326	0.12	MA	T2D	
GCKR	rs780094	0.62	MA	FG, T2D, 2 h-G, triglyceride	Increased insulin resistance derived from fasting and OGTT
IGF1	rs35767	0.90	MA	FI	Increased HOMA-IR Decreased OGTT-based insulin sensitivity
IRS1	rs2943641	0.61	CS	T2D	Increased insulin resistance derived from fasting and OGTT
	rs1260326	0.40			Increased insulin-stimulated hepatic glucose output
KLF14	rs972283	0.55	MA	T2D	Increased fasting insulin and HOMA-IR
PPARG	rs1801282	0.92	CS	T2D	Decreased insulin sensitivity derived from IVGTT and hyperinsulinemic-euglycemic clamp
Variants with unknown function					
ADCY5	rs11708067	0.78	MA	FG, 2 h-G, T2D, HOMA-B	Decreased birth weight
BCL11A	rs243021	0.46	MA	T2D	
CHCHD9	rs13292136	0.93	MA	T2D	
CRY2	rs11605924	0.54	MA	FG	
HMGA2	rs1531343	0.10	MA	T2D	
NOTCH2	rs10923931	0.11	MA	T2D	
PRC1	rs8042680	0.22	MA	T2D	
RBMS1	rs7593730		MA	T2D	Increased HOMA-IR
SLC2A2	rs11920090	0.85	MA	FG	
TP53INP1	rs896854	0.48	MA	T2D	
VPS13C	rs17271305	0.42	MA	2 h-G	
ZFAND6	rs11634397	0.56	MA	T2D	
ZBED3	rs4457053	0.26	MA	T2D	

MA=meta analysis, CS=candidate gene study, LA=linkage analysis, FG=fasting glucose, 2h-G=2 hr glucose

CDC123*/*CAMK1D, encoding cell division cycle 123 homolog and calcium/calmodulin-dependent protein kinase 1 D, is located on chromosome 10p13.

CAMK1D plays a role in granulocyte function and it may also affect beta-cell function by reducing the beta-cell mass due to enhanced apoptosis [107]. The SNP rs12779790, reported to be associated with type 2 diabetes, belongs to this genomic region. This variant was also associated with a beta-cell defect [108].

CDKAL1, encoding cyclin-dependent kinase 5 regulatory sub-unit associated protein 1-like 1, is located on chromosome 6p22.3. This gene is expressed in pancreatic islets, skeletal muscle and brain. A study based on *Cdkal1* knockout mice reported that Cdkal1 controls the first-phase insulin exocytosis by facilitating ATP generation, and altering K_{ATP} channel and Ca^{2+} channel activity [109]. It has been recently reported that it is a t-RNA modification enzyme required for the accurate translation of codons [110]. Several SNPs belonging to this gene region, mainly rs7754840, have been associated with type 2 diabetes and decreased first phase insulin secretion [13,111,112].

CDKN2A*/*CDKN2B, encoding Cyclin-dependent kinase inhibitor 2A/B, are located adjacent to each other on chromosome 9p and encode two different isoforms of cyclin-dependent kinase (CDK) inhibitors. CDKs are protein kinases that have a role in regulating the cell cycle. CDK inhibitors are mainly considered to be tumor-suppressor genes. Their function is largely unknown in type 2 diabetes, but variants of these genes, rs10811661 and rs564398, have been reported to be associated with type 2 diabetes [14,15,17]. Both proteins encoded by *CDKN2B* and *CDKN2A* are expressed in pancreatic islets and seem to be involved in the regulation of pancreatic beta-cell replication [113,114].

CENTD2 or ***ARAP1***, encoding ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1, is located on chromosome 11q13.4. It has been identified as a component of the machinery that controls the endocytic trafficking and signalling of the EGF receptor [115]. Variant rs1552224 has been identified as a risk locus for type 2 diabetes [20]. Its association with increased plasma glucose values and decreased glucose-stimulated insulin release has been recently reported [116].

DGKB, encoding diacylglycerol kinase beta, is located on chromosome 7p21.2. This enzyme regulates the intracellular concentration of the second messenger diacylglycerol and thus plays a key role in many cellular processes. It is a lipid-regulating enzyme and it catalyzes the phosphorylation of diacylglycerol to phosphatidic acid [117]. *Dgkb* knockout mice display various behavioral abnormalities and exhibit impaired GSK3B signaling [118]. Another study showed that DGK inhibition results in increased protein kinase C-alpha activity, reduced

glucose-induced insulin receptor activation, and GLUT4 translocation [119]. The rs2191349 risk allele near *DGKB*/*TMEM195*, was identified as a fasting glucose enhancing allele and it also decreased glucose-stimulated insulin secretion [18,106].

FADS1, encoding fatty acid desaturase 1, is a member of the fatty acid desaturase (*FADS*) gene family located on chromosome 11q12.2-q13.1. The enzyme plays a crucial role in desaturation and elongation of polyunsaturated fatty acids. Fatty acid desaturases are therefore considered as important factors in the pathogenesis of lipid-induced insulin resistance. It has been reported that insulin can induce the expression of *FADS1* and *FADS2* in a time- and dose-dependent manner [120]. *FADS1* SNP rs174550 has been shown to associate with fasting glucose levels [18].

G6PC2, encoding the enzyme glucose-6-phosphatase, catalytic, 2, is located on chromosome 2q24.3. This enzyme catalyses the terminal step in gluconeogenic and glycogenolytic pathways, promoting the release of glucose into the bloodstream. *G6pc2* knockout mice are reported to have reduced fasting plasma glucose levels [121]. The SNP rs560887 was reported to be associated with fasting glucose, insulin secretion and type 2 diabetes [18,122].

GCK, encoding Glucokinase, is located on chromosome 7p15.3-p15.1 and is expressed in the liver and pancreas. This enzyme catalyses the first rate limiting step in the metabolism of glucose and is termed as a glucose sensor in the pancreas. Beta-cell specific *Gck* knockout mice do not survive due to diabetes. In contrast, liver-specific *Gck* knockout mice survive and exhibit mild hyperglycemia and defective glycogen synthesis [123]. *GCK* mutations in humans have been reported to cause maturity-onset diabetes of the young, type 2 (MODY2) [124]. The risk allele of SNP rs1799884 has been associated with fasting glucose, impaired insulin secretion and type 2 diabetes [18,122].

GIPR, encoding the gastric inhibitory polypeptide (GIP) receptor, is located on chromosome 19q13.3. GIPR is a transmembrane protein G-protein coupled receptor found in pancreatic beta-cells and also other tissues. The incretin hormone, GIP, mediates early insulin secretion from the beta-cells after a meal. *Gipr* knockout mice have higher blood glucose levels with an impaired initial insulin response after oral glucose load [125]. SNP rs10423928 of *GIPR* was reported to be associated with 2h glucose [19]. Additionally, lower insulin secretion along with a diminished incretin effect has been observed in carriers of the risk-allele.

GLIS3, encoding GLIS family zinc finger 3, is a member of the GLI-similar zinc finger protein family located on chromosome 9p24.2. *GLIS3* encodes a nuclear protein with five C2H2-type zinc finger domains. This protein functions as both a repressor and activator of transcription and is specifically involved in the development of pancreatic beta-cells. It regulates insulin gene expression and interacts with various transcription factors like Pdx1, MafA that are involved in pancreatic development [126]. *Glis3* mutant mice develop neonatal diabetes, evidenced by hyperglycemia and hypoinsulinemia [127]. *GLIS3* SNP rs7034200 has been implicated in fasting glucose homeostasis [18].

HHEX, hematopoietically expressed homeobox, located on chromosome 10q23.33, encodes a transcription factor that is expressed in the embryonic ventral-lateral foregut that gives rise to the ventral pancreas and the liver. *Hhex* knockout mice display early embryonic lethality, due to impaired forebrain, liver and thyroid development [128]. The mechanism by which the *HHEX* variant alters the risk of type 2 diabetes, awaits further investigation. The variant might have a role in altered organogenesis as demonstrated by the developmental defects in *HHEX* knockout mice [129]. SNP, rs7923837, located in the 3′-flanking region of the gene has been reported to associate with glucose-stimulated insulin secretion and type 2 diabetes [130,131].

IGF2BP2, encodes insulin-like growth factor 2 (IGF-2) mRNA binding protein 2 which is a member of the IGF-2 mRNA-binding protein (IMP) family. The gene is located on chromosome 3q27.2 and the protein has been implicated in RNA localization, stability and translation that are essential for normal embryonic growth and development. It is expressed in the brain and several tissues including the pancreas [132]. The predisposition to type 2 diabetes, conferred by the SNP rs4402960, is mainly attributable to its association with first-phase insulin secretion and impaired beta-cell function [111].

HNF1A, encoding hepatocyte nuclear factor homeobox A, is located on chromosome 12q24.2. HNF1A is a transcription factor which is required for the expression of several liver-specific genes. Mutations in this gene cause maturity onset diabetes of the young type 3 (MODY3) [133]. SNP rs7957197 was reported to be associated with type 2 diabetes [20].

HNF1B, encoding hepatocyte nuclear factor 1-beta, is a member of the homeodomain-containing superfamily of transcription factors located near chromosome 17q21.3. HNF1B binds to DNA as a homodimer or a heterodimer with

a related protein, HNF1-alpha. This transcription factor is known to play a role in regulating the development of pancreas. Selective deletion of *Hnf1b* in beta-cells in mice leads to impaired glucose tolerance, dysregulated islet gene expression, and reduced glucose-stimulated insulin secretion [134]. A mutation in the *HNF1B* causes MODY5 [135]. SNP rs4430796 has been shown to have modest effects on the risk of type 2 diabetes [136].

JAZF1, Juxtaposed with another zinc finger gene 1, encodes a nuclear protein that functions as transcriptional repressor. *JAZF1* is located on chromosome 7p15. In adipocytes and liver cells, JAZF1 reduces lipid synthesis and increases lipolysis, mainly by down-regulating the levels of sterol regulatory element-binding protein 1, acetyl-coenzyme A carboxylase, and mRNA expression of fatty acid synthetase and by increasing hormone-sensitive lipase mRNA expression [137]. Jazf1 overexpression studies in mice heart, showed cardiac defects and upregulated levels of pro-apoptotic genes [138]. *JAZF1* SNP rs864745 has been associated with lower insulin secretion and elevated type 2 diabetes risk [16].

KCNJ11, encoding potassium inwardly-rectifying channel, subfamily J, member 11, is located on chromosome 11p15.1. *KCNJ11* encodes the pore-forming subunit Kir6.2 of the ATP-sensitive potassium channel of beta-cells, which couples glucose sensing with membrane depolarization and exocytosis of insulin granules. *KCNJ11* mutations prevent electrical activity and insulin release from INS-1 cells by increasing the K_{ATP} current and hyperpolarizing the beta-cell membrane [139]. The best studied and confirmed type 2 diabetes risk variant E23K (rs5219) has been shown in vitro to increase the probability of the channel's open state, to enhance its activity, and to impair its ATP sensitivity, thereby inhibiting beta-cell excitability and insulin release [140,141]. Similar associations with impaired insulin release and type 2 diabetes have been demonstrated in various population-based studies [98,142]. A previous study also reported that carriers of the rs5219 risk allele had increased hepatic insulin sensitivity [143].

KCNQ1, encodes potassium voltage-gated channel, KQT-like subfamily, member 1, that is required for repolarization phase of the action potential. *KCNQ1* is located on chromosome 11p15.5. It is likely that KCNQ1 influences K^+-dependent insulin signaling on glucose metabolism. Its overexpression in pancreatic beta-cells has led to the lowering of action potential and caused impaired insulin release [144]. Various polymorphisms of *KCNQ1*, mainly rs2237897, have been shown to associate with reduced glucose-stimulated insulin secretion and type 2 diabetes [145]. The risk

allele is further reported to be associated with higher fasting glucose and lower beta-cell function [146,147].

MADD, encodes a mitogen-activated protein-kinase (MAPK) activating death domain protein and is located on chromosome 11p11.2. This enzyme interacts with TNF-alpha receptor 1 to activate MAPK and propagates the apoptotic signal [148]. It is also a GDP/GTP Exchange Protein which cycles Rab3A between active/inactive forms. Thus, *MADD* is considered to have a role in apoptosis and exocytosis [149]. A SNP of this gene, rs7944584, was initially associated with fasting hyperglycemia [18]. Recently, it was also shown to be associated with impaired proinsulin to insulin conversion [150].

MTNR1B, encoding melatonin receptor 1B, is located on chromosome 11q21-q22. Its endogenous ligand, melatonin, is a neurohormone that mediates circadian rhythmicity and appears to influence insulin secretion and glucose levels [151,152]. Expression of melatonin receptors has been demonstrated in mouse, rat, and human pancreatic islets (MT1 in α-cells and MT2 in β-cells) [153]. Both MT1 and MT2 are G-protein coupled receptors and are proposed to exert inhibitory effects on insulin secretion. The risk allele of *MTNR1B* SNP rs10830963 has been identified as a fasting glucose enhancing allele and is associated with impaired early insulin secretion and type 2 diabetes [18,154].

PROX1, encoding Prospero homeobox 1, is a transcription factor and considered an early specific marker for liver and pancreatic development [155]. *PROX1* is located on chromosome 1q41. It has also been considered as a tumor-suppressor gene and its expression is altered in liver tumors [156]. This gene is regulated by β-catenin-TCF/LEF activation downstream of Wnt signaling [157]. The risk allele of *PROX1* SNP rs340874 has been shown to be associated with elevated fasting glucose levels and type 2 diabetes risk [18].

SLC30A8, encoding the zinc-transporter ZnT8, is located on chromosome 8q24.11. It is highly expressed in the pancreatic beta-cells and it colocalises with insulin in the beta-cell [158]. It acts as a zinc transporter in beta-cells and is important for storage and maturation of insulin within cytoplasmic secretory granules. *Slc30a8* knockout mice have a reduced Zinc content in islets and also lower plasma insulin concentrations [159]. The variant in *SLC30A8,* rs13266634, that has been reported to be associated with type 2 diabetes, may affect the function of the transporter by altering zinc accumulation in insulin granules and hence influence insulin stability and insulin trafficking [160].

TCF7L2, encoding transcription factor 7-like 2, is located on chromosome 10q25.3. TCF7L2 is a component of the bipartite transcription factor complex that is involved in the Wnt signaling pathway [161]. In human and murine islets, TCF7L2 is required for beta-cell survival and beta-cell proliferation, as well as for glucose and incretin-stimulated insulin secretion [162,163]. Interestingly, *Tcf7l2* null mice display enhanced glucose tolerance, suggesting that over-expression of this gene is associated with increased risk of type 2 diabetes [164]. *TCF7L2* SNP rs7903146 is currently the strongest risk locus reported to be associated with type 2 diabetes with an odds ratio of 1.4 and its susceptibility has been confirmed in many ethnic groups [165,166]. This variant has been associated with glucose-stimulated and incretin-stimulated insulin secretion and also with impaired proinsulin to insulin conversion [167-169].

THADA, encoding thyroid adenoma associated protein, is located on chromosome 2p21. This region is characterized by the presence of frequent structural chromosomal rearrangements in benign adenomas of the thyroid. Hence, *THADA* has been reported to play a role mainly in thyroid adenomas. Additionally, it has been implicated in the death receptor pathway suggestive of a role in apoptosis [170]. A SNP in *THADA*, rs7578597 has been reported to be associated with type 2 diabetes [16].

TSPAN8, encoding tetraspanin 8, is located on chromosome 12q14.1-q21.1. TSPAN8 is a protein belonging to the transmembrane 4 superfamily. This family of proteins mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility. *TSPAN8* has been shown to play a prominent role in metastasis and is implicated in hepatocellular carcinoma [171]. *Tspan8* deficient mice exhibited reduced body weight although no changes in fasting insulin or glucose levels were detected [172]. The SNP rs7961581 located in this gene was reported to be associated with type 2 diabetes [16].

WFS1, encoding Wolfram syndrome 1, is located on chromosome 4p16.1. It encodes an endoplasmic reticulum transmembrane protein. Mutations in the gene are associated with Wolfram syndrome that mainly affects the central nervous system. Mice with disrupted *WFS1* gene developed glucose intolerance, even overt diabetes, due to insufficient insulin secretion [173]. Recently, it was demonstrated that Wfs1 localises to the secretory granules in the pancreatic beta-cells and *Wfs1* null mice display impaired proinsulin processing [174]. SNPs in *WFS1* have been shown to be associated with decreased insulin secretion and the risk of type 2 diabetes [175,176].

2.4.2 Variants regulating insulin sensitivity

ADAMTS9, encoding ADAM metallopeptidase with thrombospondin type 1 motif, 9, is a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family. The gene encoding ADAMTS9 is located on chromosome 3p14.1. ADAMTS9 is a secreted metalloprotease that cleaves two proteoglycans, versican and aggrecan. It is expressed in several tissues, including skeletal muscle and pancreas. The first evidence for an association of a variant of this gene with type 2 diabetes emerged from a meta-analysis and a consequent study indicated that it might affect insulin sensitivity [16, 177].

DUSP9, encoding dual specificity phosphatase 9, is located on chromosome Xq28. DUSP9 is a phosphatase that negatively regulates members of the MAP kinase superfamily. DUSP9 has been reported to negatively regulate insulin signaling and contribute to the pathogenesis of insulin resistance [178]. A variant of *DUSP9*, rs5945326, has been reported to increase the risk of type 2 diabetes primarily through its effect on insulin action [20].

GCKR, encodes a glucokinase regulatory protein, and is located on chromosome 2p23. GCKR inhibits glucokinase in liver and pancreatic islet cells. *Gckr* knockout mice show a parallel loss of GCK protein levels and activity in the liver, resulting in altered glucose metabolism and impaired glycemic control [179]. *GCKR* rs780094 has been associated with susceptibility to type 2 diabetes, increased fasting plasma glucose levels, low triglycerides levels and increased HOMA-IR [180].

IGF1, encoding Insulin like growth factor 1, is located on chromosome 12q23.2. Several metabolic disorders have been demonstrated in *Igf-1* deficient transgenic mice that include lipid abnormalities, insulin resistance, and decreased bone density [181]. Liver-specific *Igf-1* deficient mice, develop insulin resistance in the liver, muscle and adipose tissues [182]. The fasting insulin–raising allele at *IGF1* rs35767 was associated with lower insulin sensitivity [183].

IRS1, Insulin receptor substrate 1, is located on chromosome 2q36 that encodes a protein which is phosphorylated by insulin receptor tyrosine kinase. *Irs1* deficient mice have been reported to display a phenotype of growth retardation and insulin resistance [184,185]. Mutations in this gene are associated with type 2 diabetes and susceptibility to insulin resistance. A genetic variant near *IRS1*, rs2943641, has been associated with insulin resistance, hyperinsulinemia and type 2 diabetes [186].

KLF14, Kruppel-like factor 14, encodes a member of the Kruppel-like zinc finger family of transcription factors located on chromosome 7q32.3. This family plays a key role in regulating cellular differentiation and tissue development [187]. KLF14 has been recently identified as a regulator of adipose gene expression [188]. In addition to type 2 diabetes, a variant in this gene region, rs972283 is associated with high-density lipoprotein (HDL) cholesterol levels [20,189].

PPARG2, encoding peroxisome proliferator-activated receptor γ (PPARγ), is located on chromosome 3p25. PPARγ is a lipid-activated nuclear receptor and transcription factor. It plays a fundamental role in adipogenesis and insulin sensitivity by regulating transcriptional activity of various genes. PPARγ belongs to the nuclear hormone receptor superfamily and is a central regulator of adipogenesis and lipogenesis [190,191]. Selective disruption of PPARγ2 in mice causes a phenotype of an overall reduction in white adipose tissue, less lipid accumulation, and decreased expression of adipogenic genes in adipose tissue and impaired insulin sensitivity [192]. The most intensely studied and confirmed type 2 diabetes risk SNP rs1801282 (Pro12Ala polymorphism) of *PPARG2* has been associated with insulin resistance [96].

2.4.3 Variants with unknown function

ADCY5, encoding a protein that belongs to the adenylate cyclase family of enzymes, is located on chromosome 3q13.2-q21. Adenylyl cyclases mediate G protein-coupled receptor signaling through the synthesis of the second messenger, cAMP. ADCY5 belongs to the calcium-inhibited family of adenylate cyclase isoforms. The risk allele of SNP rs11708067 has been associated with fasting hyperglycemia, impaired proinsulin conversion and type 2 diabetes [18,19,150].

BCL11A, B-cell CLL/lymphoma 11A, encoding a zinc-finger protein, is located on chromosome 2p16.1. BCL11A is a potent silencer of fetal hemoglobin and plays a role in coordinating the switch from fetal to adult hemoglobin [193]. BCL11A has been reported to act as a transcriptional repressor that interacts with several proteins, including BCL6, COUP-TF, and SIRT1 [194]. Variants belonging to this genic region have been associated with type 2 diabetes [16].

CHCHD9, coiled-coil-helix-coiled-coil-helix domain containing 2 pseudogene 9, is located on chromosome 9q21.31. Its function is largely unknown. A SNP of *CHCHD9*, rs13292136, has been associated with type 2 diabetes [20].

CRY2, encoding cryptochrome 2 (photolyase-like), is located on chromosome 11p11.2. The cryptochrome class of proteins has a role in circadian control of gene expression that also include cAMP signaling and hepatic gluconeogenesis [195]. *Cry1* and *Cry2* double knockout mice present with increased CREB activity, expression of gluconeogenic genes, and higher fasting blood glucose levels [196]. SNP rs11605924 has been associated with elevated fasting glucose and type 2 diabetes [18,197].

HMGA2, high mobility group AT-hook 2, is located on chromosome 12q15. The encoding protein contains structural DNA-binding domains and may act as a transcriptional regulating factor. It has also been implicated in adipocyte differentiation in a study where knockout mice demonstrated resistance to diet-induced obesity [198]. A SNP belonging to this genic region, rs1531343, is associated with type 2 diabetes [20].

NOTCH2, belongs to the Notch family that plays a role in a variety of developmental processes. The gene is located on chromosome 1p13-p11. This protein functions as a receptor for membrane bound ligands, and may play a role in vascular, renal and hepatic development. Notch-2 was recently identified as a novel target for β-catenin-dependent Wnt signaling that is involved in pancreatic development and insulin secretion [199]. *NOTCH2* SNP rs10923931 has been associated with type 2 diabetes [16].

PRC1, encoding protein regulator of cytokinesis 1, is located on chromosome 15q26.1. It is a substrate of several cyclin-dependent kinases. It has a role in microtubule formation that play a critical role in processes such as cell migration and division [200]. A SNP rs8042680 has been associated with type 2 diabetes [20].

RBMS1, encoding RNA binding motif, single stranded interacting protein 1, is located on chromosome 2q24.2. It belongs to a family of proteins that binds to single stranded RNA/DNA and has been implicated in diverse functions as DNA replication, gene transcription, cell cycle progression and apoptosis. A variant in this region, rs7593730, has been associated with type 2 diabetes, fasting glucose and HOMA-IR [201].

SLC2A2, encodes solute carrier family 2, member 2, and is located on chromosome 3q26.1-q26.2. It is a facilitated glucose transporter observed mainly in the liver and pancreatic beta-cells. Its expression is suggested to be regulated by HNF-1alpha and HNF-3beta [202]. As expected, *Slc2a2* knockout mice develop hyperglycemia and

impaired glucose tolerance [203]. SNP rs11920090 has been associated with fasting glucose levels [18].

TP53INP1, encoding tumor protein p53 inducible nuclear protein 1, is located on chromosome 8q22. Tp53inp1 has been implicated in several types of carcinoma. Overexpression of *TP53INP1* induces G1 arrest and increases p53-mediated apoptosis indicating its role in regulating cell cycle progression and apoptosis [204]. Interestingly, the p53-p53INP1 pathway has been identified as a molecular mechanism through which the other important type 2 diabetes gene, *TCF7L2*, affects beta-cell survival [205]. A variant near this genic region, rs896854, has been associated with type 2 diabetes [20].

VPS13C, encoding vacuolar protein sorting 13 homolog C, is located on chromosome 15q22.2. The VPS family of proteins are large proteins with a ubiquitous expression [206]. A variant in this region, rs17271305, has been associated with 2-h glucose levels [19].

ZFAND6, encoding zinc finger, AN1-type domain 6, is located on chromosome 15q25.1. This gene plays a role in the TNFalpha signaling pathway [207]. A SNP, rs11634397, belonging to this region has been associated with type 2 diabetes [20].

ZBED3, encodes a zinc finger, BED domain-containing 3 protein and is located on chromosome 5q13.3. It has been identified as an Axin-binding protein that is involved in modulating Wnt/beta-catenin signaling [208]. A variant from this genomic region has been reported to be associated with type 2 diabetes [20].

2.5 CARDIOVASCULAR DISEASE

CVD is a major worldwide public health problem and the number one cause of death in the industrialized countries. Cardiovascular death rates are either already high or appear to be climbing in countries where diabetes is prevalent. It has been estimated that by 2030, the mortality due to CVD will increase to almost 23.6 million people (mainly from CAD) as compared to an estimated 17.1 million in 2004 (WHO). CVD is projected to remain the single leading cause of death. Moreover, CVD remains the most important cause of morbidity and mortality in patients with type 2 diabetes.

The risk of developing CVD is significantly increased in subjects with type 2 diabetes. Adults with diabetes are two to four times more likely to have heart

disease or stroke than adults without diabetes. The risk of cardiac mortality in diabetic subjects is comparable to the risk of non-diabetic subjects after a myocardial infarction [209].

2.5.1 CVD risk factors

CVD risk factors can be classified as traditional or non-traditional risk factors (Fig 5). A majority of these risk factors are modifiable. The traditional risk factors for CVD include elevated blood pressure, smoking, high low density lipoprotein (LDL) cholesterol, low HDL cholesterol and diabetes. The non-traditional risk factors include insulin resistance, endothelial dysfunction, inflammation, microalbuminuria, impaired fibrinolysis etc. [210]. Several of these risk factors such as hypertension, hyperinsulinemia, dyslipidemia, obesity, thrombosis formation are already present in the pre-diabetic state [211]. However, these risk factors frequently cluster and interact with each other. Insulin resistance, causing various metabolic disturbances, leads to CVD and hence it is also sometimes referred to as the cardiometabolic syndrome. The most important risk factors including mainly the traditional risk factors that explain 75-90% of all CVD events are briefly described below.

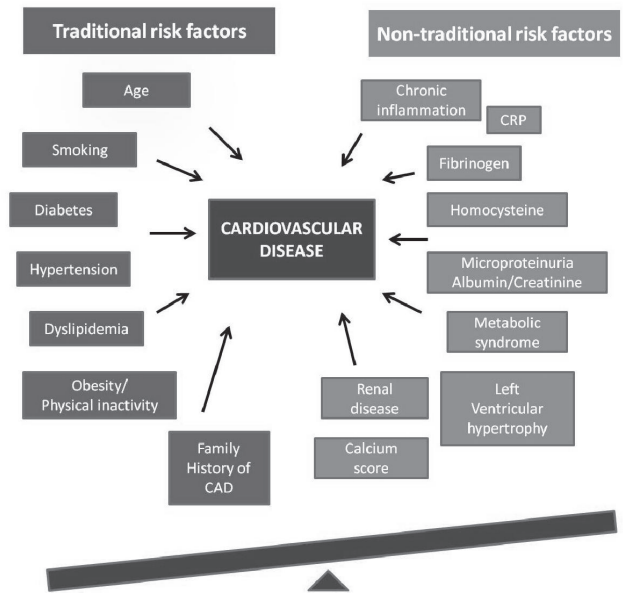

Figure 5. Traditional and non-traditional risk factors for CVD [210]

2.5.1.1 Age, gender and family history of CVD

Age, gender and family history are the major non-modifiable risk factors for CVD. Various pathophysiological features such as the increase in the size of cardiac myocytes with a corresponding decrease in their number, thickening of arterial walls, vascular stiffness, hypertension are observed in elderly subjects [212].

In men, the onset of CVD occurs earlier than in women probably as a consequence of the differences in the sex hormones that affect HDL cholesterol levels [213]. Several other CVD risk factors e.g., diabetes mellitus, previous myocardial infarction, alcohol consumption etc., are more prevalent in men until the age of 45-50 years, later they become comparable with those in age-matched women [214]. The cumulative risk of CAD in men by the age of 70 is 30% and by age of 90 years is 48%. Women typically develop CAD about 10 years later than men with a cumulative risk of 15 and 30% by the ages of 70 and 90 years, respectively [215].

CVD has a strong genetic component as demonstrated in several family and twin studies. Furthermore, animal models and genetic association studies support its strong genetic basis. Although advancing age enhances the risk, approximately 8% of the cases are diagnosed before age 50 [215]. A significant difference in concordance of CAD deaths was observed in monozygotic twins as compared with dizygotic twins in both men and women; 39 vs 26% and 44 vs 14%, respectively. Recent genome-wide association studies have identified a large number of variants associated not only with CVD risk but also with various intermediate phenotypes such as LDL, HDL, TG and total cholesterol and CRP levels [216-218].

2.5.1.2 Elevated blood pressure

Elevated blood pressure is a well-known risk factor for CVD. Smoking, obesity and a high intake of salt lead to the elevation of blood pressure. Elevated blood pressure clusters with dyslipidemia, glucose intolerance and obesity and thus significantly enhancing the risk of CVD [219]. Insulin resistance is often associated with these pathophysiological traits [220].

Systolic blood pressure is considered to be a stronger predictor of CVD risk than diastolic blood pressure [221]. High blood pressure has adverse affects on the arteries imposing a mechanical stress on the arterial walls resulting in their enlargement and thickening. Elevated blood pressure has been associated with long-term mortality from CVD [222]. Men and women with high-normal blood pressure

at baseline examination have been shown to have a high incidence of CVD than those with optimal blood pressure [223].

2.5.1.3 Smoking

Smoking is a significant and independent risk factor for the development and progression of CVD. It acts synergistically with other conventional risk factors, greatly increasing the baseline risk associated with each risk factor individually and significantly reducing the age of onset [224].

Smoking is associated with increased levels of inflammatory markers and accelerated atherosclerosis [225]. Smokers often have high levels of total cholesterol, LDL cholesterol and total triglycerides and lower levels of HDL cholesterol than non-smokers [226]. Smokers were reported to have relative risk of myocardial infarction of 2.24 times higher in females and 1.43 times higher in males, when compared with non-smokers [227]. Smoking cessation has been clearly shown to significantly improve the health status and to reduce several CVD risk factors [227]. Besides the well-documented risk for CVD, there is also evidence for an increased risk of the development of type 2 diabetes. Heavy smokers have a higher relative risk of diabetes (OR 1.94) compared with non-smokers [228-230].

2.5.1.4 Dyslipidemia

Dyslipidemia plays a major role in the pathogenesis of CVD. As a consequence of increased lipolysis, the high flux of NEFA to the liver results in hypertriglyceridemia. Elevated triglyceride level increases the risk of CVD [231]. VLDL, the major carrier of triglycerides, are hydrolyzed by lipoprotein lipase generating smaller and denser VLDL that are subsequently converted to IDL [232]. IDL particles undergo further catabolism to form LDL. Triglyceride enrichment of LDL results in the formation of small, dense LDL particles. These small and dense LDL particles are more common in subjects with CVD and these particles are considered to be highly atherogenic due to their delayed clearance [233,234].

HDL plays a major role in reverse cholesterol transport from endothelial cells to the liver which is an important anti-atherogenic role played by HDL. Additionally, HDL has anti-apoptotic activity [235] and anti-inflammatory actions [236] that are atheroprotective. In the insulin resistant state, increased transfer of triglycerides from VLDL to HDL results in the formation of HDL particles that are

smaller and denser and are cleared more rapidly, contributing to the decreased HDL levels. Therefore, low HDL cholesterol levels increase the risk of coronary heart disease [237].

The presence of small, dense LDL particles, increased levels of plasma triglycerides and decreased levels of HDL cholesterol represent the atherogenic lipoprotein phenotype. This phenotype is also observed in insulin resistant states such as in obesity and type 2 diabetes.

2.5.1.5 Type 2 diabetes

Type 2 diabetes has long been recognized as an independent risk factor for CVD. Various metabolic abnormalities that are initiated long before the diagnosis of type 2 diabetes contribute to two-fold increase in the CVD risk in impaired glucose tolerant states and to a four-fold increase in CVD risk in diabetic states [238]. Hyperglycemia, impaired insulin action and concomitant hyperinsulinemia increase the risk for CVD in subjects with pre-diabetes and diabetes [9,211,239-242].

The presence of high levels of both fasting and 2h glucose is associated with an enhanced risk of CVD. Moreover, a graded relationship between fasting and 2h glucose with cardiovascular events was apparent even for glucose levels that were below the diabetic threshold [242]. High glucose could be causally related to CVD through a number of mechanisms particularly, increased oxidative stress. Furthermore, elevations in the glucose levels are confounded by other CVD risk factors like hyperinsulinemia, hyper-triglyceridemia, obesity and hypertension [240-242] resulting in various microvascular and macrovascular complications.

Insulin resistance per se is associated with a 1.5-2 fold increase in the incidence of CVD. A clear relationship has been noted between insulin resistance and carotid intima-media thickness, a surrogate marker for CVD [243]. Insulin resistance is well correlated with central obesity, lipid abnormalities and hypertension and clustering of these metabolic abnormalities, results in atherosclerosis and other heart-related disorders.

2.5.1.6 Other cardiovascular risk factors

Physical inactivity and obesity. Physical inactivity is associated with an increased risk of CVD. A study based on meta-analysis reported a relative risk of death from coronary heart disease of 1.9 when subjects with sedentary occupations were

compared with those in active occupations [244]. Physical activity improves several CVD risk factors such as blood pressure, lipid levels and insulin sensitivity. Obesity is associated with an increased risk of morbidity and mortality as well as reduced life expectancy. A genetic predisposition, increased caloric intake or decreased energy expenditure or a combination of these traits are responsible for the current epidemic of obesity. It is an independent risk factor for CVD, and enhanced CVD risk has also been observed in obese children [245,246]. Weight loss leads to significant improvements in CVD risk factors [247-249].

C-reactive protein. Inflammation plays a key role in atherosclerosis and CVD [250]. High levels of hs-CRP have been shown to be an independent predictor of CVD risk in non-diabetic subjects and in type 2 diabetes subjects [251]. Beyond its role as a CVD risk marker, it might have a direct role in atherogenesis [252,253], as it co-localizes with the membrane attack complex in early atheromatous lesions that leads to cell lysis and death. The levels of CRP, along with other complement proteins, are substantially upregulated in the atheromatous plaque [253].

Adiponectin. Adiponectin is one of the most abundant adipokines. It has insulin sensitising effects in several tissues. Adiponectin also has anti-inflammatory and anti-atherogenic properties. Secretion of adiponectin is reduced as adipose tissue mass increases [254]. Serum adiponectin is negatively correlated with adverse features of the metabolic syndrome as well as other associated features of insulin resistance and conventional cardiovascular risk factors. Serum adiponectin levels are lower in subjects with obesity [255], in subjects with CAD, as well as in those with type 2 diabetes, compared to controls. They are especially low in patients with both type 2 diabetes and CAD [256].

3. Aims of the study

The major aim of the study was to investigate the mechanisms and genetic regulation of insulin resistance in insulin sensitive tissues, especially in the liver.

The specific aims of the study were as follows:

1) To develop a novel marker of hepatic insulin resistance,
2) To examine the effects of SNPs increasing the risk of type 2 diabetes on liver and adipose tissue insulin sensitivity,
3) To evaluate the association between liver insulin resistance and the levels of CVD risk factors,
4) To investigate the association of the SNPs regulating the levels of fasting glucose with indices of insulin release, proinsulin conversion and insulin sensitivity.

4. Subjects and methods

4.1 SUBJECTS

The EGIR-RISC study (Study I)

A sample of 368 clinically healthy non-diabetic men (n=183) and women (n=185) from the RISC study (age 43.0±8.2 years, BMI 26.0±4.0 kg/m^2; mean±SD) was used to develop and validate the surrogate index of liver IR. In this sample, 288 participants had normal glucose tolerance and 80 had either impaired fasting glucose or impaired glucose tolerance or both [257]. All participants were recruited from 19 research centres in 14 European countries. The protocol was approved by the local Ethics Committees and all participants gave written informed consent. The inclusion criteria of the study participants and the study protocol have been described previously [258].

The METSIM study (Studies II-IV)

Metabolic Syndrome in Men (METSIM) study is a population-based cross-sectional study. It includes participants (age 57.2±7.1 years, BMI 26.8±3.8 kg/m^2) who were randomly selected from the population register of Kuopio town, Eastern Finland (population of 95,000).

Study II included 8,460 non-diabetic participants. This study involved two parts: 1) genetic association analyses that included 6,733 non-diabetic men (age 57.0±6.9 years, BMI 26.8±3.8 kg/m^2; mean±SD) who were genotyped for the SNPs being investigated; 2) gene expression study that included 41 obese participants (age 44.2±8.3 years, BMI 45.5±6.1 kg/m^2, mean±SD; 11 men, total 18 subjects with type 2 diabetes) from an ongoing study, including participants who were undergoing bariatric surgery at the Kuopio University Hospital and who also underwent liver biopsy.

Studies III and IV involved 8,750 non-diabetic men. Out of 8,750 non-diabetic subjects, 3,034 (34.7%) men had normal glucose tolerance (NGT), 4,345 (49.7%) had isolated impaired fasting glucose (IFG), 312 (3.6%) had isolated impaired glucose tolerance (IGT) and 1,059 (12.1%) had IFG and IGT, according to the ADA criteria [253]. All studies were approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital, and were carried out in accordance with the Helsinki Declaration. All study participants gave written informed consent.

Table 2. Baseline characteristics of non-diabetic participants of the METSIM and RISC studies

Variable	METSIM	RISC
N	8750	368(183)*
Age, years	57.2±7.1	43±8.2
Body mass index, kg/m^2	26.8±3.8	25.9±3.9
Fasting plasma glucose, mmol/l	5.7±0.5	5.0±0.6
2h plasma glucose, mmol/l	6.0±1.7	5.7±1.4
Fasting plasma insulin, pmol/l	57.3±40.9	38.0±21.5

*(N) – male subjects

4.2 METHODS

Endogenous glucose production (Study I)

Endogenous glucose production (EGP) was measured in the RISC study with [6-6^{2}H^2] glucose in the fasting state and during the euglycemic–hyperinsulinemic clamp. A primed continuous infusion of labelled glucose was continued for 2 h and blood samples obtained during the last 20 min of the equilibration period to measure the plasma tracer concentration. EGP during the clamp was measured assuming a monocompartment model for glucose kinetics. Insulin sensitivity was measured by the euglycemic insulin clamp technique [259], using a primed infusion rate of 1 mUmin^{-1}kg^{-1}, followed by an exogenous glucose infusion.

Oral glucose tolerance test

A 2 h OGTT (75 g glucose) was performed after an overnight fast and samples for plasma glucose, insulin and NEFA were drawn at 0, 30 and 120 min.

Laboratory measurements

The plasma glucose concentration was measured by the glucose oxidase method in the EGIR-RISC study and by the enzymatic hexokinase photometric assay in METSIM study. Plasma insulin and proinsulin concentrations were measured by radioimmunoassay. Body composition was determined by bioelectrical impedance. Glucagon levels were measured by glucagon assay (developed in J. Holst's laboratory, University of Copenhagen, Denmark) and NEFA by spectrophotometry in RISC study and colorimetry in METSIM study. Total cholesterol, LDL cholesterol, HDL cholesterol, total triglycerides and alanine aminotransferase (ALT) were measured by standard colorimetric methods. Blood pressure (BP) was measured in

the sitting position after a 5-minute rest with a mercury sphygmomanometer. The average of 3 measurements was used to calculate systolic and diastolic BP. Plasma adiponectin was measured by an enzyme immunoassay method (Human Adiponectin Elisa Kit, Linco Research, St Charles, Mi, USA), apolipoprotein B by immunoturbidimetry (Konelab Systems Reagents). Serum concentrations of high-sensitivity C-reactive protein (hs-CRP) were assayed by Kinetic immunoturbidimetry (Immage Immunochemistry System, Beckman Coulter, CA, USA).

Genotyping

Genotyping of SNPs (*PPARG2* rs1801282, *KCNJ11* rs5219, *TCF7L2* rs7903146, *SLC30A8* rs13266634, *HHEX* rs1111875, rs7480010 [previously assigned to gene locus *LOC387761*], *CDKN2B* rs10811661, *IGF2BP2* rs4402960, *CDKAL1* rs7754840, *HNF1B* rs7501939, *WFS1* rs10010131, *JAZF1* rs864745, *CDC123* rs12779790, *TSPAN8* rs7961581, *THADA* rs7578597, *ADAMTS9* rs4607103, *NOTCH2* rs10923931, *KCNQ1* rs2283228) was performed with the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA, USA) and Sequenom iPlex Gold SBE assay (for *MTNR1B* rs10830963, *ADRA2A* rs10885122, *FAM148A* rs11071657, *CRY2* rs11605924, *ADCY5* rs11708067, *SLC2A2* rs11920090, *FADS1* rs174550, *DGKB* rs2191349, *PROX1* rs340874, *GCK* rs4607517, *G6PC2* rs560887, *GLIS3* rs7034200, *GCKR* rs780094, *MADD* rs7944584). The TaqMan genotyping call rate was 100%, with an error rate of 0% in 4.5% of DNA samples genotyped in duplicate. Sequenom iPlex call rate was 90.2-96.9%, and error rate 0% among 4.2% of DNA samples genotyped in duplicate. All SNPs were consistent with Hardy-Weinberg equilibrium (p>0.001).

Gene expression data

Tissue-specific expression data were obtained from Gene-Sapiens [260], version IST4, which contains expression data from 15 liver and 16 adipose tissue samples from healthy human tissue, measured with Affymetrix (Santa Clara, CA, USA) gene expression microarrays. The following mean relative expression level values from GeneSapiens were used to classify expression levels: <300 for low, 300 to 700 for medium and >700 for high expression.

Liver histological analysis

Liver biopsies were performed using Tru-Cut (Radiplast, Uppsala, Sweden) during the elective gastric bypass operation. Histological assessment of the liver was

performed as described previously [261]. Steatosis was graded into four categories (<5%, 5–32%, 33–66% and >66%).

Lipoprotein subclasses

Proton Nuclear Magnetic Resonance (NMR) spectroscopy was used to measure lipid, lipoprotein subclass and particle concentrations in native serum samples as previously described [262]. Total lipid and particle concentrations of 14 lipoprotein subclasses were measured. The measurements of these subclasses have been calibrated using high-performance liquid chromatography methods [263]. The subclasses were as follows: chylomicrons and largest VLDL particles, five different VLDL subclasses: very large VLDL, large VLDL, medium-size VLDL, small VLDL, and very small VLDL; intermediate-density lipoprotein (IDL); three LDL subclasses: large LDL, medium-size LDL, and small LDL. The following components of the lipoprotein particles were measured: phospholipids, triglycerides, cholesterol, free cholesterol and cholesterol esters. In addition, mean particle diameters of VLDL and LDL fractions were calculated on the basis of the corresponding subclass distributions (IDL particles were included in the LDL fraction).

Calculations

The trapezoidal method was used to calculate glucose and insulin AUC in an OGTT based on 0, 30 and 120 min data (AUC 0–120 min). The Liver IR index was calculated from the formula: -0.091 + (log insulin AUC 0-120 min × 0.400) + (log fat mass % × 0.346) - (log HDL Cholesterol × 0.408) + (log BMI × 0.435). The hepatic insulin resistance index proposed by Abdul-Ghani *et al*. was calculated as described [264]. The adipocyte IR index was calculated as a product of fasting NEFA and fasting insulin, $Ins_0 \times NEFA_0$, as previously reported [265]. HOMA-IR and Matsuda ISI were calculated as described earlier [266,267]. A combined genetic risk score for liver IR was calculated as the sum of type 2 diabetes risk alleles at five SNPs that were significantly or nominally associated (after adjustment for age, BMI and WHR) with Liver IR index (at *KCNJ11*, *HHEX*, *CDKN2B*, *NOTCH2* and *MTNR1B* loci), and were weighted for their effect sizes.

For Study IV, early-phase insulin release during the first 30 min of an OGTT ($InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$), HOMA-B index of basal insulin secretion, and indices of proinsulin conversion in the fasting state ($Proins_0/Ins_0$), during the first 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 were calculated as reported previously

[268]. Disposition index was calculated as $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ × Matsuda ISI. A genetic risk score was calculated as a sum of FG-increasing alleles associated ($p<0.05$) with a trait weighted for their effect sizes for each trait individually.

Statistical analysis

Pearson correlation analysis was performed between EGP×FPI and all clinical and laboratory variables such as glucose and insulin AUC from an OGTT, fasting NEFA, lipoproteins, measures of adiposity, alanine transaminase, adiponectin and glucagon (for Study I) and between Liver IR index and Matsuda ISI with CVD risk factors (for Study III). Test of equality of correlations was based on Fisher's r to z-transformation where each correlation coefficient was converted into a z-score and compared taking into account the sample size. Forward multiple stepwise regression analysis was applied to identify the statistically significant determinants of EGP×FPI. The effect of sex and obesity on Liver IR index was tested using a univariate linear regression model.

In Study II, changes in indices across the categories of glucose levels were estimated by general linear model adjusted for age, BMI and WHR. Associations between SNPs and continuous traits were presented as effect sizes (β and SE) per copy of the type 2 diabetes risk allele, estimated by linear regression adjusted primarily for age, and additionally for age, BMI and WHR.

In order to examine the variance of different CVD risk factors explained by the Liver IR index and Matsuda ISI in Study III, the coefficient of determination (adjusted R^2) for CVD risk factors was calculated from linear regression adjusted for age.

In Study IV, the effect of the genetic risk score (GRS) on $Proins_0/Ins_0$ and $ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$ ratios was analyzed by linear regression adjusted for age, BMI, and Matsuda ISI. Hardy-Weinberg equilibrium was tested by χ^2-test. In all of the above studies, the variables were log-transformed to correct for their skewed distribution, except for Liver IR index, total cholesterol and LDL cholesterol that were normally distributed. Statistical power calculations were performed using Bioconductor's GeneticsDesign package version 1.14 [269]. $p<0.05$ was considered nominally significant in all studies. The thresholds for statistical significance after correction for multiple testing in each of the studies were as follows: $p<0.0013$ (Study II), $p<0.0019$ (Study III) and $p<8.68\text{x}10^{-5}$ (Study IV).

5. Results

5.1 A NOVEL SURROGATE INDEX FOR HEPATIC INSULIN RESISTANCE (*Study I*)

Baseline characteristics. The study sample included in this analyses comprised of 368 clinically healthy non-diabetic men and women from the RISC study (183 males; age 43±8.22; BMI 25.94±3.95), wherein a total of 288 subjects had normal glucose tolerance and 80 either impaired fasting glucose, impaired glucose tolerance, or both as per the ADA criteria.

Validation of Liver IR index. Correlation analyses were performed between EGPxFPI and all clinical and laboratory variables such as glucose and insulin AUC from an OGTT, fasting NEFA, lipoproteins, measures of adiposity, alanine transaminase, adiponectin and glucagon. Variables having correlation (r) of $r>0.2$ with EGP×FPI, including BMI, waist, fat mass %, HDL cholesterol, total triglycerides, adiponectin, glucose AUC 0-120 min, insulin AUC 0-120 min, were included in the multiple stepwise linear regression analysis. Four of these variables remained statistically significant in the regression model and were used to develop a novel surrogate marker of hepatic insulin resistance, Liver IR index as follows.

Liver IR index = -0.091 + (log Insulin AUC 0-120 × 0.400) +
(log fat mass % × 0.346) - (log HDL cholesterol × 0.408) + (log BMI × 0.435)

Liver IR index correlated significantly with EGP×FPI (r=0.65, p<0.001)

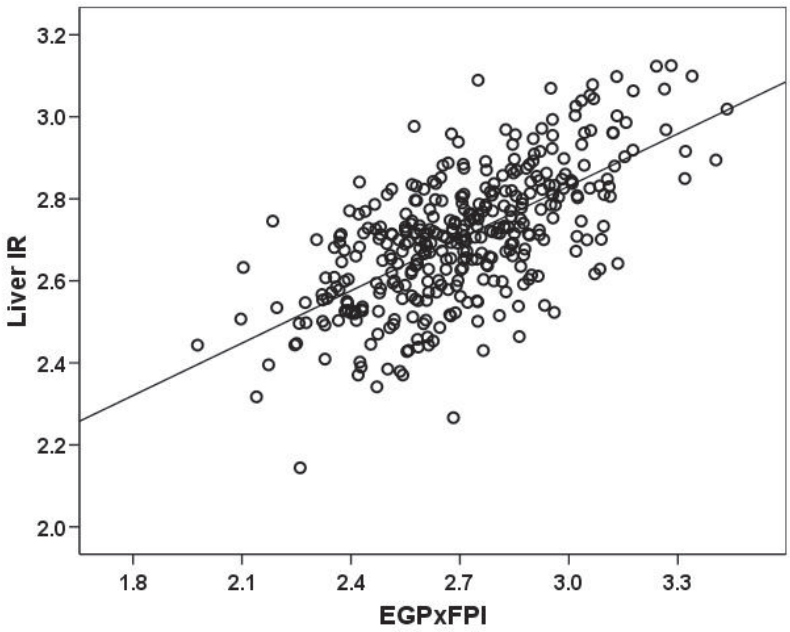

Figure 6. Pearson correlation of Liver IR index with endogenous glucose production (EGP) multiplied by fasting plasma insulin (FPI) (r=0.65, p<0.001)

Liver IR index exhibited a slightly stronger correlation with EGP×FPI in glucose intolerant subjects (r=0.69) than in normoglycemic subjects (r=0.62). Liver IR index correlated strongly in obese subjects (BMI>25, n=206, r=0.62, p<0.001) than in lean subjects (BMI≤25, n=162, r=0.53, p<0.001).

The correlation of Liver IR index with EGPxFPI was similar when compared with basal EGP and EGP during clamp. The correlation coefficients of the Liver IR index with EGP×FPI were similar for men and women (r=0.65).

5.2 ASSOCIATION OF SNPs ASSOCIATED WITH THE RISK OF TYPE 2 DIABETES WITH LIVER AND ADIPOSE TISSUE-SPECIFIC INSULIN RESISTANCE (*Study II*)

Baseline characteristics. Among the 8,460 non-diabetic subjects examined in this study, a total of 2,951 subjects had normal glucose tolerance, 4,181 isolated IFG, 302 isolated IGT, and 1,026 both IFG and IGT according to the ADA criteria.

Association of Liver IR and Adipocyte IR indices with glucose levels. Liver IR index was significantly associated with both FPG (increase up to 1.4%) and 2hPG

levels (increase up to 3.5%), p=3.1×10^{-12} and 5.4×10^{-103}, respectively. Similarly, Adipocyte IR index also showed a significant association with both FPG (increase up to +88%) and 2hPG (increase up to +108%), p=2.9×10^{-122} and 1.0×10^{-219}, respectively.

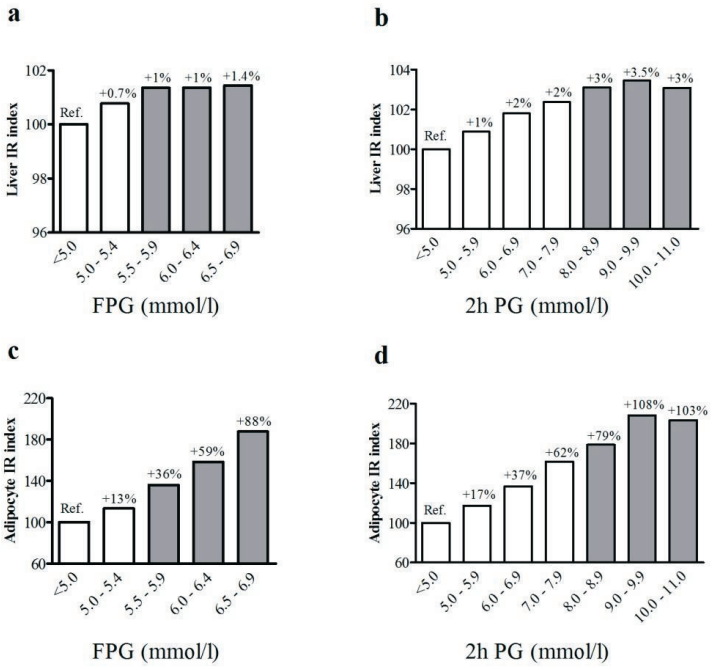

Figure 7. Liver IR (**a**,**b**) and Adipocyte IR (**c,d**) indices across the categories of fasting (FPG) and 2-hour plasma glucose (2hPG) levels within the non-diabetic range. Bars display values relative to the reference category (FPG < 5.0 mmol/l, 2hPG < 5.0 mmol/l). White bars indicate normal glucose levels (NFG and NGT) and grey bars indicate impaired glucose levels (IFG and IGT). Calculations were based on geometric means, adjusted for age, BMI and WHR with the general linear model and p values were obtained as follows: (**a**)3.1×10^{-12}, (**b**)5.4×10^{-103}, (**c**)2.9×10^{-122} and (**d**)1.0×10^{-219}

Association of type 2 diabetes SNPs with Liver IR index. *HHEX* rs1111875 and *MTNR1B* rs10830963 were significantly and four other SNPs (at *KCNJ11*, *TCF7L2*, *CDC123* and *NOTCH2* loci) nominally associated with Liver IR index in analyses adjusted for age only. When additional adjustments were done for BMI and WHR, risk alleles at five SNPs decreased Liver IR either significantly, *HHEX* rs1111875 (p=5.4×10^{-5}) and *KCNJ11* rs5219 (p=1.8×10^{-4}), or nominally, *CDKN2B* rs10811661 (p=0.013), *NOTCH2* rs10923931 (p=0.016) and *MTNR1B* rs10830963 (p=0.009). The *Pro12* allele (rs1801282) of *PPARG2* was associated with a higher Liver IR (p=0.039). All effect sizes were less than 0.3% per risk allele.

Table 3. Association of 19 SNPs with Liver IR index in 6,733 non-diabetic men

Gene SNP	Allele	MAF%	Liver IR index							Expression in liver
			adjusted for age only			adjusted for age, BMI and WHR				
			β	SE	*p* value	β	SE	%	*p* value	
PPARG2 rs1801282	C[a]/G	15.3	-0.0001	0.004	0.981	0.006	0.003	0.21	**0.039**	Low
KCNJ11 rs5219	G/A[a]	47.4	-0.009	0.003	**0.002**	-0.008	0.002	-0.27	**1.8E-04**	Low
TCF7L2 rs7903146	C/T[a]	17.5	-0.008	0.004	**0.034**	-0.004	0.003	-0.16	0.090	High
SLC30A8 rs13266634	C[a]/T	39.3	-0.003	0.003	0.359	0.0003	0.002	0.01	0.879	Low
HHEX rs1111875	C[a]/T	46.6	-0.010	0.003	**3.5E-04**	-0.008	0.002	-0.29	**5.4E-05**	High
rs7480010	A/G[a]	17.4	-0.007	0.004	0.083	-0.003	0.003	-0.10	0.288	-
CDKN2B rs10811661	A[a]/G	14.8	-0.005	0.004	0.191	-0.007	0.003	-0.25	**0.013**	Low
IGF2BP2 rs4402960	C/A[a]	32.0	-0.002	0.003	0.529	-0.001	0.002	-0.04	0.600	Low
CDKAL1 rs7754840	G/C[a]	36.9	-0.006	0.003	0.053	-0.003	0.002	-0.12	0.125	Low
WFS1 rs10010131	G[a]/A	44.6	-0.002	0.003	0.567	-0.001	0.002	-0.05	0.465	Medium
HNF1B rs7501939	C/T[a]	27.3	0.004	0.003	0.173	-0.0003	0.002	-0.01	0.865	High
JAZF1 rs864745	A[a]/G	48.4	0.004	0.003	0.192	0.004	0.002	0.14	0.054	Low
CDC123 rs12779790	A/G[a]	21.3	-0.007	0.003	**0.034**	-0.003	0.002	-0.11	0.212	High
TSPAN8 rs7961581	A/G[a]	19.9	-0.001	0.004	0.864	0.003	0.003	0.10	0.282	Medium
THADA rs7578597	A[a]/G	5.1	-0.005	0.007	0.427	-0.007	0.005	-0.24	0.142	Medium
ADAMTS9 rs4607103	G[a]/A	25.8	-0.001	0.003	0.792	-0.001	0.002	-0.04	0.600	Medium
NOTCH2 rs10923931	C/A[a]	14.0	-0.009	0.004	**0.023**	-0.007	0.003	-0.26	**0.016**	Medium
KCNQ1 rs2283228	A[a]/C	6.1	-0.002	0.006	0.785	-0.002	0.004	-0.06	0.704	Low
MTNR1B rs10830963	C/G[a]	35.8	-0.010	0.003	**0.001**	-0.005	0.002	-0.20	**0.009**	Low

Effect sizes (β and SE and % of β from mean) and corresponding *p* values per type 2 diabetes risk allele are shown. Results for the additive model are presented based on adjustments as indicated. Tissue-specific expression of respective genes was obtained from GeneSapiens database [260] [a] type 2 diabetes risk allele.

Further analyses revealed that additional adjustment for insulin secretion ($InsAUC_{0\text{-}30}$/$GluAUC_{0\text{-}30}$) abolished the associations of *PPARG*, *KCNJ11*, and *NOTCH2* with Liver IR, and weakened the association of *HHEX* with Liver IR (p=0.003) to a nominally significant level.

Combined effect of risk alleles on Liver IR index. The risk alleles of 5 SNPs that significantly or nominally reduced Liver IR (*KCNJ11* rs5219, *HHEX* rs1111875, *CDKN2B* rs10811661, *NOTCH2* rs10923931 and *MTNR1B* rs10830963) in adjusted analyses were combined to evaluate their joint effects. There was a small but consistent decrease, up to 2% (p=6.9×10^{-9}), in the Liver IR with an increasing number of risk alleles.

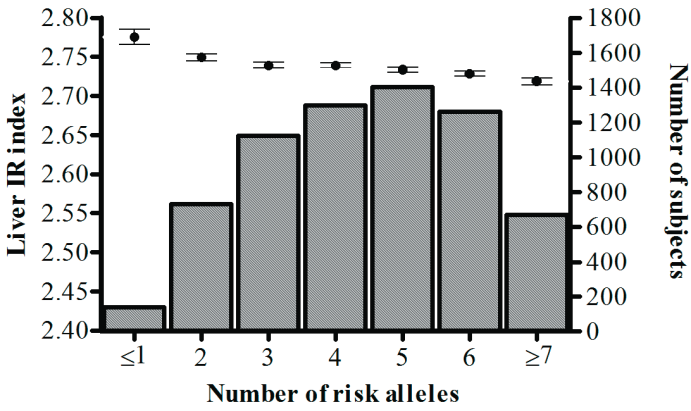

Figure 8. Liver IR index according to the number of type 2 diabetes risk alleles combined for five SNPs (*KCNJ11* rs5219, *HHEX* rs1111875, *CDKN2B* rs10811661, *NOTCH2* rs10923931 and *MTNR1B* rs10830963). Data are adjusted for age, BMI and WHR. Black circles with error bars indicate means and SE; grey bars show the number of participants in each category. The effect of the number of risk alleles on Liver IR index was significant (p = 6.9x10^{-9})

Association of type 2 diabetes SNPs with Adipocyte IR index. *PPARG2* rs1801282, *TCF7L2* rs7903146 and *NOTCH2* rs10923931 (p=0.026, 0.038 and 0.044, respectively) showed nominally significant association with adipocyte IR index when adjusted for age only. Additional adjustment for BMI and WHR strengthened the association of the *Pro12* allele of *PPARG2* with higher adipocyte IR index (p=6.2×10^{-5}, effect size +7% per allele) to a significant level, and abolished the other associations.

Table 4. Association of 19 SNPs with Adipocyte IR index in 6,733 non-diabetic men

Gene SNP	Allele	MAF%	Adipocyte IR index							Expression in adipose tissue
			adjusted for age only			adjusted for age, BMI and WHR				
			β	SE	*p* value	β	SE	%	*p* value	
PPARG2 rs1801282	C[a]/G	15.3	0.824	0.402	**0.026**	1.226	0.349	6.58	**6.2E-05**	High
KCNJ11 rs5219	G/A[a]	47.4	-0.634	0.290	0.081	-0.517	0.251	-2.77	0.124	Low
TCF7L2 rs7903146	C/T[a]	17.5	-0.582	0.377	**0.038**	-0.344	0.327	-1.85	0.089	High
SLC30A8 rs13266634	C[a]/T	39.3	-0.498	0.295	0.080	-0.300	0.256	-1.61	0.213	Low
HHEX rs1111875	C[a]/T	46.6	-0.559	0.287	0.098	-0.426	0.249	-2.29	0.169	High
rs7480010	A/G[a]	17.4	-0.896	0.383	0.071	-0.602	0.332	-3.23	0.236	-
CDKN2B rs10811661	A[a]/G	14.8	0.736	0.407	0.384	0.614	0.353	3.29	0.482	Low
IGF2BP2 rs4402960	C/A[a]	32.0	-0.462	0.307	0.310	-0.419	0.266	-2.25	0.278	Low
CDKAL1 rs7754840	G/C[a]	36.9	-0.233	0.299	0.119	-0.021	0.259	-0.11	0.361	Low
WFS1 rs10010131	G[a]/A	44.6	-0.495	0.289	0.297	-0.483	0.25	-2.59	0.224	High
HNF1B rs7501939	C/T[a]	27.3	0.241	0.333	0.510	-0.100	0.289	-0.54	0.546	Medium
JAZF1 rs864745	A[a]/G	48.4	0.247	0.286	0.126	0.233	0.248	1.25	0.076	Medium
CDC123 rs12779790	A/G[a]	21.3	-0.490	0.350	0.231	-0.194	0.304	-1.04	0.730	High
TSPAN8 rs7961581	A/G[a]	19.9	-0.319	0.361	0.767	-0.075	0.312	-0.40	0.614	Low
THADA rs7578597	A[a]/G	5.1	-0.111	0.657	0.960	-0.217	0.569	-1.16	0.907	Medium
ADAMTS9 rs4607103	G[a]/A	25.8	-0.475	0.332	0.168	-0.518	0.288	-2.78	0.075	Medium
NOTCH2 rs10923931	C/A[a]	14.0	-0.937	0.418	**0.044**	-0.771	0.362	-4.14	0.063	High
KCNQ1 rs2283228	A[a]/C	6.1	-0.079	0.600	0.861	-0.126	0.519	-0.68	0.762	Low
MTNR1B rs10830963	C/G[a]	35.8	-0.435	0.299	0.212	-0.162	0.259	-0.87	0.795	Low

Effect sizes (β and SE and % of β from mean) and corresponding *p* values per type 2 diabetes-risk allele (underlined) are shown. Results for the additive model are presented based on adjustments as indicated. *p* values for Adipocyte IR index were calculated using log-transformed variable due to its skewed distribution. Tissue-specific expression of the respective genes was obtained from GeneSapiens database [260]. [a] type 2 diabetes risk allele.

5.3 LIVER INSULIN RESISTANCE AND CARDIOVASCULAR RISK FACTORS (*Study III*)

Baseline characteristics. Among 8,750 non-diabetic men included in the analyses from the Metabolic Syndrome In Men (METSIM) Study, a total of 3,034 (34.7%) men had NGT, 4,345 (49.7%) had isolated IFG, 312 (3.6%) had isolated IGT and 1,059 (12.1%) had IFG and IGT, according to the ADA criteria.

Correlations of Liver IR index and Matsuda ISI with CVD risk factors. Liver IR index and Matsuda ISI correlated significantly ($p<0.0019$) with all CVD risk factors examined except for LDL cholesterol (both indices), and total cholesterol (Matsuda ISI). Liver IR index correlated significantly more strongly than Matsuda ISI with age

(*r*=0.161 *vs*. -0.067), total cholesterol (*r*=-0.088 *vs*. 0.020), hs-CRP (*r*=0.284 *vs*. -0.219), and total triglycerides (*r*=0.507 *vs*. -0.477, nominally significant difference $p<0.05$). In contrast, compared to Liver IR index, Matsuda ISI showed nominally stronger correlations with systolic and diastolic BP (*r*=-0.234 and -0.275 *vs*. *r*=0.202 and 0.239, respectively).

Table 5. Pearson correlation coefficients of Liver IR index and Matsuda ISI with various CVD risk factor levels in non-diabetic men

CVD risk factor	Liver IR index		Matsuda ISI		P^{comp}
	r	*p* value	*r*	*p* value	
Age	0.161	$<1\times10^{-6}$	-0.067	$<1\times10^{-6}$	$<1\times10^{-6}$
Systolic Blood Pressure	0.202	$<1\times10^{-6}$	-0.234	$<1\times10^{-6}$	0.027
Diastolic Blood Pressure	0.239	$<1\times10^{-6}$	-0.275	$<1\times10^{-6}$	0.011
Total cholesterol	-0.088	$<1\times10^{-6}$	0.020	0.066	$<1\times10^{-6}$
LDL cholesterol	0.011	0.297	-0.012	0.269	0.947
Total triglycerides	0.507	$<1\times10^{-6}$	-0.477	$<1\times10^{-6}$	0.009
Apolipoprotein B	0.251	$<1\times10^{-6}$	-0.230	$<1\times10^{-6}$	0.142
C-reactive protein	0.284	$<1\times10^{-6}$	-0.219	$<1\times10^{-6}$	$<1\times10^{-6}$
Plasma adiponectin	-0.340	$<1\times10^{-6}$	0.338	$<1\times10^{-6}$	0.882

Pearson correlation coefficients *r* and the corresponding *p* values of Liver IR index and Matsuda ISI with CVD risk factor levels are shown. P^{comp} represents *p* value indicating the difference between the two correlation coefficients.

Extent of variance of the CVD risk factors explained by Liver IR index and Matsuda ISI. The coefficient of determination, R^2, obtained from linear regression analyses to examine the extent of variance of CVD risk factors explained by both indices demonstrated that the Liver IR index explained more variance for the majority of CVD risk factors than did Matsuda ISI. The age-adjusted variance explained by Liver IR index was largest for total triglycerides (28%), whereas the corresponding percentage for Matsuda ISI was 24%. In order to determine the variance explained by the Liver IR index, independent of Matsuda ISI, both variables were included in the same model. These analyses revealed that the variance explained increased when Liver IR index was additionally included with Matsuda ISI in the model. Furthermore, the percent variance based on the R^2 change, including both the indices, indicated that Liver IR index explained a substantially higher proportion of the variation of total triglycerides, plasma adiponectin (11.6 *vs*. 0.2%), hs-CRP (8.1 *vs*. 0.6%) and apolipoprotein B (6.3 *vs*. 0.1%) than did Matsuda ISI.

Table 6. Variance of cardiovascular (CVD) risk factors explained by Liver IR index and Matsuda ISI in non-diabetic subjects

CVD risk factor	Liver IR index adjusted for age			Matsuda ISI adjusted for age			Liver IR index adjusted for age and Matsuda ISI		
	R^2	*p value*	*PV*	R^2	*p value*	*PV*	R^2	*p value*	*PV**
Systolic Blood Pressure	0.090	$<1\times10^{-6}$	4.1	0.110	$<1\times10^{-6}$	5.5	0.116	$<1\times10^{-6}$	0.5/4.8
Diastolic Blood Pressure	0.080	$<1\times10^{-6}$	5.7	0.093	$<1\times10^{-6}$	7.5	0.093	0.102	-/7.6
Total cholesterol	0.027	$<1\times10^{-6}$	0.8	0.023	0.364	-	0.038	$<1\times10^{-6}$	2.7/1.1
LDL cholesterol	0.029	0.0003	-	0.028	0.029	-	0.029	0.0002	0.1/-
Total triglycerides	0.284	$<1\times10^{-6}$	25.7	0.240	$<1\times10^{-6}$	22.7	0.285	$<1\times10^{-6}$	25.7/0.1
Apolipoprotein B	0.114	$<1\times10^{-6}$	6.3	0.092	$<1\times10^{-6}$	5.3	0.115	$<1\times10^{-6}$	6.3/0.1
hs-C reactive protein	0.080	$<1\times10^{-6}$	8.0	0.050	$<1\times10^{-6}$	4.8	0.086	$<1\times10^{-6}$	8.1/0.6
Plasma adiponectin	0.151	$<1\times10^{-6}$	11.6	0.138	$<1\times10^{-6}$	11.4	0.153	$<1\times10^{-6}$	11.6/0.2

Adjusted R squared (R^2) and *P* values obtained from linear regression analyses using CVD risk factor as dependent variable and independent variables as indicated. PV indicates percent variance of the CVD risk factor explained by the respective indices alone. PV* indicates the individual contribution of Liver IR index/Matsuda ISI, when both were included in the model together. (The percent variance is based on the R-squared change). '-' indicates that the respective index was not significant in the model (indicating that in such cases, age alone explained a larger variation).

5.4 ASSOCIATION OF SNPs INCREASING FASTING GLUCOSE WITH INDICES OF INSULIN RELEASE, PROINSULIN CONVERSION AND INSULIN SENSITIVITY (*Study IV*)

The association of 16 SNPs with $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ as an index of insulin release, Matsuda ISI as a measure for insulin sensitivity, disposition index and $Proins_0/Ins_0$ as an index for proinsulin processing were as follows.

Association with insulin release (Table 7). FG-increasing alleles at 8 SNPs (in or near *CRY2*, *DGKB*, *FADS1*, *GCK*, *GCKR*, *MTNR1B*, *SLC30A8*, and *TCF7L2*) were associated with the lower $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$ index of early-phase insulin release. Associations of *MTNR1B* and *TCF7L2* remained significant after correction for multiple testing and adjustment for BMI and Matsuda ISI in addition to age (effect sizes -6.5 and -4.2% per FG-increasing allele). Associations of *CRY2*, *FADS1* and *GCK* with insulin release were nominally significant after adjustments and *SLC30A8* persisted before and after adjustments (effect size magnitudes <5%), whereas associations of *DGKB* and *GCKR* disappeared after adjustment for Matsuda ISI (adjustment for BMI did not affect the association, data not shown). FG-increasing alleles at 13 SNPs (*ADRA2A*, *ADCY5*, *CRY2*, *DGKB*, *FADS1*, *GCK*, *GLIS3*, *G6PC2*, *MADD*, *MTNR1B*, *SLC2A2*, *SLC30A8*, *TCF7L2*) were associated with lower HOMA-B

index of basal insulin secretion after adjustment for age, BMI and Matsuda ISI. The effects of *DGKB*, *G6PC2* and *MTNR1B* remained significant after correction for multiple testing (effect sizes 4-6% per FG-increasing allele).

Association with insulin sensitivity (Table 7). A nominal association for *GCKR* rs780094, with an effect size of -2.6% in Matsuda ISI per FG-increasing allele was observed among the 16 SNPs examined.

Association with Disposition index (Table 7)**.** Six SNPs associated with lower disposition index (*CRY2*, *FADS1*, *GCK*, *MTNR1B*, *SLC30A8*, *TCF7L2*). Only association of *MTNR1B* remained significant after correction for multiple testing, with effect size of 6.6 per FG-increasing allele (adjusted for age and BMI).

Association with proinsulin conversion (Table 7)**.** FG-increasing alleles at 3 SNPs (*MADD*, *SLC30A8*, and *TCF7L2*) were significantly and consistently associated with the higher proinsulin/insulin ratio in the fasting state ($Proins_0/Ins_0$) and similarly in the glucose-stimulated state (not shown here). *MADD* showed the most prominent associations, with effect sizes of 7-10% per allele. The FG-increasing allele at the *GCKR* locus was nominally associated with a lower proinsulin/insulin ratio in the fasting state and during an OGTT (all indices) although this effect weakened after adjustment for Matsuda ISI, suggesting a possible compensatory change tin response to insulin resistance rather than a causal effect. Furthermore, adjustment for BMI and Matsuda ISI revealed nominal associations of *ADCY5* and GLIS3 loci with $Proins_0/Ins_0$.

Table 7. Associations of 16 SNPs increasing fasting glucose level with early-phase insulin release ($InsAUC_{0-30}/GluAUC_{0-30}$), basal insulin secretion (HOMA-beta), insulin sensitivity (Matsuda ISI), disposition index (DI= $InsAUC_{0-30}/GluAUC_{0-30}$ x Matsuda ISI) and proinsulin/insulin ratio in fasting state ($Proins_0/Ins_0$) in 8,750 non-diabetic Finnish men

Gene locus, SNP	Alleles	EAF	$InsAUC_{0-30}/GluAUC_{0-30}$				HOMA-beta				Matsuda ISI				Disposition index				$Proins_0/Ins_0$			
			Effect size				Effect size				Effect size				Effect size				Effect size			
		(%)	β	SE	*P*	*P*†	β	SE	*P*	*P*†	β	SE	*P*	*P**	β	SE	*P*	*P**	β	SE	*P*	*P**
ADCY5 rs11708067	A/G	15.9	0.135	0.566	0.870	0.994	-1.476	1.396	0.129	**0.002**	-0.069	0.112	0.848	0.860	-0.569	1.978	0.774	0.865	0.682	0.471	0.184	**0.045**
ADRA2A rs10885122	G/T	15.5	-0.748	0.584	0.673	0.406	-2.706	1.435	0.225	**0.011**	-0.066	0.116	0.891	0.850	-3.339	2.040	0.102	0.092	-0.766	0.483	0.233	0.110
CRY2 rs11605924	A/C	46.9	-0.417	0.429	0.192	**0.009**	-0.457	1.064	0.195	**4.3E-04**	-0.032	0.086	0.582	0.505	-3.146	1.505	**0.037**	0.084	0.183	0.355	0.913	0.277
DGKB rs2191349	G/T	43.1	-0.846	0.422	**0.027**	0.202	-4.396	1.043	**1.6E-04**	**2.9E-05**	0.126	0.084	0.066	0.089	-0.085	1.477	0.954	0.811	0.346	0.349	0.123	0.242
FADS1 rs174550	T/C	42.8	-0.625	0.423	0.076	**0.002**	-0.375	1.037	0.334	**0.049**	0.027	0.084	0.716	0.165	-4.052	1.477	**0.006**	**0.002**	0.306	0.348	0.863	0.641
FAM148A rs11071657	A/G	30.5	-0.279	0.449	0.849	0.155	0.236	1.110	0.593	0.682	-0.099	0.089	0.338	0.443	-2.960	1.567	0.059	0.072	0.006	0.372	0.851	0.453
GCK rs4607517	G/A	9.8	-0.951	0.701	0.334	**0.005**	-1.243	1.728	0.398	**0.002**	-0.194	0.139	0.239	0.372	-6.275	2.452	**0.011**	**0.015**	-1.022	0.584	0.170	0.721
GCKR rs780094	C/T	37.7	0.608	0.425	**0.042**	0.705	0.618	1.045	0.090	0.639	-0.161	0.084	**0.021**	**0.001**	-0.266	1.496	0.859	0.755	-1.004	0.351	**0.005**	**0.046**
GLIS3 rs7034200	C/A	48.8	0.010	0.416	0.897	0.482	-0.534	1.029	0.252	**0.004**	-0.077	0.082	0.689	0.797	-1.027	1.454	0.480	0.526	0.580	0.335	0.150	**0.024**
G6PC2 rs560887	C/T	27.8	0.890	0.461	0.168	0.215	-3.864	1.135	**9.2E-05**	**8.0E-16**	-0.042	0.092	0.474	0.098	1.461	1.613	0.365	0.493	0.152	0.384	0.947	0.874
MADD rs7944584	A/T	18	0.026	0.539	0.838	0.852	-1.372	1.327	0.364	**0.007**	-0.078	0.107	0.657	0.808	-0.326	1.888	0.863	0.941	2.309	0.449	**1.3E-09**	**1.1E-16**
MTNR1B rs10830963	C/G	35.8	-2.181	0.373	**1.4E-10**	**1.2E-19**	-4.456	0.899	**3.1E-09**	**7.1E-16**	0.028	0.074	0.466	0.828	-10.416	1.288	**7.0E-16**	**5.2E-18**	0.677	0.312	**0.045**	0.179
PROX1 rs340874	T/C	39.7	-0.114	0.423	0.424	0.308	-0.179	1.043	0.867	0.727	0.004	0.084	0.857	0.545	-1.370	1.478	0.354	0.403	0.322	0.352	0.289	0.286
SLC2A2 rs11920090	T/A	13.1	1.172	0.622	0.173	0.271	-1.151	1.531	0.384	**0.003**	-0.083	0.124	0.389	0.467	0.901	2.176	0.679	0.597	-0.025	0.515	0.600	0.256
SLC30A8 rs13266634	C/T	39.3	-1.018	0.370	**0.003**	**0.001**	-2.656	0.889	**0.004**	**2.7E-04**	0.036	0.073	0.272	0.782	-3.203	1.278	**0.012**	**0.004**	1.244	0.310	**1.6E-05**	**4.4E-06**
TCF7L2 rs7903146	C/T	17.5	-1.649	0.466	**4.1E-05**	**2.0E-06**	-3.590	1.111	**0.002**	**0.001**	0.098	0.091	0.191	0.661	-5.307	1.595	**8.8E-04**	**1.4E-04**	1.384	0.386	**3.3E-05**	**3.9E-06**

Effect size shown is β-coefficient and SE per copy of fasting glucose increasing allele (underlined), and was calculated using untransformed variables adjusted for age by linear regression. *P* values were calculated using log-transformed variables by linear regression. *P* values are adjusted for age; $P^{\dagger}$ values are adjusted for age, BMI, and Matsuda ISI; *P** values are adjusted for age and BMI. In the entire cohort, means±SE of examined parameters were as follows: $InsAUC_{0-30}/GluAUC_{0-30}$ 30.70±0.26 pmol/mmol, HOMA-beta 74.75±0.61, Matsuda ISI 6.96±0.05 [mg/dl, mU/l], Disposition index 163.7±0.9 and $Proins_0/Ins_0$ 32.60±0.22. Nominally significant *P* values ($P<0.05$) are in bold, *P* values significant after correction for multiple testing ($P<8.68\times10^{-5}$) are in bold and underlined. Results for the additive model are presented. EAF – effect allele frequency.

Association with fasting glucose (Table 8). Eleven SNPs (in or near *ADCY5*, *CRY2*, *DGKB*, *FADS1*, *GCK*, *GCKR*, *G6PC2*, *MTNR1B*, *SLC2A2*, *SLC30A8*, *TCF7L2*) were associated with higher FG levels, although only associations of *G6PC2* and *MTNR1B* remained statistically significant after correction for multiple testing. Adjustment for age and BMI did not affect the results. The effect sizes were modest with *MTNR1B* showing the largest effect (0.088 mmol/l or 1.5% increase in FG per risk allele).

Table 8. Association of 16 SNPs with fasting glucose levels in 8,750 non-diabetic Finnish men

			Fasting glucose (mmol/l)			
Gene locus, SNP	**Alleles**	**EAF**	**Effect size**			
		(%)	**β**	**SE**	***P***	***P* ***
ADCY5 rs11708067	A/G	15.9	0.028	0.013	**0.034**	**0.038**
ADRA2A rs10885122	G/T	15.5	0.021	0.014	0.105	0.098
CRY2 rs11605924	A/C	46.9	0.031	0.010	**0.004**	**0.010**
DGKB rs2191349	G/T	43.1	0.031	0.010	**0.002**	**0.001**
FADS1 rs174550	T/C	42.8	0.025	0.010	**0.012**	**0.004**
FAM148A rs11071657	A/G	30.5	0.010	0.011	0.280	0.319
GCK rs4607517	G/A	9.8	0.055	0.016	**0.001**	**0.001**
GCKR rs780094	C/T	37.7	0.029	0.010	**0.004**	**0.002**
GLIS3 rs7034200	C/A	48.8	0.018	0.010	0.057	0.068
G6PC2 rs560887	C/T	27.8	0.071	0.011	**3.5E-11**	**1.9E-12**
MADD rs7944584	A/T	18.0	0.022	0.013	0.094	0.108
MTNR1B rs10830963	C/G	35.8	0.088	0.009	**5.1E-24**	**2.6E-27**
PROX1 rs340874	T/C	39.7	0.011	0.010	0.277	0.318
SLC2A2 rs11920090	T/A	13.1	0.034	0.015	**0.017**	**0.019**
SLC30A8 rs13266634	C/T	39.3	0.027	0.009	**0.001**	**2.7E-04**
TCF7L2 rs7903146	C/T	17.5	0.027	0.011	**0.016**	**0.004**

Effect size shown is β-coefficient (SE) per copy of fasting glucose increasing allele (underlined), and was calculated by linear regression adjusted for age. *P* values were adjusted for age, P^* values were adjusted for age and BMI. In the entire cohort, mean±SE of fasting glucose was 5.70±0.48. Nominally significant *P* values ($P<0.05$) are in bold, *P* values significant after correction for multiple testing ($P<8.68\times10^{-5}$) are in bold and underlined. Results for the additive model are presented. EAF – effect allele frequency.

Analysis of traits according to the genetic risk scores (Figure 9)**.** By combining the risk alleles at SNPs, nominally or significantly associated with the trait of interest, a GRS for each individual trait was calculated. An increasing number of FG-increasing alleles was associated with a gradual decrease in $InsAUC_{0\text{-}30}/GluAUC_{0\text{-}30}$, HOMA-B and the disposition index by 20, 24 and 24%, respectively, in carriers with the largest number of risk alleles. Although the effects of the GRS on basal and glucose-stimulated insulin secretion were comparable, the increase in the proinsulin/insulin ratio during an OGTT ($ProinsAUC_{0\text{-}30}/InsAUC_{0\text{-}30}$) was considerably larger than that in the fasting state (42 vs. 27%). In contrast, the GRS had only a rather modest effect on the FG level (a 7% increase in the carriers of ≥19 risk alleles compared with the carriers of ≤8 risk alleles at 11 SNPs).

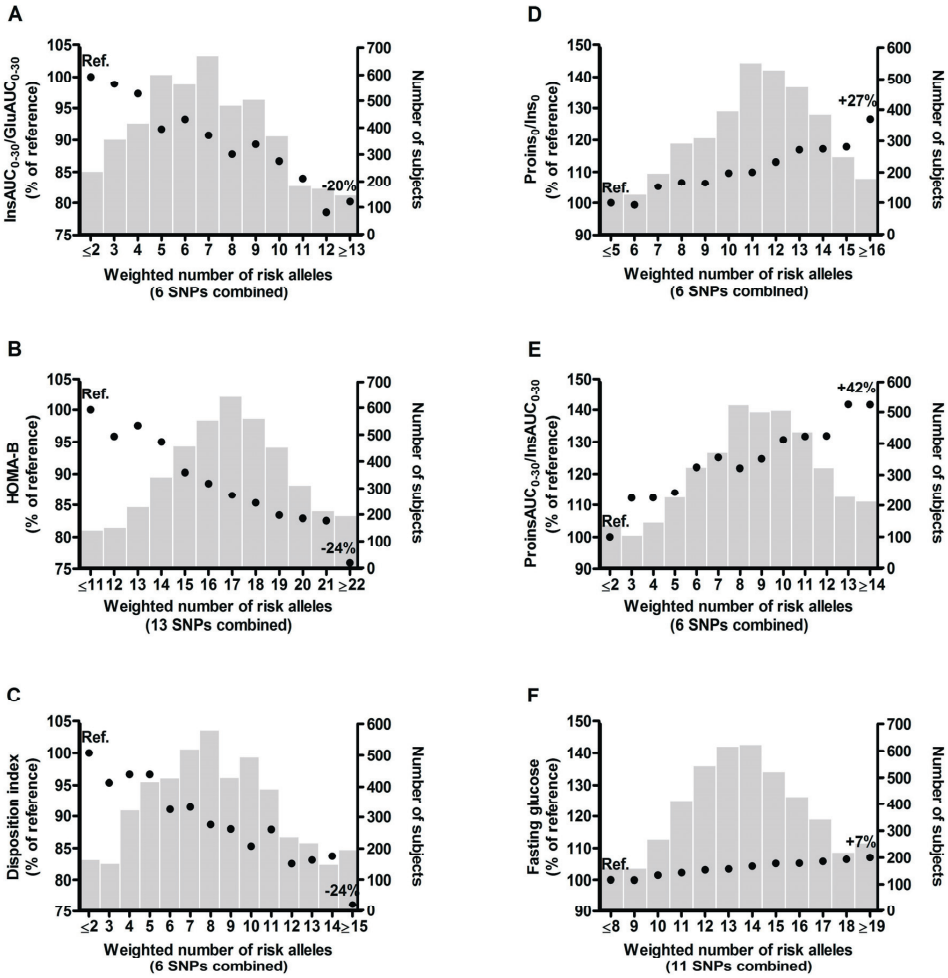

Figure 9. Combined effects of associated (P<0.05) SNPs on the glucose- and insulin-related traits as follows: **A** – early-phase insulin release ($InsAUC_{0-30}/GluAUC_{0-30}$), **B** – basal insulin secretion (HOMA-B), **C** – Disposition index, **D** - index of proinsulin-to-insulin conversion at fasting state ($Proins_0/Ins_0$), **E** - index of proinsulin-to-insulin conversion during 0-30 min of an OGTT ($ProinsAUC_{0-30}/InsAUC_{0-30}$), **F** – fasting glucose. Genetic risk scores were calculated as the number of FG-increasing (risk) alleles in SNPs associated (P<0.05) with the respective trait weighted for their effect sizes. Filled circles display the value of the respective trait relative to the reference category with the lowest number of risk alleles. Calculations were based on geometric means, adjusted for age, BMI, (Matsuda ISI) with the general linear model. Bars show the numbers of subjects in each category.

6. Discussion

6.1 REPRESENTATIVENESS OF THE STUDY SUBJECTS

The study involved two well-characterized and large study samples 1) the European Group for the study of Insulin Resistance-Relationship between Insulin Sensitivity and Cardiovascular disease (EGIR-RISC) study 2) the Metabolic Syndrome In Men (METSIM) study.

Study I included 368 healthy subjects from the EGIR-RISC study cohort. The study includes data pooled from euglycemic hyperinsulinemic clamp experiments performed in 21 centres across Europe which makes it one of the largest database available with clamp data. Baseline measurements of glucose tolerance and insulin sensitivity were performed by an OGTT and the euglycemic insulin clamp. In this study, hepatic insulin resistance was measured by using the glucose tracer technique (tritiated glucose) and calculated as the product of endogenous glucose production and fasting plasma insulin (EGPxFPI), considered to be the gold-standard measure. Measurement of hepatic insulin resistance with the most accurate gold-standard method in this study was ideal towards achieving our objective of developing a novel surrogate index for assessing insulin resistance in the liver. One limitation of the study is that though it includes data from different centers, they are all of European origin, therefore the genetic differences in the participants are likely to be small.

Studies II – IV included >6700 Finnish middle-aged non-diabetic subjects from the Kuopio region who were participants in the METSIM study. The study population has been well-characterised and the subjects were carefully phenotyped. Baseline measurements of glucose tolerance and insulin sensitivity were measured by an OGTT. Measurement of insulin sensitivity with glucose clamp, the gold-standard method was not feasible, owing to the large sample size. However, the OGTT-derived indices employed in the study have been validated and are being used in several studies. Furthermore, most of the reported genetic variants/polymorphisms related to type 2 diabetes have been genotyped in the entire study population. The effects of genetic variants on type 2 diabetes were modest. The effects of risk loci on insulin sensitivity were not as strong as their effects on insulin secretion. One of the limitations of the study is the inclusion of only men. However, no consistent differences have been reported between men and women in terms of genetic associations.

6.2 HEPATIC INSULIN RESISTANCE (*Studies I-II*)

The inability of insulin to suppress endogenous glucose production (EGP) and to promote glucose uptake is a major metabolic consequence of insulin resistance in the liver. As the diabetic state worsens, an increase in basal hepatic glucose production and/or its decreased suppression by insulin are the major factors responsible for a progressive rise in the fasting glucose levels.

A linear relationship exists between the rise of the fasting plasma insulin (FPI) level and the decline in the rate of basal EGP in healthy subjects. Endogenous glucose production primarily reflects hepatic glucose production as >80% of it originates in the liver [270]. Thus, the higher the rate of EGP and the level of FPI, the greater is the severity of hepatic insulin resistance. Based on the above principle, the concomitant use of isotopes in clamp studies has revealed that hepatic glucose production is insufficiently inhibited by insulin, demonstrating the presence of insulin resistance in the liver and therefore, a product of endogenous glucose production and fasting plasma insulin (EGPxFPI) is widely accepted as the golden standard measure for hepatic insulin resistance [259].

6.2.1 Development of Liver IR index (*Study I*)

We developed a novel surrogate marker, Liver IR index, for hepatic insulin resistance that correlated strongly with EGPxFPI. Unlike other markers that are based mainly on OGTT-derived measures of glucose and insulin, this index takes into consideration various metabolic traits that are related to hepatic insulin resistance, e.g. BMI, fatmass and HDL cholesterol. Obesity is a known risk factor for hepatic insulin resistance. Moreover, intra-abdominal visceral fat and abdominal subcutaneous fat also correlate positively with hepatic insulin resistance [271]. Increased BMI and fatmass elevate the NEFA levels that directly promote liver insulin resistance. Hepatic insulin resistance has also been described to reduce the HDL cholesterol level [272]. Furthermore, it has been reported in a large study involving non-diabetic subjects that estimates of obesity (BMI and waist circumference) and dyslipidemia (TG/HDL-C ratio) are associated with features of liver disease e.g., non-alcoholic fatty liver disease (NAFLD), where liver insulin resistance is a characteristic feature [273].

Previously only one study reported a surrogate marker specific for liver insulin resistance [264]. It was calculated as product of glucose and insulin AUCs during 0-30 min. However, the index showed a lower correlation (r=0.58, p<0.001)

when compared to the Liver IR index (r=0.65, p<0.001) in our study. Moreover, the reported index was based on a study population that included mainly obese individuals.

Therefore, in this study a novel surrogate index for hepatic insulin resistance was developed and it was found to correlate well with the gold standard measure in non-diabetic subjects, irrespective of obesity and glucose tolerance status. However, further studies need to be conducted in other ethnic groups and in women.

6.2.2 Hyperglycemia and hepatic and adipocyte insulin resistance (*Study II*)

Since insulin resistance manifests itself in different tissues, some of the known type 2 diabetes risk variants could have tissue-specific effects on insulin action. Therefore, the Liver IR index and additionally a previously reported adipocyte IR index [268] were used to investigate hepatic and adipose tissue specific insulin resistance in the current study.

Insulin resistance in the adipose tissue leads to an impairment in insulin's antilipolytic effect, contributing to an increased flux of NEFAs into the circulation, which in turn stimulates gluconeogenesis, decreases glucose uptake, induces hepatic and muscle IR, and impairs insulin secretion [274,275]. The adipocyte insulin resistance index (product of fasting plasma NEFA and FPI) was reported to be significantly increased in both IFG and IGT subjects as compared to individuals with NGT [265]. The adipocyte IR index increased by ≥88% with increasing FPG and 2hPG levels within the nondiabetic range of glycemia. An increased adipocyte IR index indicates a loss of the antilipolytic effect of insulin.

Insulin resistance in the liver results in impaired suppression of hepatic glucose production, contributing to elevated glucose levels in fasting and postprandial states [77,78]. Our observation of a gradual increase of the Liver IR index by 1.4 to 3.5% with higher levels of FPG and 2hPG agrees with these findings. These associations with FPG and 2hPG levels are evidence that these indices are likely to be reliable indicators of IR in the liver and adipose tissue.

SNPs regulating liver insulin resistance

The type 2 diabetes risk allele, rs5219 of *KCNJ11*, was significantly associated with a lower Liver IR index. Similar results have been reported in a study showing that

homozygous carriers of the rs5219 risk allele of *KCNJ11* displayed a ~40% increase in hepatic insulin sensitivity as measured by the clamp and tracer infusion, in addition to an insulin secretion defect [143]. A nominal association of this variant with increased Matsuda ISI had been previously reported in our METSIM study [268]. Thus, this variant might influence type 2 diabetes risk by altering insulin secretion and also liver insulin sensitivity.

The type 2 diabetes risk allele of *HHEX* also showed a significant association with lower liver IR. There is only one study reporting the association of *HHEX* rs1111875 with greater insulin sensitivity measured by Matsuda ISI [268]. The mechanisms by which *HHEX* affects liver insulin sensitivity are unknown, but hepatocyte nuclear factor 1α (HNF1α) could be involved, as *HHEX*, which is abundantly expressed in the liver, has been shown to directly activate HNF1α in mammalian hepatocytes [276]. Furthermore, *HHEX* is required for the organogenesis of the ventral pancreas, indicating that early developmental defects due to this gene variant might be plausible [277].

Additionally, SNPs of *MTNR1B*, *CDKN2B*, *NOTCH2* and *PPARG2* showed nominally significant associations with the Liver IR index. In agreement with our results, a different SNP of *MTNR1B* has been reported to be associated with decreased HOMA-IR [278]. In contrast, a study based on the hyperinsulinemic-euglycemic clamp showed that the *MTNR1B* risk allele reduced suppression of hepatic glucose production, suggesting that it could be associated with hepatic IR [279]. *CDKN2B* polymorphism rs10811661 has been recently reported to be associated with insulin release but not with insulin sensitivity as measured by hyperinsulinemic euglycemic glucose clamp or OGTT [280]. There have been no reports of an association of *NOTCH2* rs10923931 with insulin sensitivity, however both *NOTCH2* and *CDKN2B* have a role in the development of the liver [281,282].

Combined analyses of the five SNPs in *KCNJ11*, *HHEX*, *CDKN2B*, *NOTCH2* and *MTNR1B* detected a significant decrease in the Liver IR index (2%), although these SNPs individually had an effect size of <0.3%. In an earlier study, we reported that SNPs in or near *HHEX*, *CDKN2B*, *MTNR1B* and *KCNJ11* were associated with lower glucose-stimulated insulin release and showed comparatively larger effect sizes from 1.2% to 6.7% per risk allele [268]. We believe that the variants of these genes primarily affect insulin secretion and that their effect on liver insulin sensitivity is of less importance and might be a compensatory mechanism.

The associations between risk SNPs and Liver IR index either lost their statistical significance (*KCNJ11*, *CDKN2B*, *NOTCH2*) or weakened (*HHEX*) when adjusted for insulin secretion (in addition to age, BMI and WHR). Furthermore, previous studies have shown that *HNF1A* mutation carriers who have MODY [283] and carriers of the E1506K mutation of *ABCC8* [284] display increased insulin sensitivity, although they have a severe defect in insulin secretion. Another study showed that high insulin sensitivity, evaluated by the euglycemic clamp, reflecting mostly skeletal muscle insulin sensitivity, could protect against the detrimental effect of several genes on insulin secretion [285].

Based on the above observations in conjunction with the results from our study it was hypothesized that the increased liver insulin sensitivity conferred by these risk alleles could be a mechanism to counterbalance the impaired insulin secretion. However, these compensatory mechanisms might be effective only in the non-diabetic range of glycemia, and fail when frank hyperglycemia develops.

SNPs regulating adipocyte insulin resistance

The Pro12Ala polymorphism of *PPARG2* was significantly associated with a higher adipocyte IR index. The Ala allele of rs1801282 has been previously shown to be associated with insulin sensitivity [96,286,287]. The Ala isoform due to its lower transactivation capacity may lead to less efficient stimulation of PPARγ target genes and predispose to lower levels of adipose tissue mass accumulation, which in turn may be responsible for improved insulin sensitivity. In contrast, the more common proline allele (~85% frequency) is known to be associated with a modest (1.25-fold) but statistically significant increase in the risk of diabetes.

PPARγ is a nuclear factor regulating the transcription of various genes, particularly adipose specific genes involved in adipocyte differentiation; it also contributes to the regulation of NEFA metabolism by stimulating uptake, storage and oxidation of NEFA in adipocytes [288]. Since the Pro12Ala polymorphism is present only in *PPARG2*, which is found prominently in adipose tissue [289], it is probable that this SNP exerts its effect on insulin sensitivity directly in adipose tissue. Increased release of NEFA from insulin-resistant adipose tissue may further impair insulin sensitivity in the liver and this might well explain its weak association with Liver IR index.

6.3 LIVER INSULIN RESISTANCE AND CVD RISK FACTORS (*Study III*)

There is a significant crosstalk between the liver, muscle and adipose tissue and various factors that affect cardiovascular health. Therefore, it is likely that the pathophysiological state of these organs is reflected also in risk factors for CVD. Under insulin resistant conditions, the metabolic imbalance causes abnormal production of several hormones, cytokines, inflammatory factors etc. depending on the respective tissue and adversely affects cardiovascular health.

The association of Liver IR index and Matsuda ISI with various CVD risk factor levels was investigated systematically in a large population-based sample of non-diabetic men. It was found that Liver IR index correlated more strongly with total triglyceride levels than with Matsuda ISI, although the difference was only nominally significant. Total triglyceride levels increased up to 110% across the quintiles of Liver IR index (explaining 28% of variation) and decreased to -51% across the quintiles of Matsuda ISI. A meta-analysis including 29 studies reported that elevated triglyceride levels were associated with increased coronary heart disease risk which supports our observation [290]. Another study reported that a progressive increase in the incidence of major coronary events was associated with progressively increased levels of fasting triglyceride levels over eight years of follow-up [291]. However, there is still controversy on the causal role of total triglycerides as a risk factor for CVD events.

Various inflammatory factors/adipokines that are CVD risk factors are secreted directly from adipocytes and adipose tissue-derived macrophages. These in turn contribute to inflammation and the increased synthesis of inflammatory proteins, e.g. CRP in the liver. In agreement with these observations, it was noticed that hs-CRP levels correlated more closely with the Liver IR index than with the Matsuda ISI, and that hs-CRP increased significantly (up to +104%) across the quintiles of Liver IR index. A recent meta-analysis comprising >7 000 patients with coronary heart disease events showed that subjects with hs-CRP in the upper tertile had a 50% increased risk for the development of acute CVD events [292].

A larger change in adiponectin levels was observed across the quintiles of Matsuda ISI than across the quintiles of Liver IR index (+53 vs. -36%) which is in agreement with the studies indicating that hyperinsulinemia decreases adiponectin levels [293]. Adiponectin might directly modify hepatic insulin sensitivity since a

negative correlation has been reported between plasma adiponectin level and endogenous glucose production [294].

Systolic and diastolic BP are important CVD risk factors often associated with insulin resistance. In subjects with hypertension the major site of insulin resistance is in peripheral insulin sensitivity tissues (mainly in skeletal muscle) and not in the liver [295], which could explain nominally stronger correlations of systolic and diastolic BP with Matsuda ISI than with Liver IR index in the current study.

The conclusion drawn from Study III is that the Liver IR index correlates more strongly with the majority of CVD risk factors than does Matsuda ISI which reflects whole body insulin sensitivity. Therefore, it is likely that insulin resistance especially in the liver plays a major role in the adverse changes in CVD risk factors, particularly those related to dyslipidemia and inflammation.

6.4 EFFECTS OF SNPS ELEVATING GLUCOSE ON INSULIN SECRETION AND INSULIN SENSITIVITY (*Study IV*)

Fasting hyperglycemia is one of the hallmarks of liver insulin resistance and type 2 diabetes. To investigate the mechanisms underlying the elevation of fasting glucose by the reported FG-elevating SNPs, we tested the association of these SNPs with various indices of insulin secretion and insulin sensitivity. We found that several FG-related loci had an effect on insulin release (basal or glucose-stimulated) and/or on indices of proinsulin conversion, whereas only one locus (*GCKR*) was nominally associated with insulin sensitivity.

6.4.1 Effects on insulin secretion

Basal insulin secretion

The balance between glucose uptake and glucose production in the liver maintains FG levels. Hepatic glucose production is stimulated by glucagon, whereas insulin prevents excessive release of glucose by the liver. Therefore, the association of 16 FG-increasing loci with decreased HOMA-B (reflecting basal insulin secretion) reported in a previous study [18] was not unexpected. Our results are in agreement with these findings, although most of the associations were nominally significant, and only the results of *G6PC2*, *MTNR1B*, and *DGKB* remained significant after correction for multiple testing.

SNPs of the three genes in *G6PC2*, *MTNR1B*, and *DGKB* displayed the largest effects on HOMA-B (5-6% per allele) in this study. *G6PC2* encodes glucose-6-phosphatase catalytic subunit 2, which catalyses dephosphorylation of glucose-6-phosphate, thus opposing the action of glucokinase, a glucose-sensor of the beta-cell modulating insulin secretion. *MTNR1B* encodes the receptor 1B for melatonin, a hormone playing an important role in the regulation of circadian rhythms with widespread biological functions. Melatonin has an inhibitory effect on insulin secretion [296]. It is likely that these variants enhance the expression of the gene or activity of the protein, thereby increasing glucose levels. *DGKB* encodes for diacylglycerol kinase β, which phosphorylates diacylglycerol (DAG). In the beta-cell, DAG activates protein kinase C (PKC), which further leads to an increase in insulin secretion, whereas phosphorylation of DAG terminates its action on PKC [297].

Glucose-stimulated insulin secretion

A defective first-phase insulin release to glucose stimulation is considered as a characteristic feature of type 2 diabetes. The FG-increasing alleles at the *MTNR1B* and *TCF7L2* loci were significantly associated with impaired glucose-induced insulin secretion. In addition, nominally significant associations with impaired glucose-stimulated insulin secretion were found for *CRY2*, *FADS1*, *GCK*, and *SLC30A8* (after adjustment for age, BMI and Matsuda ISI). *MTNR1B*, *TCF7L2* and *SLC30A8*, the established type 2 diabetes risk loci, have been previously shown in several studies to affect insulin secretion in OGTT or intravenous glucose-tolerance tests [154,298,299]. Similar associations of *FADS1* and *GCK* with an early-phase insulin release during an OGTT (insulinogenic index) have also been reported [183].

GCK encodes glucokinase, which catalyzes the phosphorylation of glucose in the beta-cell and liver. In the beta-cell, glucokinase catalyses the first step of the glycolytic pathway and thereby functions as a glucose sensor regulating insulin secretion [300]. Mutations in *GCK* have been implicated in several disorders of glucose metabolism such as maturity-onset diabetes of the young (MODY) and permanent neonatal diabetes [301]. The SNP of *GCK* examined in this study has also been shown to increase the risk of type 2 diabetes [18]. *FADS1* encodes fatty acid desaturase 1, regulating the unsaturation of fatty acids. Non-esterified fatty acids potentiate glucose-stimulated insulin secretion in murine beta-cells in vitro [302,303]. Interestingly, *FADS1* had a larger effect on glucose-stimulated ($InsAUC_{0-30}/GluAUC_{0-30}$) than on basal (HOMA-B) insulin secretion in the current study (2.4 vs 0.8% per risk allele). *CRY2* encodes cryptochrome 2 (Cry2), which is implicated in the

regulation of the circadian feedback loop, and could therefore modulate the circadian rhythm of insulin secretion. Interestingly, mice lacking Cry1 and Cry2 proteins exhibited impaired glucose tolerance due to decreased insulin secretion [195-197].

Proinsulin processing

The effects on proinsulin processing, in addition to effects on insulin secretion, have been previously reported for *TCF7L2*, *SLC30A8*, and *MTNR1B* [154,167,268,304]. In the present study, FG-increasing alleles at the *TCF7L2* and *SLC30A8* loci were significantly associated with the high proinsulin/insulin ratio in the fasting state. Impaired proinsulin processing is likely to be one of the mechanisms contributing to impaired insulin secretion associated with these loci, although an increase in the proinsulin/insulin ratio secondary to other abnormalities cannot be excluded. Additionally, the *MADD* locus had the most prominent effects on proinsulin conversion, with effect sizes ~2-times larger than those of *TCF7L2*. *MADD* encodes MAP-kinase activating death domain, which is involved in the regulation of cell proliferation, survival and death, and is expressed especially in neoplastic cells [305]. Although the mechanism by which the *MADD* locus regulates proinsulin processing is unclear, there could also be a role of the neighboring genes.

GCKR was nominally associated with decreased proinsulin/insulin ratio. Since *GCKR* appears to regulate insulin sensitivity, decreased proinsulin/insulin ratio could reflect higher demand for insulin to compensate for insulin resistance. An inverse relationship between insulin resistance and the low proinsulin/insulin ratio in non-diabetic subjects has been reported previously [39].

6.4.2 Effects on insulin sensitivity

FG levels also depend on insulin sensitivity, particularly in the liver. A nominally significant reduction in Matsuda ISI was detected for an FG-increasing allele of rs780094 of *GCKR*. This finding agrees with the results of previous studies [15,306,307] and a meta-analysis of GWA studies, where *GCKR* was the sole FG-related locus associated with HOMA-IR [18] and OGTT-derived indices of insulin sensitivity [183]. *GCKR* encodes the glucokinase regulatory protein (GKRP) that inhibits the effects of glucokinase on glycogen synthesis and gluconeogenesis in the liver [122,308]. Moreover, impaired regulation of glucokinase by GKRP has been implicated in the pathophysiology of the *MODY2* [124,309], characterized by impaired insulin secretion and increased hepatic glucose production [180,310].

Surprisingly, no significant association was found with Liver IR index probably because of the very modest effects of these loci that might need even larger studies with larger statistical power to be detected.

The effects of the tested SNPs on the FG level in our study were rather similar to those reported in the meta-analysis [18], although only the effects of *G6PC2* and *MTNR1B* were statistically significant. It was not possible to confirm associations for 5 SNPs (*ADRA2A*, *FAM148A*, *GLIS3*, *MADD*, and *PROX1*) probably due to a limited statistical power of our study. We observed a larger effect of the genetic risk score on the proinsulin/insulin ratio during an OGTT (increase by 42%) than in the fasting state (increase by 27%) suggesting that the defect(s) in the conversion of proinsulin to insulin becomes more apparent during glucose-stimulation than in the fasting state, and could therefore be underestimated by fasting measurements alone.

In summary, our study demonstrated that several newly confirmed candidate loci for fasting hyperglycemia were associated especially with early-phase insulin release and/or with indices of proinsulin-to-insulin conversion.

6.5 CONCLUDING REMARKS

Insulin resistance is one of the major pathophysiological features of type 2 diabetes. It is known to be present in the prediabetic state several years before the development of type 2 diabetes. Insulin resistance also regulates significantly the levels of several CVD risk factors.

Insulin resistance can be observed in several tissues, especially in the muscle, liver and adipose tissue. Although there are accurate methods to measure tissue-specific insulin resistance with the glucose clamp technique combined with tracers, these methods are cumbersome and not feasible for application in large population-based studies. Therefore, the availability of surrogate markers of tissue-specific insulin resistance provides us with tools basing on which it is possible to investigate the pathophysiology of insulin resistance at the population level.

During the last five years, GWA studies have identified a large number of candidate genes and several risk loci that increase the risk of type 2 diabetes and hyperglycemia. Many of these genes were novel and not predicted to play a significant role in the pathophysiology of type 2 diabetes. Surprisingly, most of the gene variants reported so far have been associated with impaired insulin secretion

and a very few with insulin resistance. Few of the several reasons might be 1) the lower heritability of insulin resistance, as lifestyle and the environment seems to play a major role, 2) lack of good markers of insulin resistance that are able to capture the modest effects of insulin resistance in different tissues and 3) lack statistical power to detect these modest associations.

The current study led to the development of a novel Liver IR index that correlated strongly with the gold standard measure for hepatic insulin resistance. The index performed well irrespective of the obesity and glucose tolerance status in the non-diabetic range. By applying this Liver IR index in the METSIM study, it was possible to identify that type 2 diabetes risk loci in *KCNJ11* and *HHEX* confer increased hepatic insulin sensitivity. Furthermore, the Pro12 allele in *PPARG* was found to confer predominantly adipocyte insulin resistance. These results demonstrate that evaluation of tissue-specific insulin resistance is very likely to increase the number of gene variants regulating insulin resistance. Our findings also demonstrated the close connection between tissue-specific insulin resistance and insulin secretion.

Insulin resistance significantly increases the risk of CVD. This association was based on studies which applied whole-body level measures of insulin resistance (euglycemic clamp or Matsuda ISI). No previous study has examined the association of tissue-specific insulin resistance in a large population based study. The current study demonstrated that insulin resistance, especially in the liver, affects CVD risk factors, especially those involved in low-grade inflammation and dyslipidemia. We further demonstrated that some of the SNPs elevating fasting glucose also regulate proinsulin processing and insulin secretion. However, we could not detect any significant association of these SNPs with Liver IR index probably as their effect sizes are very modest. Their role as CVD risk factors remain to be elucidated.

In spite of the development of new technologies of genetics during the last few years, common variants explain only a small proportion of the genetic variation of different clinical or laboratory traits. In the future, the application of exome and whole genome sequencing might substantially increase our understanding of the role of genetic factors regulating insulin secretion and insulin resistance and how these modify the risk of type 2 diabetes.

7. Summary

The main findings of the Studies I-IV were as follows:

Study I: A novel surrogate index for hepatic insulin resistance was developed that correlates strongly with the gold-standard measure of hepatic insulin resistance in non-diabetic subjects, irrespective of glucose tolerance and obesity status.

Study II: Among the type 2 diabetes risk loci, *KCNJ11* rs5219 and *HHEX* rs1111875 were associated with increased liver insulin sensitivity whereas *PPARG* rs1801282 was associated with higher adipocyte insulin resistance. These results indicate that type 2 diabetes risk loci might have tissue-specific effects.

Study III: Liver IR index reflecting hepatic insulin resistance correlated more strongly with several cardiovascular risk factors than did Matsuda insulin sensitivity index that reflects whole body insulin sensitivity. These results highlight the important role of the liver in the risk of developing cardiovascular disease.

Study IV: Several confirmed candidate loci for fasting hyperglycemia were associated especially with early-phase insulin release or with indices of proinsulin-to-insulin conversion emphasizing the important role of impaired insulin secretion in fasting hyperglycemia.

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Jagadish Vangipurapu

Liver Insulin Resistance and Hyperglycemia

Genetic Determinants and Association with Cardiovascular Risk Factors

Insulin resistance is a major metabolic disturbance in type 2 diabetes and is observed primarily in the muscle, liver and adipose tissue. This thesis introduces a novel surrogate index to estimate hepatic insulin resistance. It further examines the tissue specificity of reported type 2 diabetes risk loci in the liver and adipose tissue insulin resistance. The study compares the association of liver and muscle insulin resistance indices with cardiovascular risk factors. Insulin resistance in the liver correlates more strongly with various cardiovascular risk factors revealing a critical role of the liver in cardiovascular health. Finally, the thesis examines mainly insulin release and proinsulin processing as possible mechanisms for the association of several genetic risk loci with hyperglycemia.

UNIVERSITY OF EASTERN FINLAND

Publications of the University of Eastern Finland
Dissertations in Health Sciences

ISBN 978-952-61-0843-8