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Plasma Cell Glycoprotein-1, Interleukin-6 and Tumor Necrosis Factor-α as Candidate Genes for Insulin Resistance, Obesity and Type 2 Diabetes

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L3, Canthia building, University of Kuopio, on Friday 26th March 2004, at 12 noon

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ABSTRACT

Type 2 diabetes is an inherited disorder characterized by defects in insulin secretion and insulin action. However, the genetic basis of type 2 diabetes is known only in rare cases. In this study the plasma cell glycoprotein-1 (PC-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) genes were investigated as candidate genes for type 2 diabetes. All genes were screened by restriction fragment length polymorphism or single strand conformational polymorphism methods. The 121Q allele (genotypes K121Q and Q121Q combined) was associated with higher fasting insulin levels than the K121K genotype in Finnish healthy normoglycemic subjects. Moreover, compared with subjects having the K121K genotype, subjects carrying the 121Q allele had lower insulin sensitivity as measured by the hyperinsulinemic euglycemic clamp. There was no significant difference between the 121Q allele carriers and subjects with the K121K genotype in first phase insulin secretion. Spanish subjects with the 121Q allele had higher leptin and triglycerides levels than subjects with the K121K genotype. In elderly Finns the effect of the K121Q polymorphism of the PC-1 gene on insulin levels and insulin sensitivity was dependent on birth length, since insulin resistance was highest in subjects carrying the 121Q allele who were small at birth. Furthermore, the interaction between the K121Q polymorphism of the PC-1 gene and birth length increased susceptibility to type 2 diabetes and hypertension in adulthood. Healthy Finnish subjects with the C-174C genotype of the IL-6 gene had a significantly lower energy expenditure than subjects with the G-174C or G-174G genotypes. Moreover, subjects with the C174C genotype had lower rates of whole body glucose uptake than carriers of the -174G allele, and both the rates of oxidative and non-oxidative glucose disposal were significantly affected by the IL-6 promoter polymorphism. In the Finnish Diabetes Prevention Study, the -308A allele of the TNF-α gene was a predictor for the conversion from impaired glucose tolerance to type 2 diabetes. Furthermore, this polymorphism had a gene-gene interaction with the C-174G polymorphism of the IL-6 gene, and a gene-environmental interaction with lifestyle changes.

In summary, the PC-1, IL-6 and TNF-α genes are important candidate genes for insulin resistance and type 2 diabetes. Therefore, genes regulating the insulin signaling pathway and cytokine response should be further studied as susceptibility genes for type 2 diabetes.

National Library of Medicine Classification: WK 810, WK 820
Medical Subject Headings: diabetes mellitus, type II/genetics; insulin resistance/genetics; obesity; polymorphism, restriction fragment length; polymorphism, single-stranded conformational; Finland; insulin/secretion; insulin resistance; interleukin-6/genetics; tumor necrosis factor/genetics; glycoproteins; Spain

Everything should be made as simple as possible,
but not simpler
-Albert Einstein

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Warsaw, January 2004
Agata Kubaszek

ABBREVIATIONS

AUC	area under the insulin response curve
ATP	adenosine triphosphate
βIRKO	β-cell-specific knock-out mice of the IR gene
BMI	body mass index
BMR	basal metabolic rate
bp	base pair
C/EBP alpha	CCAAT/enhancer-binding protein
CAPN10	calpain-10
CRP	C-reactive protein
dCTP	deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
EE	energy expenditure
FDPS	Finnish Diabetes Prevention Study
FFA	free-fatty acids
FFM	fat free mass
FIRKO	fat-specific knock-out mice of the IR gene
FPG	fasting plasma glucose
GLUT	glucose transporter
HDL	high-density lipoprotein
HNF	hepatocyte nuclear factor
HOMA	homeostasis assessment model
HSL	hormone-sensitive lipase
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-6	interleukin-6
IR	insulin receptor
IRS	insulin- receptor substrate
IVGTT	intravenous glucose tolerance test
Kir6.2	ATP-sensitive potassium channel gene
LAR	leukocyte common antigen-related protein
LDL	low-density lipoprotein
LIRKO	liver-specific knock-out mice of the IR gene
LPL	lipoprotein lipase
MIRKO	muscle-specific knock-out mice of the IR gene
MODY	maturity-onset diabetes of the young
NCEP	National Cholesterol Education Program
NFkB	nuclear factor
NIRKO	neurons-specific knock-out mice of the IR gene
NOD	non-obese diabetic transgenic mice
OGTT	oral glucose tolerance test
PAI-1	plasminogen activator inhibitor-1
PC-1	plasma cell glycoprotein 1
PCR	polymerase chain reaction
PI-3	phosphatidylinositol 3-kinase
PKC	protein kinase C
PPAR-γ	peroxisome proliferator-activated receptor
PTP-1B	protein tyrosine phosphatase
RFLP	restriction fragment length polymorphism
RQ	respiratory quotient
SIRS	Spanish Insulin Resistance Study
SOCS-3	suppressors of cytokine signaling-3
SSCP	single strand conformational polymorphism
SREBP-1	sterol regulatory element-binding protein 1
TNF-α	tumor necrosis factor-α
WBGU	rates of whole body glucose uptake
WHO	World Health Organization
WHR	waist-to-hip ratio

LIST OF ORIGINAL PUBLICATIONS

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

I Kubaszek A, Pihlajamäki J, Karhapää P, Vauhkonen I, Laakso M: The K121Q polymorphism of the PC-1 gene is associated with insulin resistance but not with dyslipidemia. *Diabetes Care* 26:464-7, 2003

II González-Sánchez JL, Martínez-Larrad MT, Fernández-Perez C, Kubaszek A, Laakso M, Serrano-Ríos M: K121Q PC-1 gene polymorphism is not associated with insulin resistance in a Spanish population. *Obes Res* 11:603-5, 2003

III Kubaszek A, Markkanen A, Eriksson JG, Forsen T, Osmond C, Barker DJP, Laakso M: The association of the K121Q polymorphism of the PC-1 gene with type 2 diabetes and hypertension depends on size at birth. *Submitted*

IV Kubaszek A, Pihlajamäki J, Punnonen K, Karhapää P, Vauhkonen I, Laakso M: The C-174G promoter polymorphism of the IL-6 gene affects energy expenditure and insulin sensitivity. *Diabetes* 52:558-61, 2003

V Kubaszek A, Pihlajamäki J, Komarovski V, Lindi V, Lindström J, Eriksson J, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Uusitupa M, Laakso M: Promoter polymorphisms of the TNF-α (G-308A) and IL-6 (C-174G) genes predict the conversion from impaired glucose tolerance to type 2 diabetes: The Finnish Diabetes Prevention Study. *Diabetes* 52:1872-6, 2003

CONTENTS

1 INTRODUCTION

Type 2 diabetes is a common metabolic disorder, affecting about 3-5% of Western populations (1). This complex disease is characterized by chronic hyperglycemia, due to decreased insulin action and impaired insulin secretion (2). Type 2 diabetes comprises 80-90% of all diabetic cases, and its prevalence increases with aging (3). It has a strong familial aggregation and a non-Mendelian mode of inheritance (4). Type 2 diabetes is genetically heterogeneous, and strongly influenced by environmental and life-style factors, such as obesity, central obesity and low physical activity.

The genetic basis of type 2 diabetes is known only in relatively rare cases. For example, maturity-onset diabetes of the young (MODY) is a genetic subgroup of diabetes characterized by an autosomal dominant mode of inheritance and an early onset of diabetes. Altogether five subtypes of MODY (MODY 1-5) have been identified so far (5-9). In contrast, the genetic basis of common type 2 diabetes is largely unknown, but it is probably caused by interaction of several susceptibility genes with lifestyle and environment. Insulin resistance in skeletal muscle, liver and adipose tissue is crucial in the pathogenesis of common type 2 diabetes (10). Recent studies also indicate a significant role of insulin sensing in the pancreas and brain (11,12). For all these reasons proteins that interrupt insulin signaling pathway, and therefore contribute to insulin resistance, play an important role in the development of type 2 diabetes. For example molecules secreted from adipose tissue, so called adipokines, can influence insulin sensitivity in fat and other tissues (13). Consequently, proteins that contribute to obesity and increase the amount of body fat can also be important in mediating insulin resistance.

One of the latest hypotheses to explain the pathogenesis of type 2 diabetes is the fetal origin hypothesis (14). According to this hypothesis, insulin and glucose metabolism are determined during fetal growth, and therefore small size at birth is associated with a high incidence of type 2 diabetes in adult life (15,16).

Additionally, type 2 diabetes has been linked to low-grade cytokine-associated acute phase proteins (17). Previous studies have shown that proteins interacting with insulin receptor (i.e. plasma cell glycoprotein 1, PC-1) and also cytokines (i.e. tumor necrosis

factor (TNF) -α, interleukin-6 (IL-6)) may induce insulin resistance (18-20). Therefore, functional variants in these genes may contribute to the risk of type 2 diabetes.

In this study common polymorphisms of the PC-1 gene, the IL-6 gene and the TNF-α gene were investigated as candidate genes for insulin resistance, obesity and type 2 diabetes.

2 REVIEW OF THE LITERATURE

2.1 DEFINITION OF TYPE 2 DIABETES

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia. The diagnostic criteria for diabetes mellitus are fasting plasma glucose (FPG) ≥7.0 mmol/L, or 2-hour glucose ≥11.1 mmol/L in an oral glucose tolerance test (OGTT), or casual plasma glucose ≥11.1 mmol/L, confirmed on a subsequent day (21). Nondiabetic individuals with FPG ≥6.1 mmol/L but <7.0 mmol/L are considered to have impaired fasting glucose (IFG), and those with 2-h glucose level in the OGTT ≥7.8 mmol/L but <11.1 mmol/L are defined as having impaired glucose tolerance (IGT). Both IFG and IGT represent a prediabetic state, and are risk factors for future diabetes.

2.2 NATURAL HISTORY OF TYPE 2 DIABETES

Type 2 diabetes results from interaction between genetic and environmental factors. In most individuals, insulin resistance is the first abnormality in glucose metabolism (22). Insulin resistance is accompanied by increased insulin secretion from the pancreatic β-cells leading to compensatory hyperinsulinemia (Figure 1). However, when the β-cells of genetically susceptible individuals are unable to compensate for insulin resistance postprandial hyperglycemia and later on fasting hyperglycemia develop. Chronic hyperglycemia contributes to further impairment in pancreatic insulin secretion capacity and also to insulin resistance (23). Therefore, type 2 diabetes is a progressive disease, and the control of glycemia needs increasing doses of oral antidiabetic drugs and the addition of insulin (24).

In the prediabetic state fasting glucose levels are in or near the normal range. Subjects with IGT or IFG are at a significantly higher risk of developing diabetes and cardiovascular disease than are those with normal glucose tolerance. About 30% of IGT subjects progress to type 2 diabetes in 10 years (25). The benefit of lifestyle intervention in subjects with glucose intolerance has been lately a focus of interest. Large studies in China, Finland and US have shown that intensive lifestyle intervention reduces the risk of type 2 diabetes in overweight subjects with IGT (26-28).

Type 2 diabetes is frequently undiagnosed for years, and therefore, macrovascular complications often precede the diagnosis of diabetes (29,30). Patients with type 2 diabetes have an increased incidence of all atherosclerotic complications (coronary heart disease, cerebrovascular disease, peripheral vascular disease) (31,32). This excess of cardiovascular diseases is the most important long-term complication of type 2 diabetes. Other complications include retinopathy with a potential loss of vision, nephropathy, which can lead to renal failure, peripheral neuropathy, foot ulcers, amputation, Charcot joints, and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction (30).

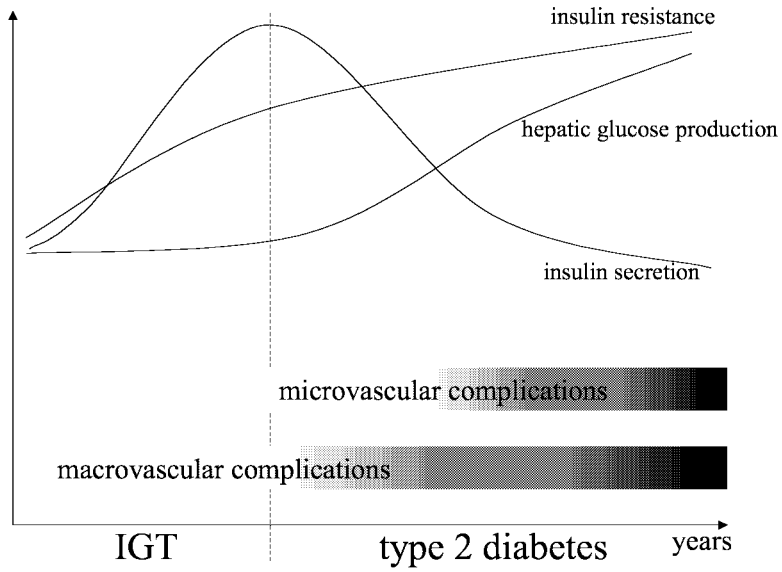

Figure 1. Natural history of type 2 diabetes (modified from (33))

2.3 RISK FACTORS FOR TYPE 2 DIABETES

2.3.1 Obesity

Obesity, especially central obesity, is a well known risk factor for type 2 diabetes, and about 60-90% of type 2 diabetic patients are obese (34). The degree of obesity is defined according to the WHO guidelines as follows: normal body mass index (BMI),

18.5-24.9 kg/m^2; overweight, 25-29.9 kg/m^2; mild obesity, 30-34.9 kg/m^2; severe obesity, 35-39.9 kg/m^2; and morbid obesity, ≥40 kg/m^2 (35). Obesity is an increasing problem in western societies, and it affects about 10-25% of persons in European populations (35). Obesity results from an imbalance between energy intake, energy expenditure (EE) and relative proportions of lipid and carbohydrates oxidized. Subjects gain weight when energy intake exceeds EE. The components of EE are basal metabolic rate (BMR), the thermic effect of food, and physical activity. Low BMR, low physical activity, a low ratio of lipid oxidation, low leptin levels, low activity of the sympathetic nervous system and high insulin sensitivity are all predictors of weight gain (36).

Obesity itself causes insulin resistance (37), and a loss of weight is associated with an improvement in insulin sensitivity (38) and secretion (39). The mechanisms how obesity causes insulin resistance are not clear, but mediators secreted from adipocytes may interrupt insulin signaling. High free fatty acid (FFA) release from adipose tissue has been suggested to cause insulin resistance in skeletal muscle according to Randle's hypothesis (40). Adipose tissue is now recognized as an endocrine organ, not only as a tissue of energy storage (41). The most important substances secreted from adipose tissue (adipokines) are leptin, adiponectin, and cytokines such as TNF-α and IL-6. They are bioactive particles, which change function of other tissues, for example skeletal muscle and brain, but also adipose tissue itself. Plasma concentrations of cytokines are elevated in obese people, and this may play an important role in the pathogenesis of insulin resistance (42,43). Therefore, obesity contributes to a state of chronic low-grade inflammation.

There is strong evidence for a genetic component in the development of obesity (44). However, only in very rare cases is severe obesity due to a single gene mutation. For example, mutations in the leptin gene (45), in the leptin receptor gene (46) and in the melanocortin receptor 4 gene (47) can cause a monogenic subtype of obesity. Candidate genes for polygenic obesity (48) involve genes that play a role in adipocyte differentiation (peroxisome proliferator-activated receptor (PPAR-γ)), sympathetic nervous activity (β3 adrenergic receptor), cytokines and other adipokines, energy metabolism, lipid metabolism and insulin sensitivity (49).

2.3.2 Other risk factors

Major known risk factors for the development of type 2 diabetes include a previous history of abnormal glucose tolerance, hyperinsulinemia, hypertension, physical inactivity, and a family history of diabetes (50). Modifiable risk factors include physical inactivity, low fiber and high saturated fat intake and a lack of alcohol consumption. Non-modifiable factors are age, sex, family history of type 2 diabetes, prior gestational diabetes, impaired glucose tolerance, history of hypertension or dyslipidemias. Additionally, type 2 diabetes has been linked to low-grade cytokine-associated inflammation (17). Also low birth weight has been associated with increased rates of type 2 diabetes (14,51).

2.4 PATHOPHYSIOLOGY OF TYPE 2 DIABETES

Type 2 diabetes involves abnormalities in insulin secretion and in insulin action in its target tissues (52). Typically, the first pathogenetic change in the development of type 2 diabetes is insulin resistance, leading to compensatory hyperinsulinemia, and later on to β-cell dysfunction (Figure 1). Environmental factors, such as excessive energy intake, sedentary lifestyle and low physical activity, jointly with genetic factors contribute to the pathogenesis of type 2 diabetes. The genetic basis of common type 2 diabetes has remained largely unknown, but it is most likely polygenic, due to several susceptibility genes. When genetically susceptible individuals become insulin resistant, for example due to obesity or physical inactivity, they usually develop IGT, a precursor phase of type 2 diabetes. When pancreatic β-cells fail to secrete more insulin to compensate for insulin resistance, manifest type 2 diabetes develops (10,53). Therefore, progression from normal glucose metabolism to diabetes usually needs defects in both insulin action and insulin secretion. However, primary defects in insulin secretion alone can also cause diabetes (54).

2.4.1 Insulin resistance

2.4.1.1 Insulin signaling pathway

Insulin is secreted from pancreatic β-cells in response to an increased glucose level in the blood. Insulin signaling is initiated when insulin binds to its receptor on the plasma membrane. Insulin receptor (IR) is the first crucial protein to transmit the signal. IR belongs to a subfamily of receptor tyrosine kinases and it is widely expressed in different tissues. It is a tetrameric glycoprotein that contains two extracellular α-subunits and two transmembrane β-subunits. Insulin binding to the α-subunit leads to autophosphorylation of the β-subunit, and intracellular target protein phosphorylations, for example that of insulin-receptor substrate (IRS) (55). Phosphorylation reactions transmit the signal to final biological effectors, such as glucose transporters, lipoprotein lipase (LPL), hormone sensitive lipase (HSL), glycogen synthase, and regulators of growth and gene expression (Figure 2). Thus, insulin controls gene expression and activities of many metabolic enzymes, leading to an increase of glucose uptake in skeletal muscle and fat, and to inhibition of hepatic glucose production. Additionally, insulin stimulates lipogenesis, glycogen and protein synthesis and inhibits lipolysis, glycogenolysis and protein breakdown. Moreover, it stimulates cell growth and differentiation (56).

2.4.1.2 Definition of insulin resistance

Insulin resistance is a condition that is characterized by decreased tissue sensitivity to normal actions of insulin (57). Insulin resistance leads to a compensatory increase in insulin secretion, hyperinsulinemia, which is a very important risk factor for type 2 diabetes and cardiovascular disease (58). Insulin resistance usually precedes type 2 diabetes for years (59). Both genetic and environmental factors contribute to insulin resistance. However, insulin resistance itself is neither necessary nor sufficient to cause diabetes.

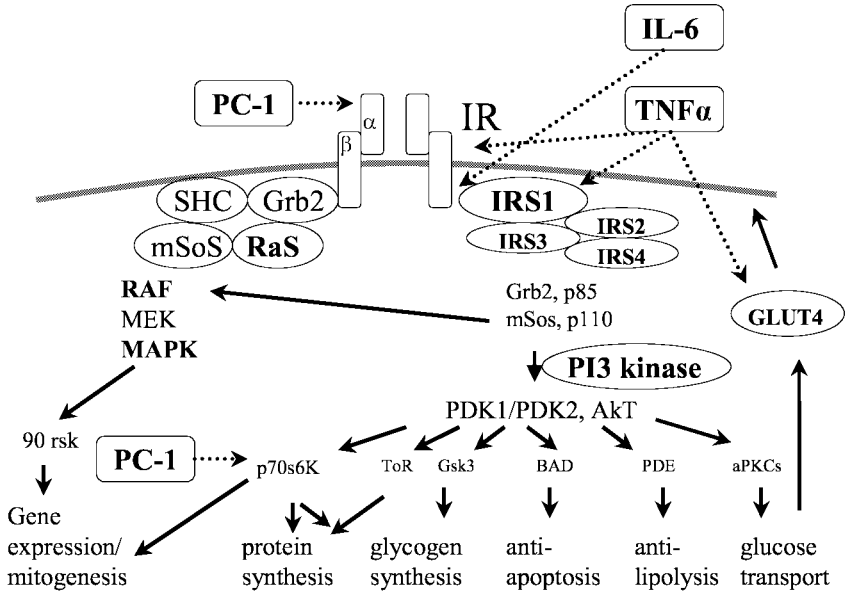

Figure 2. Insulin signaling pathway and sites of action of the PC-1, IL-6 and TNF-α (modified from (56))

Insulin delivery and its binding to its receptor, as well as normal insulin signaling, seem to be crucial in the maintaining of normal insulin sensitivity. Several animal models have been generated to investigate the role of IR in glucose homeostasis. Mice lacking IR are born without apparent metabolic disturbances, but soon after birth the glucose level increases and insulin level rises very dramatically (60). Some days later β-cell failure occurs and these mice develop diabetes and die of ketoacidosis.

In humans IR mutations cause severe intrauterine growth retardation, failure to thrive, postprandial hyperglycemia and fasting hypoglycemia (61). This indicates that the normal insulin signaling pathway plays a crucial role in normal fetal growth and glucose homeostasis in humans.

2.4.1.3 Tissue specificity and animal models

Insulin resistance in subjects with type 2 diabetes involves several organs, particularly skeletal muscle, adipose tissue and liver. Typically non-oxidative insulin-stimulated

glucose metabolism (mainly glycogen synthesis) in skeletal muscle is impaired in the prediabetic state (62). Both decreased rates of glucose uptake in skeletal muscle (63) and impaired suppression of hepatic glucose production due to liver insulin resistance (64) lead to hyperglycemia. On the basis of animal models targeting a specific impairment in insulin action, potential molecular mechanisms for insulin resistance have been identified. On the other hand, animal models have demonstrated that combined minor defects in insulin secretion and insulin action lead to diabetes, pointing out to the importance of interactions of different genetic loci (65).

In humans skeletal muscle is the main site of insulin dependent glucose metabolism (66). Insulin sensitivity in skeletal muscle is independent of obesity, although obesity can cause it to deteriorate (67). Skeletal muscle was previously thought to be the most important site of insulin action (68). However, mice with muscle-specific inactivation of the IR gene (MIRKO) are normoglycemic, but have increased fat mass (69). On the other hand, heterozygous mice with the disruption of glucose transporter 4 (GLUT4) have impaired glucose uptake in skeletal muscle and diabetes (70), which supports the importance of this tissue for glucose metabolism.

Adipose tissue account only for about 10% of the rates of whole body glucose uptake (71,72). In adipose tissue the main role of insulin is the inhibition of catecholamine-induced lipolysis. Insulin inactivates HSL (73) and stimulates LPL (74), resulting in the release of free-fatty acids (FFA) from lipoproteins and in the increase in FFA uptake into adipocytes (75). In insulin-resistant states FFA levels are increased, and they have a lipotoxic influence on β-cells function (76). Mice with fat-specific knock-out of the IR gene (FIRKO) have a low fat mass, and a loss of the normal relationship between plasma leptin and body weight. These mice are protected against obesity, and obesity-related glucose intolerance (77). FIRKO mice also exhibit polarization of adipocytes into populations of large and small cells, which differ in gene expression of fatty acid synthase, C/EBP alpha, and SREBP-1. Thus, insulin signaling in adipocytes is critical for the development of obesity and obesity-associated metabolic abnormalities.

The liver plays a central role in glucose homeostasis. The loss of ability to suppress hepatic glucose uptake by insulin is closely associated with fasting hyperglycemia.

Liver-specific knock-out mice of the IR gene (LIRKO) have severe insulin resistance, glucose intolerance and elevated glucose production (78).

Mice with tissue-specific knock-out of the insulin receptor have demonstrated the importance of insulin action in pancreatic β-cells and brain. β-cell-specific knock-out mice of the IR gene (βIRKO) have a selective impairment of glucose-induced insulin release, which leads to the development of glucose intolerance and diabetes (11). It remains still unknown how insulin controls its own secretion, but normal insulin signaling in the pancreas seems to be critical. Although neurons do not need insulin-mediated glucose uptake, the disruption of IR in mice in brain (NIRKO) results in increased food intake, moderate obesity and reduced fertility, which suggests that insulin has a role in the control of appetite and reproduction (12).

2.4.1.4 Molecular mechanisms

Mechanisms leading to insulin resistance at the molecular level could be genetic or acquired. Mutations in genes regulating insulin signaling, for example in IR, are relatively rare (61). Also mutations that change expression level, for example in the promoter region of IR, cause insulin resistance (61). Acquired mechanisms are often associated with obesity or are induced by the environment. For example, in obesity there are decreased levels of signaling proteins (for example IR, IRS-1, IRS-2) decreased tyrosine phosphorylation, and increased serine phosphorylation of the IR (79-81). Excessive insulin receptor degradation impairs insulin signaling. Increased activity of proteins such as protein tyrosine phosphatase (PTP-1B), leukocyte common antigen-related protein (LAR) and PC-1 cause decreased tyrosine phosphorylation (82-85). PC-1 protein can interact with the β-subunit of the IR (18) (Figure 2), and it is overexpressed in tissues of insulin resistant subjects (85,86). Additionally, PC-1 may impair insulin signaling at the post-receptor site at the p70s6 kinase level (Figure 2) (87). TNF-α was found to increase serine phosphorylation of IR. In obesity there is an activation of protein kinase C (PKC) and inhibition of nuclear factor NFkB kinase (88-90). All these acquired mechanisms play a role in human insulin resistant states.

Adipocytes are crucial in the development of insulin resistance. Elevated FFAs inhibit glucose uptake and increase glucose output from the liver (91). Fat tissue

produces adipokines, which are hormones influencing energy metabolism (13). For example, TNF-α, which is produced by adipocytes, is overexpressed in skeletal muscle and adipocytes of insulin resistant subjects (92).

TNF-α induces insulin resistance by several mechanisms. First, it reduces the expression of GLUT4 (93) and affects insulin signaling by reducing tyrosine autophosphorylation of IR (94), increasing serine phosphorylation of IRS-1 (95), and decreasing C/EBP (93) and PPAR-γ synthesis (96). TNF-α stimulates HSL expression and thus lipolysis (97), and decreases LPL activity (98). Proinflammatory cytokines have deleterious effects on both β-cell function (99) and on the disruption of the insulin signaling pathway (19). High circulating IL-6 levels have been found to be associated with insulin resistance (43). IL-6 receptors are found in adipose tissue, and IL-6 has been shown to impair insulin signaling in hepatic cell lines (20).

2.4.1.5 Measurement of insulin sensitivity

A direct method of measuring insulin sensitivity is the euglycemic hyperinsulinemic clamp, which is considered the golden standard (100). This technique can be also used in combination with indirect calorimetry to evaluate the intracellular metabolism of glucose (101). However, this method is time consuming and difficult to use in large population-based studies.

Other common methods to measure insulin sensitivity are based on an indirect approach. The simplest test is the measurement of fasting plasma insulin, which gives an acceptable estimate of insulin sensitivity in healthy individuals (102). An alternative method is the homeostasis model assessment (HOMA) index of insulin resistance, which is calculated from fasting glucose and insulin levels (103). This index shows a quite good correlation with more reliable measures of insulin sensitivity, such as the euglycemic hyperinsulinemic clamp (104). The HOMA model can be used as a surrogate measure of insulin resistance in population-based studies. Bergman's Minimal Model, based on frequently sampled glucose and insulin levels during an intravenous glucose tolerance test (IVGTT), gives an estimate of insulin sensitivity that correlates well with results of the hyperinsulinemic euglycemic clamp (105,106).

2.4.1.6 Insulin resistance syndrome

The clustering of abdominal obesity, hypertension, dyslipidemia and glucose intolerance is called the metabolic syndrome or the insulin resistance syndrome (107). The WHO criteria are the coexistence insulin resistance (measured as insulin sensitivity by the euglycemic hyperinsulinemic clamp in the lowest quartile) or FPG ≥6.1 mmol/L and at least two of the following criteria: hypertension (≥160/90 mmHg or on antihypertensive treatment), dyslipidemia (serum triglycerides ≥1.7 mmol/L and/or decreased high-density lipoprotein (HDL) cholesterol, <0.9 mmol/L for men and <1.0 mmol/L for women), central obesity (waist-to-hip-ratio (WHR) >0.9 for men and >0.85 for women and/or BMI >30 kg/m^2 or waist girth ≥94 cm) or microalbuminuria (urinary albumin excretion rate ≥20 μg/min or albumin/creatinine ≥20 mg/g on at least two different occasions). The National Cholesterol Education Program (NCEP) criteria are at least 3 of the following traits: FPG ≥6.1 mmol/L, abdominal obesity (waist girth>102 cm or >94 cm), serum triglycerides ≥1.7 mmol/L, serum HDL cholesterol <1.0 mmol/L and blood pressure ≥130/85 mmHg or antihypertensive medication (108,109). With increasing prevalence of obesity, especially central obesity, which contributes to insulin resistance, the prevalence of insulin resistance syndrome also increases. The underlying cause of all the features of this syndrome is likely to be insulin resistance. Other typical metabolic abnormalities are small, dense low-density lipoprotein (LDL) particles, enhanced postprandial lipemia, increased uric acid and elevated plasminogen activator inhibitor-1 (PAI-1) (110-113). Using the 1998 WHO proposed definition of metabolic syndrome, studies of a Scandinavian population revealed that 10% of those with normal glucose tolerance, 50% of those with IGT, and 80% of type 2 diabetic patients had the metabolic syndrome (114). Moreover, it has also been found that the presence of the metabolic syndrome is associated with a 3-fold increased risk of coronary heart disease, myocardial infarction, and stroke and a 3-5-fold increased risk of cardiovascular death (115).

2.4.2 Impaired insulin secretion

Insulin secretion from the pancreatic β-cells in the islets of Langerhans is mainly regulated by glucose entry via its transporter. The intracellular glucose metabolism

induces a rise in the ATP/ADP ratio, which causes a closure of ATP-sensitive potassium channels, inducing a higher intracellular K^+ level (116,117). This, in turn, depolarizes the membrane and opens voltage-sensitive calcium channels. Ca^{2+} entry triggers insulin secretion from secretory granules (118). Like many other hormones, insulin is released in a pulsatile manner, which results in oscillatory concentrations in peripheral blood. In contrast, type 2 diabetic subjects exhibit irregular oscillations of basal plasma insulin (119). Disturbed pulsatile insulin release is a typical feature in first-degree relatives of patients with type 2 diabetes (119). The ability to secrete adequate amounts of insulin is determined by functional integrity of β-cells and their overall mass (53). In the natural course of type 2 diabetes insulin resistance leads to compensatory insulin oversecretion. In type 2 diabetic patients also early phase insulin secretion is impaired (2). When a patient develops diabetes, chronic hyperglycemia (glucotoxicity) further deteriorates insulin sensitivity and insulin secretion (23). FFAs aggravate this effect, and this mechanism is known as lipotoxicity (120). Early in the diabetic state β-cells are unresponsive to glucose, but later on there is also a reduction in β-cell mass (121). β-cell dysfunction can be caused by either qualitative or quantitative changes in molecules regulating insulin synthesis or secretion. Genetically programmed apoptosis may be responsible for β-cell loss, as well as β-cell exhaustion from long-term hypersecretion (122,123) and the deposition of amyloid-like material in the islets (124). The βIRKO mice have demonstrated progressively impaired glucose tolerance and decreased β-cell mass, suggesting a central role of insulin sensing in the pancreas (11).

Low birth weight has also been found to be associated with impaired insulin secretion in some, but not all studies (125). Also the inhibitory effect of TNF-α and IL-1 on β-cell function may play an important role in islet destruction (126,127).

2.4.3 Fetal origin hypothesis

The fetal origin hypothesis (Barker's hypothesis) proposes that impaired fetal development results in adaptations to malnutrition that permanently change metabolism and predispose to metabolic and cardiovascular diseases in adult life (14). It has been proposed that similar mechanisms that produce low birth weight in the fetus lead to type 2 diabetes and the metabolic syndrome (128,129). Alterations during fetal life are

reduced capacity for insulin secretion and insulin resistance, which in combination with environmental and life-style factors determine the risk for type 2 diabetes in adulthood. As fetal adaptations include reduced growth, size at birth is a marker of the intrauterine environment, and both low birth weight and length are associated with high rates of diabetes and hypertension. Since insulin is one of the growth factors regulating fetal growth (130), adaptations to undernutrition can involve alteration in insulin action. As result insulin resistance can be initiated during intrauterine growth. Furthermore, fetal development may influence gene expression, and therefore genes associated with insulin resistance could have different effects depending on size at birth. Indeed, the effect of the Pro12Ala polymorphism of the PPAR-γ2 gene on insulin sensitivity is modified by size at birth (131). However, genes can also regulate insulin-mediated fetal growth directly, as shown for the glucokinase gene (132). Many studies worldwide have confirmed initial epidemiological findings for the fetal origin hypothesis, although the findings have varied between the studies (16,133,134). The relationship with insulin resistance seems to be obvious, but the relationship with insulin secretion has remained unclear (125).

The alternative hypothesis proposes that genetically mediated insulin resistance results in poor fetal growth, as well as in susceptibility to type 2 diabetes and cardiovascular disease in adulthood (135).

2.4.4 Role of low-grade inflammation

Diabetic patients have been shown to have increased levels of inflammatory markers, and therefore the alterations in the inflammatory system could modify the risk of diabetes. Circulating levels of cytokines, such as TNF-α and IL-6, and acute phase proteins, such as C-reactive protein (CRP) are elevated in obesity, metabolic syndrome and type 2 diabetes (42,136). In a prospective study elevated concentrations of cytokines and acute-phase proteins have been associated with the development of type 2 diabetes (137-139), and also cardiovascular diseases (140-142). CRP has been found to be a marker of adiposity and insulin resistance, as it correlates with BMI, WHR and insulin levels (143). However, it is a predictor for type 2 diabetes even in subjects with low BMI (144). The main physiological abnormalities seen in type 2 diabetes, insulin

resistance and impaired insulin secretion, are both exaggerated by cytokines (99,145). Accumulating evidence suggests a link between altered cytokine production and diabetes. Circulating levels of CRP and IL-6 have been found to predict type 2 diabetes in a prospective study (137), and subjects with IGT have been reported to have elevated circulating IL-6 and CRP levels (146,147). Moreover, in healthy subjects CRP correlates both with IL-6 and TNF-α levels, which suggests a tight link between these cytokines (148). Cytokines, especially IL-6, stimulate hepatic protein synthesis, lipoprotein metabolism and the hypothalamic-pituitary axis (149-152). Other acute phase proteins, which are elevated in type 2 diabetes are fibrinogen, complement proteins and PAI-1 (153,154).

Additionally, inflammatory markers predict diabetes-associated macrovascular and microvascular complications (155). Cytokines induce adhesion molecule synthesis, stimulate smooth muscle growth and increase endothelial permeability, and thus promote atherosclerosis (156-158).

2.5 GENETICS OF TYPE 2 DIABETES

2.5.1 Heritability

Genetic susceptibility plays a major role in type 2 diabetes. Type 2 diabetes strongly aggregates in families (4), but the mode of inheritance has remained unclear (159). Risk for type 2 diabetes varies among the populations, and is highest in Pima Indians (160). There is a high concordance for type 2 diabetes approaching 90% in monozygotic twins (161), and also the offspring of diabetic parents have a high risk of type 2 diabetes (162,163). Insulin resistance and impaired insulin secretion seem to be inherited, as they are often present even in normoglycemic offspring of type 2 diabetic patients (164,165). However, despite accumulating evidence for the role of genetic factors in the etiology of type 2 diabetes, the high prevalence of this disease in the general population suggests that there are several susceptibility genes, but their contribution to the risk of type 2 diabetes is difficult to verify. Type 2 diabetes manifests itself late in life, and therefore subjects carrying risk genotypes are not easily distinguished from healthy relatives.

Furthermore, type 2 diabetes is associated with premature mortality, and therefore the collection of families of these patients has a selection bias.

Type 2 diabetes is genetically a heterogeneous disease. It is not known at this moment if type 2 diabetes is caused by many predisposing minor genes or a limited number of major genes, but the first alternative is most likely (166). Until now significant progress has been made only in the genetics of the rare, autosomal dominant forms of type 2 diabetes, which account for only 5% of the disease. Mutations in five genes (glucokinase, hepatocyte nuclear factor (HNF)-1α, HNF-4α, HNF-1β, and insulin promoter factor 1) have been found to cause MODY (maturity onset diabetes of the young) (167). Mitochondrial type 2 diabetes is caused by various mutations in the mtDNA (168). The genes contributing to the common form of type 2 diabetes have remained largely unknown. Moreover, interactions between environmental and genetic factors are likely to play a key role in the pathophysiology of type 2 diabetes.

2.5.2 Methods of identifying genes for type 2 diabetes

2.5.2.1 Genome-wide random search

A genome-wide random search is a method to identify new chromosomal regions (susceptibility loci) for type 2 diabetes (169). There are no a priori knowledge or assumptions about the importance of specific genes or chromosomal regions. The entire human genome is screened with highly polymorphic markers, with a marker density less than 10-15 cM for polygenic diseases. When the region having the linkage has been identified, a denser marker map is used and the attractive candidate gene in this region is screened. Therefore, genome-wide scans can help to find the most important chromosomal regions of interest. Finally the gene is identified by positional cloning (170).

So far several genome scans for type 2 diabetes have been completed in several populations. Almost all studies have reported linkage to different regions of the genome, suggesting that several susceptibility genes contribute to the etiology of type 2 diabetes in these populations. For example, genome-wide scans in Mexican-Americans reported the linkage to chromosomal region 2q37.3 (171), in Finnish subjects to 12q and

20q (172,173), in Pima Indians to 11q (174), in Utah families of Northern European ancestry to 1q21-23 (175), and in French subjects to 3q (176). The genome-wide scan carried out in Mexican-Americans in Stan County, Texas, reported the linkage to chromosome 2 (NIDDM1). The gene identified in this locus (2q37.3) was calpain-10 (CAPN10), which is the first and only gene for type 2 diabetes even identified by positional cloning (177).

2.5.2.2 Candidate gene approach

Candidate gene approach is based on the current understanding of the pathophysiology of type 2 diabetes (178). For example, genes involved in insulin signaling, insulin secretion, glucose metabolism, obesity, and fuel metabolism have been a focus of interest. Major sources of knowledge to identify promising candidate genes include metabolic studies, cell studies, and studies on genetically transformed animals. Variations in genes coding these molecules are then tested for the association with type 2 diabetes.

The Finnish population is very homogenous, and therefore suitable for studies of complex diseases. Small founder population and relative genetic homogeneity is helpful in finding susceptibility genes for type 2 diabetes (179).

2.5.3 Candidate genes of the present study

Several candidate genes have been screened so far as candidate genes for type 2 diabetes, including particularly genes regulating insulin resistance, insulin secretion and obesity. Specifically, these include genes controlling insulin production and delivery, the insulin receptor, insulin signaling pathway, glucose and energy metabolism, FFA and lipid metabolism, adipocyte metabolism, cytokines and genes taking part in the regulation of feeding. Their influence on insulin sensitivity, insulin secretion, obesity and on the risk of type 2 diabetes has been extensively studied in several populations. The most promising susceptibility genes for type 2 diabetes are CAPN10, PPAR-γ and Kir6.2 genes (180-182). Furthermore, genes regulating insulin signaling, for example PC-1, PTP-1B, IR and IRS, are promising candidate genes for type 2 diabetes (61,183-186).

Proinflammatory cytokines are important regulators of inflammatory processes. Cytokines are expressed in several tissues, including skeletal muscle and adipose tissue and central nervous system, which all play a role in energy metabolism. Genes regulating cytokine responses are possible candidate genes that may predispose to type 2 diabetes.

2.5.3.1 PC-1

Plasma cell glycoprotein 1 (PC-1, ENPP1) encodes ectonucleotide pyrophosphatase/ phosphodiesterase -1 and is localized on chromosome 6q22-q23. PC-1 is expressed in several tissues, and it is situated in plasma membranes of the cells. It is also found inside cells, in plasma reticulum, and can also be detected extracellulary in soluble form in the bloodstream. PC-1 activity is increased in fibroblasts from patients with common type 2 diabetes. It may inhibit IR by interacting directly with the α-subunit of IR (18). PC-1 binds to the connecting domain of the IR that moves the two β-subunits together to transactivate them (187,188). Thus, PC-1 inhibits autophosphorylation of IR (18) and impairs insulin signaling downstream of IR at the level of p70s6 kinase (87). Human studies show an association of increased adipose tissue (86) and skeletal muscle (85) PC-1 content and decreased plasma levels of the soluble form of PC-1 (189) with insulin resistance.

The 121Q allele (Gln121) in exon 4 of the PC-1 gene has been shown to interact with IR, and has a greater inhibitory action on the insulin receptor than does the K121K genotype (Lys121) (190). Indeed, the K121Q genotype has been shown to be associated with insulin resistance in Caucasian Sicilians (183) and with higher glucose and insulin levels in Finnish and Swedish subjects (191). However, it was not associated with type 2 diabetes in Danish Caucasians (192) or in Oji Cree (193). Moreover, the chromosomal region 6q22-q23 has been found to harbor a major gene with strong effects on obesity and lipid levels, including leptin concentrations in Mexican-American families (the LOD score for traits-specific insulin and leptin was 4.7) (194). Additionally, a cluster of three single nucleotide polymorphisms in the 3'-untranslated region of the PC-1 gene has been found to stabilize PC-1 mRNA and to be associated with increased PC-1 protein content and insulin resistance (195). The PC-1 codon 121 polymorphism also

contributes to diabetic complications as carriers of the 121Q allele had increased risk for developing end-stage renal disease early in the course of type 1 diabetes (196,197). As shown in Table 1 both positive and negative associations have been reported between the K121Q polymorphism of the PC-1 gene with different measures of glucose metabolism.

Table 1. The associations of the K121Q polymorphism of the PC-1 gene with different measures of glucose metabolism (numbers in parentheses refers to different studies)

	Yes	No
Insulin resistance:		
euglycemic clamp	(183)	
insulin levels	(191)	
Diabetes		(191-193)
OGTT		(192)

2.5.3.2 IL-6

Interleukin-6 (IL-6, interferon-β2) is a multifunctional cytokine expressed in many tissues, including adipose tissue, skeletal muscle and hypothalamus, which are involved in the regulation of body energy balance. One third of circulating IL-6 concentration originates from adipose tissue (198) and IL-6 level correlates with obesity (43). IL-6 is refered to as an endocrine cytokine (151), as it circulates and plays its role systemically. The IL-6 gene is located on chromosomal region 7p21. IL-6 is the main inducer of hepatic production of acute phase proteins, and it is one of several pro-inflammatory cytokines implicated in insulin resistance during infection, cachexia, and obesity (199,200). The functional receptor for IL-6 consist of two transmembrane glycoproteins (gp130 and IL-6 receptor) that are members of the class I cytokine receptor superfamily.

The mechanisms, by which IL-6 causes insulin resistance have remained largely unknown. IL-6 inhibits insulin signaling in hepatocytes, and therefore the induction of suppressors of cytokine signaling (SOCS)-3 in the liver may be an important mechanism in IL-6-mediated insulin resistance (20,201). Based on these studies IL-6 plays a direct role in insulin resistance at the cellular level.

IL-6 has been found to stimulate insulin production and insulin secretion in vitro, and it stimulates insulin gene expression and insulin secretion from β-cells (202,203). IL-6 may also protect β-cells as non-obese diabetic (NOD) transgenic mice overexpressing IL-6 in pancreas have delayed onset of diabetes when compared to other NOD mice (204). In IL-6 null mice the lack of circulating IL-6 has been found to be associated with obesity and low energy expenditure (205). Moreover, IL-6 deficient mice exhibited high leptin levels and leptin insensitivity, and did not lose weight or decrease food intake after leptin treatment.

The promoter of the IL-6 gene is dynamically regulated at many sites, including multiple response element (–173- -145), which is responsive to IL-1, TNF-α and other factors (206). The recently described C-174G promoter polymorphism of the IL-6 gene has been found to influence transcriptional regulation (206,207) and plasma IL-6 levels in patients with systemic-onset juvenile chronic arthritis and in patients with primary Sjögren's syndrome (207,208). Subjects with the -174C allele have been shown to have higher CRP levels (209). Moreover, the C-164G polymorphism has been found to be associated with insulin resistance measured by frequently sampled IVGTT in one previous study (210). In Native Americans and Caucasians the G-174C promoter polymorphism of the IL-6 gene has been found to be associated with type 2 diabetes (211).

As shown in Table 2, both positive and negative associations have been reported between the C-174G polymorphism of the IL-6 gene with different measures of insulin resistance.

Table 2. The associations of the C-174G polymorphism of the IL-6 gene with different measures of glucose metabolism (numbers in parentheses refers to different studies)

	Yes	No
Insulin Sensitivity: Euglycemic Clamp	(210)	
Type 2 Diabetes	(211)	(212)
Type 1 Diabetes	(213)	

2.5.3.3 TNF-α

TNF-α is a proinflammatory cytokine having effects on lipid metabolism, coagulation, insulin resistance and endothelial function. The gene encoding TNF-α is located on chromosome 6p21.3 in the central region of the major histocompatibility complex.

There are two receptors for TNF-α, TNFRI and TNFRII. Recent studies suggests that TNFRI is most important for circulating TNF-α, while membrane-bound TNF-α associates with TNFRII.

Circulating TNF-α concentrations have been found to be positively correlated with insulin resistance across the spectrum of glucose tolerance (214). TNF-α inhibits insulin signaling and leads to insulin resistance (19) and impaired insulin secretion (99,126). Furthermore, it inhibits glucose-induced insulin secretion from β-cells, and it is toxic to pancreatic islets (127). TNF-α contributes to altered glucose metabolism also by increasing the rates of hepatic glucose output (215). TNF-α plays a significant role in obesity-related insulin resistance, as TNF-α deficient obese mice show an increase in insulin sensitivity compared with other models of obese mice (216).

Single-nucleotide polymorphisms in regulatory regions of the TNF-α gene have been associated with susceptibility to a number of complex disorders. The G-308A polymorphism of the TNF-α gene has been shown to interfere with plasma levels and influence transcriptional regulation of TNF-α (217,218). The subjects carrying the -308A allele were found to have a higher percentage of body fat and leptin levels and a lower insulin sensitivity index (219). In several studies this polymorphism has been associated with obesity, insulin resistance and body fat content (220-225). However, this variant of TNF-α was not associated with features of the insulin resistance syndrome or alterations in birth weight in other studies (226-229). The TNF-α gene has been associated with a predisposition to progression to insulin dependency in GADab/DRB1*1502-DQB1*0601-positive diabetic patients initially diagnosed with type 2 diabetes (230). Therefore TNF-α may have genetic interactions with genes in HLA region.

As shown in Table 3, both positive and negative associations have been reported between the A-308G polymorphism of the TNF-α gene with different measures of insulin resistance and with type 2 diabetes.

Table 3. The association of the A-308G polymorphism of the TNF-α gene with different measures of glucose metabolism, obesity, and type 2 diabetes (numbers in parentheses refers to different studies)

	Yes	No
Insulin resistance	(219,221,225,231)	(226-229)
Obesity	(220,222,223)	
Type 2 Diabetes	(232)	(233-235)

The cytokines always work in network, and there are several interactions in their action. Because TNF-α and IL-6 both are expressed at the same site they have several interactions. TNF-α is known to regulate IL-6 expression (236) and on the other hand, IL-6 can downregulate TNF-α (237). These findings suggest that both cytokines may interact to regulate insulin action.

3 AIMS OF THE STUDY

The study was undertaken to investigate the role of the PC-1, IL-6, and TNF-α genes in insulin resistance, obesity and type 2 diabetes.

The specific aims of the study were:

1. Is the K121Q polymorphism of the PC-1 gene associated with insulin resistance or impaired insulin secretion?

2. Is the K121Q polymorphism of the PC-1 gene associated with obesity and other characteristics of the metabolic syndrome?

3. Is the K121Q polymorphism of the PC-1 gene associated with low birth weight?

4. Is the C-174G polymorphism of the IL-6 gene associated with insulin resistance or energy expenditure?

5. Are the C-174G polymorphism of the IL-6 gene and the G-308A polymorphism of the TNF-α gene associated with the risk of type 2 diabetes?

4 SUBJECTS AND METHODS

4.1 STUDY POPULATIONS

4.1.1 Finnish subjects with normal glucose tolerance

Studies I and IV: A total number of 110 unrelated healthy normoglycemic subjects from our two previous population studies (82 men and 28 women, age 52±8 years, BMI 26.4±4.1 kg/m^2) participated in these studies (238,239). In addition to 110 subjects, in Study IV we included 14 unrelated healthy women, who also fulfilled the same criteria. All subjects were randomly selected among healthy, unrelated, middle-aged Finns from eastern part of Finland. All had normal glucose tolerance test. They did not have any chronic diseases, including diabetes, hypertension, or obesity, or any signs or symptoms of coronary heart disease according to the Rose cardiovascular questionnaire or ECG. Each subject had normal liver, kidney, and thyroid function and no history of excessive alcohol consumption. Additionally, they were not receiving any continuous drug treatment, which could affect carbohydrate metabolism.

Study I: We studied insulin secretion in a separate sample of 295 healthy normoglycemic subjects (150 men and 145 women, age 44±12 years, BMI 25.6±3.7 kg/m^2) (240). All subjects were living in eastern Finland. All of them had normal glucose tolerance according to the WHO criteria and they did not have any chronic diseases or continuous drug treatment that could affect carbohydrate metabolism.

4.1.2 Spanish subjects

Study II: This Study consisted of 293 (131 men, 162 women, age 48 ±8 years, BMI 27.7±4.7 kg/m^2) unrelated adults randomly selected from The Spanish Insulin Resistance Study (SIRS), whose DNA was available (241). SIRS was designed as a cross-sectional, population-based study of the prevalence of type 2 diabetes and cardiovascular risk factors. It was conducted in seven towns across Spain (Arévalo, Talavera de la Reina, Guadalajara, La Coruña, Avilés, Vic, Alicante, and Mérida) from March 1995 to April 1998. From a targeted population of 348,980 inhabitants, 3172

men and nonpregnant women aged 34–69 years were interviewed. Of those, 121 (3.8%) were excluded because they met one or more of the following exclusion criteria: abdominal hernia, overt heart or hepatic failure, surgery during the previous year, weight change >5 kg within the previous 6 months, or hospitalization. Final study population included 2949 participants. Subjects with BMI≥30 kg/m^2 were classified as obese (n=91, 35 men and 56 women). Subjects were classified as having normal glucose tolerance (n=238, 81.2%), IFG (n=21, 7.4%), IGT (n=16, 5.7%), and type 2 diabetes mellitus (n=18, 6.4%).

4.1.3 Elderly Finnish subjects

Study III: Elderly subjects were drawn from the original epidemiological study of 7086 men and women, born as singletons at the Helsinki University Hospital during 1924-33 (51). A total of 647 subjects from the original cohort were invited to attend a clinical study after an overnight fast. DNA samples were available for 489 subjects (180 men and 309 women, mean age 70±3 years, mean BMI 26.5±4.0) of the 500 participants who came to the clinic. Altogether 94 subjects had type 2 diabetes (they had medication or were diagnosed at clinical visit) and 56 of them also had medication for hypertension.

4.1.4 The Finnish Diabetes Prevention Study

Study V: Finnish Diabetes Prevention Study (FDPS) is a multi-centre, longitudinal study performed in 5 centres in Finland, comprising 522 subjects (27). DNA was available from 490 subjects (161 men and 329 women, age 55.4±7.1 years, mean BMI 31.2±4.6 kg/m^2). The study population was recruited with a special emphasis on individuals at high risk for type 2 diabetes, such as first-degree relatives of type 2 diabetic patients and overweight subjects. Study subjects had IGT, overweight (with BMI of 25 kg/m^2 or higher) and age in the range of 40 to 65 years in the recruitment period. The subjects were randomly assigned to an intervention and a control group to evaluate the impact of life-style changes on the incidence of diabetes. Criteria for exclusion were previously diagnosed diabetes other than gestational diabetes; a chronic disease making 6-year survival improbable or other unbalanced clinical condition that could interfere with the study; regular vigorous exercise or making dietary changes; or drug treatment that

would affect blood glucose level. In the FDPS medical examinations were done on a yearly basis. The intervention program in the FDPS included frequent face-to-face meetings, individual guidance by nutritionist with the goals of reducing weight and a personal exercise program. The subjects in the control group received only general oral and written information about positive effects of changing life-style with respect to diet, exercise and weight reduction (27).

4.2 METHODS

4.2.1 Clinical and laboratory examinations

4.2.1.1 Anthropometric measurements

In all studies weight and height were measured in light clothing, and BMI was calculated. Waist and hip circumferences were measured, and the waist-to-hip ratio was calculated as an indicator of body fat distribution. In Study V the medical history and physical examination were done at baseline and during annual follow-up visits. In this study we used measurements at baseline and at the 3-year examination for those who did not convert to type 2 diabetes, including height, weight, BMI, waist and hip circumference, and waist-to-hip ratio. For the subjects who developed diabetes before the 3-year examination we used baseline weight and weight measured at the visit when diabetes was established

4.2.1.2 Oral glucose tolerance test

All subjects underwent an OGTT after 12-hour fast (75 g of glucose). Plasma glucose and insulin levels were measured in the fasting state and at 60 and 120 min after a glucose ingestion.

4.2.1.3 Intravenous glucose tolerance test

Altogether 295 subjects from Study I underwent an IVGTT (an intravenous injection of glucose bolus of 0.3 g glucose/kg) after 12-hour overnight fast (240). The first-phase insulin secretion was estimated by calculating the area under the insulin response curve

(AUC) during the first 10 min of the IVGTT (samples taken at 4, 6, 8 and 10 min after the glucose bolus).

4.2.1.4 Euglycemic hyperinsulinemic clamp and indirect calorimetry

Subjects from Study I and IV participated in a euglycemic hyperinsulinemic clamp after a 12-hour overnight fast (100,238). After blood was drawn at baseline, a priming dose of short-acting human insulin (Actrapid 100 IU/mL, Novo Nordisk) was administered during the initial 10 minutes. Insulin was later maintained by a continuous insulin infusion of 480 pmol/m^2 body surface area/min at the desired level. Under these conditions hepatic glucose production is completely suppressed in healthy subjects. Blood glucose was clampedat 5.0 mmol/L for the next 180 minutes by infusion of 20% glucose at varying rates, according to blood glucose measurements performed at 5 minutes intervals. The mean value for the period from 120 to 180 minute (last hour) was used to calculate the rates of whole body glucose uptake (WBGU), which reflect the degree of insulin sensitivity.

Subjects from Study I and IV underwent indirect calorimetry to evaluate energy expenditure, and oxidative and non-oxidative glucose disposal (242,243). Indirect calorimetry was performed with a computerized flow-through canopy-gas analysis system (DELTATRAC, TM Datex, Helsinki, Finland) in connection with the euglycemic clamp to assess the rates of carbohydrate and lipid oxidation and basal metabolic rate. Gas exchange (measurement of O_2 consumption and CO_2 production) was measured for 30 min after a 12-hour fast before clamp and during the last 30 min of clamp. The mean value of last 20 min was used in calculations. The rates of glucose, protein and lipid oxidation were calculated according to the formulas by Ferranini (101). The rates of nonoxidative glucose disposal were calculating by subtracting the rates of carbohydrate oxidation from the rates of WBGU.

4.2.1.5 Analytical measurements

In all the studies serum lipids and lipoproteins, plasma glucose values and serum insulin were measured using standard methods. Total cholesterol, high-density lipoprotein-cholesterol, and triglycerides were determined by enzymatic methods (Boehringer

Mannheim, Mannheim, Germany). LDL-cholesterol was calculated by the Friedewald formula. The plasma glucose level in the fasting state was measured by the glucose oxidase method (2300 Stat Plus, yellow Springs Instrument Co. Inc. OH, USA). Plasma insulin concentration was determined by a commercial double antibody solid phase radioimmunoassay (Phasedeph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden) in Studies I, III-V. In Study II plasma insulin and leptin concentrations were measured by highly specific sensitive radioimmunoassay kits (Linco Research, St. Louis, MO). In Study IV IL-6 concentration was measured by an enzyme-linked immunosorbent assay (Quantikine kit, high sensitivity, RD Systems, Minneapolis, US). The minimum detectable concentration using this assay is 0.094 pg/ml. Non-protein urinary nitrogen was measured by an automated Kjedahl method.

In Studies II, III and V insulin sensitivity was estimated using the HOMA method and insulin resistance index (HOMA IR) was calculated using the following formula: fasting plasma glucose (mmol/L)×fasting serum insulin (mU/L) / 22.5. HOMA insulin secretion (HOMA IS) was calculated as follows: 20×fasting serum insulin (mU/L) / (fasting plasma glucose (mmol/L)-3.5) (103).

4.2.2 Screening of candidate genes

DNA extraction and PCR. Genomic DNA was extracted from peripheral blood leucocytes by the proteinase K-phenol-chloroform extraction method. All genes (the exon 4 of the PC-1 gene, and the promoter regions of the IL-6 and TNF-α genes) were amplified by polymerase chain reaction (PCR) in thermo cyclers (PTC-100 Programmable Therman controller, MJ-Research Inc, Watertown, MA, USA, or Uno Thermoblock, Biometra, Gottingen, Germany). The reaction for PC-1 gene was performed in a total volume of 20 µl containing 50 ng of genomic DNA, primers (0.5 µmol/µl), 0.375 U DNA polymerase (DynaZyme, Finnzymes, Espoo, Finland) and 100 µmol/l dNTP. The reaction for TNF-α gene was performed in a total volume of 10 µl containing 50 ng of genomic DNA, primers (0.5 µmol/µl), 0.125 U DNA polymerase (DynaZyme, Finnzymes, Espoo, Finland) and 90 µmol/l dNTP. The radioactive PCR for the IL-6 gene reaction was performed in a total volume of 6 µl, and

1.0 μCi of alpha-^{32}P dCTP (Study IV, V) was added to the mixture. PCR incubation conditions and primers are presented in Tables 4 and 5.

Table 4. Primers used in the amplification of the exon 4 of the PC-1 gene, the promoter region of the IL-6 gene and promoter region of the TNF-α gene

Gene	Primer (5' to 3')		Product size (bp)
PC-1	F	CTGTGTTCACTTTGGACATGTTG	238
	R	GACGTTGGAAGATACCAGGTTG	
IL-6	F	TGACTTCAGCTTTACTCTTGTA	198
	R	CTGATTGGAAACCTTATTAAG	
TNF-α	F	AGGCAATAGGTTTTGAGGGCCAT	107
	R	TCCTCCCTGCTCCGATTCCG	

Table 5. PCR conditions for the amplification of the exon 4 of the PC-1 gene, the promoter region of the IL-6 gene and promoter region of the TNF-α gene

	Predenaturation		Denaturation		Annealing		Extension		Extra extension		Cycles
	T (^{0}C)	Time (min)	T (°C)	Time (sec)	T (°C)	Time (sec)	T (°C)	Time (sec)	T (°C)	Time (min)	
PC-1	94	4	94	40	62	40	72	40	4	4	35
IL-6	94	5	94	30	58	30	72	30	10	10	35
TNF-α	94	4	94	40	55	45	72	40	4	4	35

Restriction fragment length polymorphism (Studies I, II, III, V). The K121Q polymorphism was screened by the Eco 47I (Ava II) restriction enzyme, and the G-308A polymorphism of the TNF-α gene by the NcoI restriction enzyme, followed by 5% polyacrylamide non-denaturating gel electrophoresis, containing 1.0 μL/mL ethidium bromide, of the digested PCR products. The genotypes were distinguished based on the digestion pattern observed under ultraviolet illumination.

Single-strand conformation polymorphism analysis (Studies IV and V). The C-174G polymorphism of the IL-6 gene was screened by the single-strand conformation polymorphism method (SSCP). PCR products were first diluted 5-20 fold with 1% SDS and 10 mmol/L EDTA and then diluted (1:1) with the loading mix (95% formamide, 20mml/L EDTA, 0.05% bromphenolblue, 0.05% xylene cyanol). After denaturation at 98°C for 3 min samples were cooled on ice. Two μL of each sample were electrophoresed on a 5% polyacrylamide gel that was 0.4 mm thick. Samples were run far from the wells at 2 different temperatures, 38°C and 29°C. The gels were dried on filter paper and autoradiographed at -70°C with intensifying screens.

4.2.3 Statistical analyses

Data were analyzed with the SPSS/Win programs (version 10.0, SPSS Inc, Chicago, Illinois, USA). Data are given as means ±SD. Students t-test for independent samples and analysis of variance with covariates were used to compare the effect of the polymorphism on continuous variables over the genotypes. Chi square test was used for comparison of categorical variables. In Study III multiple linear regression was applied to compare the effect of the polymorphism on continuous variables after adjustment for age, sex, and current BMI. Plasma glucose, insulin, proinsulin, HDL cholesterol, triglyceride and IL-6 values were log transformed before statistical analyses to achieve a normal distribution. Comparisons were made within length categories and within genotypes. In Study V univariate analysis of variance was used to compare the changes in HOMA IR and HOMA IS during the 3-year follow-up. Logistic regression analysis was performed to evaluate if the TNF-α or IL-6 polymorphisms predict the development of type 2 diabetes. In logistic regression models the genotypes were coded as follows: TNF-α: 0=GG, 1=AG or AA; IL-6: 0=GG or GC, 1=CC and study groups were coded as 0=intervention group and 1=control group.

4.3 APPROVAL OF THE ETHICS COMMITTEE

Informed consent was obtained from all subjects after the purpose and potential risks of the study were explained to them. Protocols for Studies I and IV were approved by the Ethics Committee of the University of Kuopio, and they were in accordance with the

Helsinki Declaration. Study II protocol was approved by Ethics Committee of the Hospital Clinico San Carlos of Madrid. Studies III and V protocols were approved by the Ethics Committee of the National Public Health Institute in Helsinki, Finland.

5 RESULTS

5.1 PC-1 GENE

5.1.1 The K121Q polymorphism, insulin sensitivity and insulin secretion (Study I)

We screened the K121Q polymorphism of the PC-1 gene in 110 healthy individuals who underwent the euglycemic hyperinsulinemic clamp and in a separate sample of 295 subjects who underwent an IVGTT. The frequency of the 121Q allele was 10.5% in clamped subjects (Group I) and 9.8% in subjects who underwent an IVGTT (Group II). The frequencies of genotypes in both groups (Group I: K121K 80.0 %, K121Q 19.1 %, Q121Q 0.9 %; Group II: K121K 81.4 %, K121Q 17.6 %, Q121Q 1.0 %) were in Hardy-Weinberg equilibrium. The subjects with the Q121Q genotype were combined with the K121Q genotype in all statistical analyses because of a small number of these subjects (4 of 405 subjects). BMI, systolic and diastolic blood pressure, lipids and lipoproteins did not differ between subjects with the K121K genotype and subjects with the 121Q allele in both groups, but subjects carrying the Q allele in Group II were older ($p=0.044$).

In Group I age- and gender-adjusted fasting plasma glucose levels (5.9±0.5 vs. 5.4±0.5 mmol/L, $p=0.002$), glucose AUC (811±176 vs. 731±140 mmol/L*min, $p=0.034$) and fasting insulin levels (69.6±45.6 vs. 51.9±28.4 pmol/L, $p=0.050$) were significantly higher in subjects with the 121Q allele compared to subjects with the K121K genotype. In Group II fasting insulin levels were significantly higher in subjects with the 121Q allele compared to the subjects with the K121K genotype (66.6±38.8 vs. 53.8±26.6 pmol/L, $p=0.009$) but no differences in fasting glucose levels and glucose and insulin AUC in the OGTT were observed. When all the subjects were pooled ($n=405$) age- and gender-adjusted fasting insulin levels (67.4±40.6 vs. 53.3±27.0 pmol/L, $p=0.001$) and insulin AUC (38164±31027 vs. 31099±21180 pmol/L*min, $p=0.024$) were higher in subjects with the 121Q allele than in subjects with the K121K genotype, but no difference between the groups was observed with respect to fasting

and 2-h glucose levels or glucose AUC. Further adjustment for BMI did not change the results (p value for fasting insulin 0.001, and for insulin AUC 0.025).

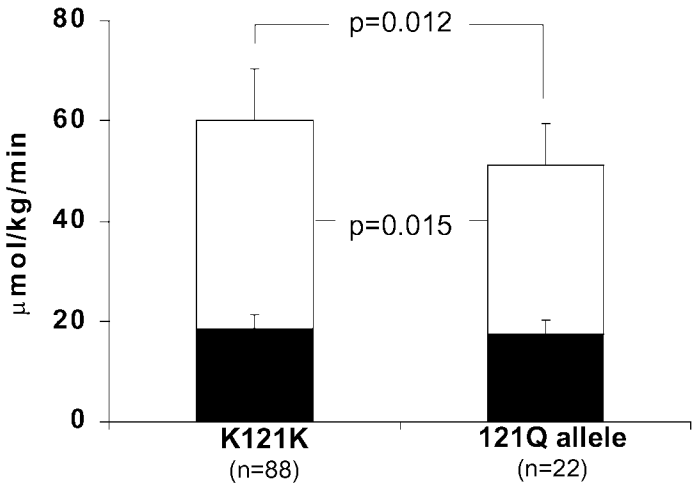

Figure 3. Insulin sensitivity measured as the rates of whole body glucose uptake, glucose oxidation (black bars) and nonoxidative glucose disposal (white bars) during the hyperinsulinemic euglycemic clamp in 110 healthy normoglycemic subjects (Group I) according to the K121Q polymorphism of the PC-1 gene

In Group I the age- and gender-adjusted rates of whole body glucose uptake (WBGU) were lower in subjects with the 121Q allele than in subjects with the K121K genotype (51.17±12.07 vs. 60.12±14.86 µmol/kg/min, p=0.012, Figure 3). In these subjects the PC-1 polymorphism affected the rates of non-oxidative glucose disposal (33.71±10.51 vs. 41.51±13.36 µmol/kg/min, p=0.015), but not significantly the rates of glucose oxidation (17.47±3.89 vs. 18.58±3.19 µmol/kg/min, p=0.148). FFA levels in the fasting state and during the clamp, the rates of lipid oxidation, respiratory quotient (RQ) and EE were similar in subjects with the K121K genotype and in subjects with the 121Q allele (data not shown).

In Group II the acute phase insulin secretion, measured as the peak insulin concentration at 4 min (422.4±323.6 vs. 355.9±220.5 pmol/L, p=0.360) and total insulin AUC during the first 10 min of the IVGTT (2973±2224 vs. 2520±1492 pmol/L*min,

p=0.415, Figure 4), and insulin AUC above basal fasting insulin (2408±2007 vs. 2014±1381 pmol/L*min, p=0.386) were not significantly different in subjects having the 121Q allele compared with the subjects having the K121K genotype. Similarly, total glucose AUC during the first 10 min of an IVGTT (103.9±10.2 vs. 104.4±10.6 mmol/L*min, p=0.772) did not differ between the groups.

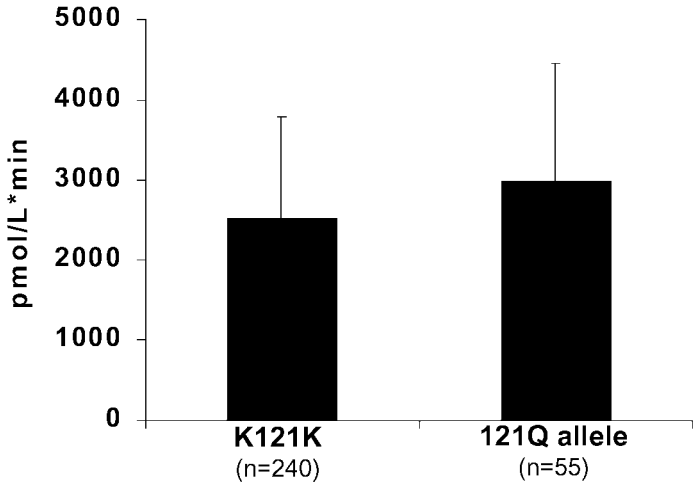

Figure 4. Total insulin secretion measured as the area under the insulin curve during the first 10 min of the intravenous glucose tolerance test in a separate sample of 295 healthy normoglycemic subjects (Group II) according to the K121Q polymorphism of the PC-1 gene

5.1.2 The K121Q polymorphism and characteristics of the metabolic syndrome (Study II)

The frequency of the K121Q genotype in the whole population was 24.6%, and it was similar in obese and non-obese subjects. No subjects with the Q121Q genotype were found. BMI, WHR, diastolic and systolic blood pressure, lipids and lipoproteins were not different between the subjects with different genotypes (Table 6). There were no significant differences with regard to plasma glucose, insulin, proinsulin levels and HOMA IR between carriers and noncarriers of the 121Q allele. Leptin and triglyceride levels were significantly higher in subjects carrying the K121Q genotype when

compared with subjects with the K121K genotype (14.7±1.6 vs. 10.6±0.7 μg/L, p=0.01, 126±11.6 vs. 106±4.7 mg/dL, p=0.06, Table 6), and both differences remained statistically significant even after adjustment for sex, age, BMI and degree of glucose tolerance (p=0.02 and p=0.010, respectively).

Table 6. Characteristics of subjects from Study II according to the K121Q polymorphism of the PC-1 gene

	K121K	K121Q	p
	(n=221)	(n=72)	
Waist to hip ratio	0.96±0.1	0.95±0.1	NS
Body mass index (kg/m^2)	27.5±4.6	27.9±4.6	NS
Systolic blood pressure (mmHg)	126±22	122±21	NS
Diastolic blood pressure (mmHg)	79±13	78±13	NS
Fasting glucose (mmol/L)	5.4±0.9 (212)	5.4±1.6 (70)	NS
Fasting insulin (pmol/L)	77.4±6.6 (217)	71.4±0.7 (70)	NS
2-hour glucose (mmol/l)	5.4±1.7 (184)	5.3±1.5 (63)	NS
2-hour insulin (pmol/l)	154±13 (162)	149±22 (50)	NS
Fasting proinsulin (pmol/l)	14.6±24.6 (74)	14.8±14.5 (21)	NS
HOMA IR	3.2±0.2 (210)	2.9±0.2 (68)	NS
Leptin (μg/l)	10.7±9.7(183)	14.7±12.5 (61)	0.01
Triglycerides (mg/dL)	106±68 (211)	126±96 (70)	0.06

Values are mean±SD.

5.1.3 The K121Q polymorphism, birth size and insulin sensitivity (Study III)

The frequency of the 121Q allele was 12.9% in the study subjects. The frequency distribution of the genotypes was in Hardy-Weinberg equilibrium. In all statistical analyses the subjects having the Q121Q genotype (n=9) were combined with the subjects with the K121Q genotype because of the small number of these subjects.

Subjects carrying the 121Q allele had a lower ponderal index (26.5±2.4 vs. 27.0±2.3 kg/m^3, p=0.04) compared with subjects who had the K121K genotype, but there were no differences in birth weight and length. BMI, systolic and diastolic blood pressure, and lipids and lipoproteins did not differ between subjects with the K121K genotype and with the 121Q allele. Fasting and 2-h glucose and insulin and HOMA IR did not

differ between subjects with the K121K genotype and subjects with the 121Q allele in the whole study group. The effect of the K121Q polymorphism on insulin sensitivity measured as fasting insulin levels or HOMA IR depended on birth length (subjects were divided into five length categories) (p for interaction 0.04 and 0.05, Table 7). Fasting insulin levels and HOMA IR were highest in subjects carrying the 121Q allele who were small for gestational age at birth. In subjects normal for gestational age, there was no effect of the 121Q allele on insulin sensitivity.

Next we investigated the simultaneous effects of the PC-1 gene polymorphism and birth length on the occurrence of type 2 diabetes and hypertension, as both of these diseases cluster in subjects with the insulin resistance syndrome. To this aim we divided our study group into two length categories, ≤49cm and >49 cm. When we compared subjects with the 121Q allele with subjects with the K121K genotype there was a 2-fold higher prevalence of type 2 diabetes in subjects below 49 cm at birth (Figure 5, 36.8 vs.

Table 7. Mean fasting insulin concentration and HOMA-IR index according to the K121Q polymorphism of the PC-1 gene and birth length

	Birth length (cm)											
	-48		-49		-50		-51		>51		p^a	p^b
Fasting insulin (pmol/L)												
K121K (n)	64	(52)	69	(79)	66	(107)	64	(64)	70	(52)	0.45	
K121Q/Q121Q (n)	79	(21)	74	(11)	75	(35)	69	(26)	57	(16)	0.06	
												0.04
p^c	0.34		0.87		0.32		0.37		0.42			
HOMA-IR index (mU×mmol/L^2)												
K121K (n)	16.1	(52)	16.8	(79)	16.6	(107)	16.0	(64)	16.9	(52)	0.60	
K121Q/Q121Q (n)	20.5	(21)	20.5	(11)	19.0	(35)	16.2	(26)	14.8	(16)	**0.05**	
												0.05
p^c	0.28		0.35		0.40		0.77		0.62			

p^a for a trend with birth length; for p^b interaction; for p^c difference between genotypes; all adjusted for age, sex and BMI

16.5%, p=0.005). Subjects who were short at birth and who had the 121Q allele had the highest incidence (31.6%) of type 2 diabetes together with hypertension (Figure 5, p=0.002 for interaction of genotype and birth length). In this group altogether 86% of patients with diabetes also had hypertension. In subjects whose birth length was above 49 cm, the 121Q allele was not associated with type 2 diabetes or hypertension. Subjects with the 121Q allele who had diabetes and hypertension and who were below 49 cm at birth were most insulin resistant (HOMA IR=32.3±7.7, p=0.007 for interaction between birth length and HOMA IR, Figure 6).

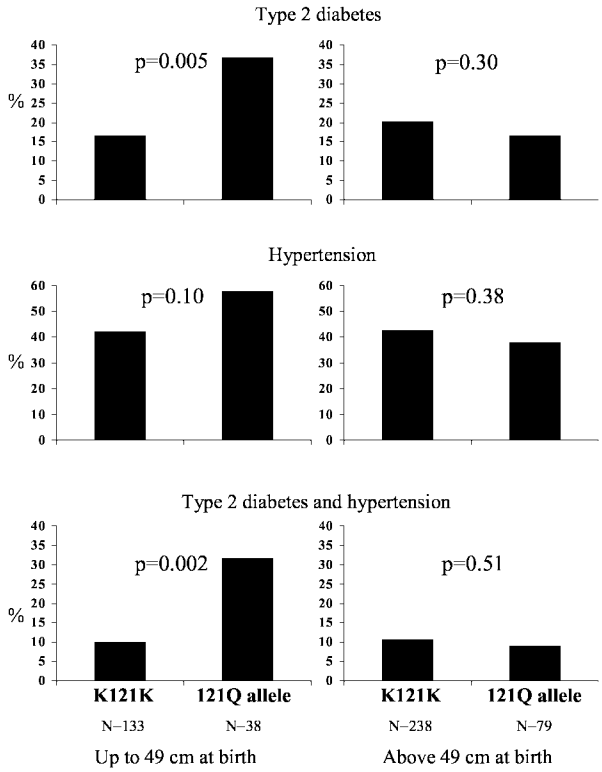

Figure 5. Prevalence (%) of type 2 diabetes, hypertension and both conditions according to length at birth and the PC-1 gene polymorphism

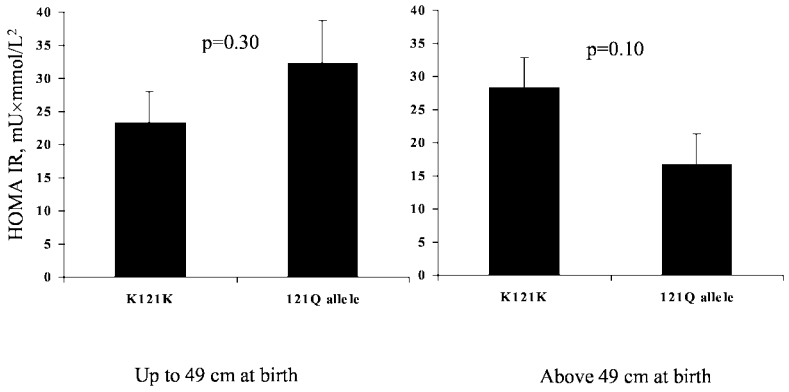

Figure 6. HOMA IR in subjects with type 2 diabetes and concomitant hypertension according to length at birth and the PC-1 gene polymorphism

5.2 CYTOKINES

5.2.1 The effect of the C-174G polymorphism of the IL-6 gene on energy expenditure and insulin sensitivity (Study IV)

The frequency of the C-174C genotype was 30%, the C-174G genotype 44%, and the G-174G genotype 26% in 124 study subjects and were in Hardy-Weinberg equilibrium. Age, BMI and WHR, systolic and diastolic blood pressure, fasting plasma glucose and insulin levels and IL-6 concentration (n=72) did not differ among the genotypes.

Fasting EE was 8% lower in subjects with the C-174C genotype than in subjects with the C-174G or G-174G genotypes (13.68±1.98, 14.73±1.57, 14.81±2.01 kcal/kg/min, respectively, p=0.012 over the genotypes, Figure 7). Similarly, the promoter polymorphism was associated with EE during the hyperinsulinemic clamp (C-174C: 15.24±2.05, C-174G: 16.62±2.06, G-174G: 16.66±2.50 kcal/kg/min, p=0.007). Both fasting EE and EE during the clamp were significantly lower among subjects with the C-174C genotype compared to carriers of the -174G allele even after adjustment for BMI, age, and gender (p=0.035, and p=0.024, respectively). BMR estimated by O_2 consumption during indirect calorimetry was 7.4% and 8% lower in

subjects with the C-174C genotype both while fasting and during the hyperinsulinemic clamp (fasting: C-174C: 2.94±0.42, C-174G: 3.17±0.34, G-174G: 3.18±0.43 ml/min/kg, p=0.009 among the three genotypes, during clamp: C-174C: C-174G: 3.46±0.42, G-174G: 3.46±0.51 ml/min/kg, p=0.008). The polymorphism did not affect the RQ during the clamp (C-174C: 0.96±0.05, C-174G: 0.96±0.04, G-174G: 0.97±0.04, p=0.505) indicating that similar proportions of fat and carbohydrates as fuel substrates were used independently of the genotypes.

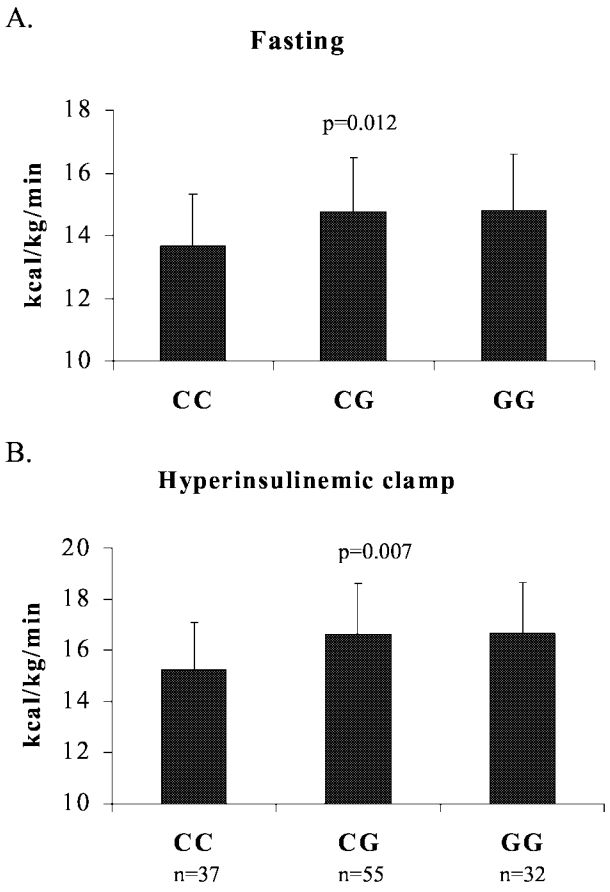

Figure 7. Energy expenditure in the fasting state (A) and during the hyperinsulinemic clamp (B) in 124 normoglycemic subjects according to the C-174G polymorphism of the interleukin-6 gene (CC, CG, and GG genotypes)

The rates of WBGU were 14% lower in subjects with the C-174C genotype than in the -174G allele carriers (C-174C: 50.95±13.91, C-174G: 59.40±14.17, G-174G:

59.21±15.93 μmol/kg/min, p=0.016, Figure 8). Even after adjustment for BMI, age and gender the subjects with the C-174C genotype had lower rates of WBGU than carriers of the -174G allele (p=0.041). When the results were expressed as μmol/kg of fat free mass (FFM)/min in the 30 subjects in whom the percent body fat was measured, the differences between the subjects with the C-174C genotype and subjects with the -174G allele were statistically significant (C-174C: 71.70±14.1, C-174G: 78.70±11.6, G-174G

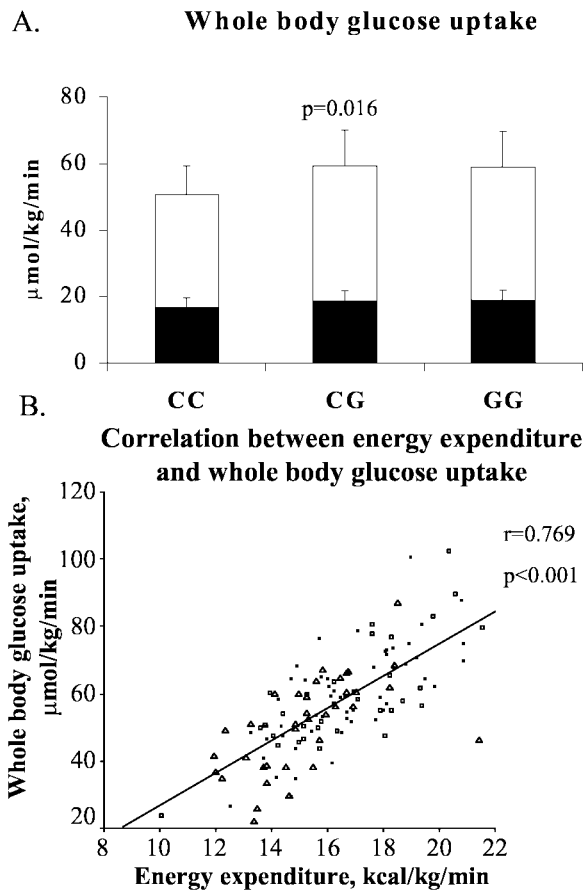

Figure 8. The rates of whole body glucose uptake, glucose oxidation (black bars) and nonoxidative glucose disposal (white bars) during the hyperinsulinemic clamp (A) in 124 normoglycemic subjects according to the C-174G polymorphism of the interleukin-6 gene (CC, CG, and GG genotypes). The correlation between energy expenditure and the rates of whole body glucose uptake during the hyperinsulinemic clamp (B) (white triangles=CC, black squares=CG, white squares=GG)

94.49±26.6 μmol/kg of FFM/min, p=0.032). The polymorphism affected both the rate of glucose oxidation (16.79±3.06, 18.67±3.25, 18.80±3.38 μmol/kg/min, p=0.013) and the rate of non-oxidative glucose disposal (33.83±12.94, 40.73±12.59, 40.41±13.73 μmol/kg/min, p=0.034). Insulin sensitivity and EE correlated strongly in the fasting state (Pearson correlation coefficient r=0.544, p<0.001) and during the clamp (r=0.769, p<0.001) in all study subjects. When calculated according to the genotypes, the correlations remained significant (fasting: C-174C, r=0.499; C-174G, r=0.478; G-174G, r=0.545; during clamp: C-174C, r=0.752; C-174G, r=0.703; G-174G, r=0.769; all p<0.01, Figure 8). IL-6 levels did not correlate with fasting EE (r=-0.046), EE during the clamp (r=0.024), or with the rates of WBGU (r=0.015). During the clamp the rates of lipid oxidation and FFA levels were unaffected by the IL–6 promoter polymorphism (data not shown).

5.2.2 The promoter polymorphisms of the TNF-α (G-308A) and IL-6 (C-174G) genes and the risk of type 2 diabetes (Study V)

We screened the polymorphisms of the TNF-α (G-308A) and IL-6 (C-174G) genes in the FDPS. In all study subjects the frequencies of the genotypes were for the TNF-α gene promoter polymorphism: G-308G 74%, A-308G 25% and A-308A 1%, and for the IL-6 gene promoter polymorphism: C-174C 32%, C-174G 46% and G-174G 22%. The frequencies did not differ between the intervention group and the control group, and were in Hardy-Weinberg equilibrium. As only six subjects had the A-308A genotype of the TNF-α gene, they were combined with subjects having the A-308G genotype. There were no differences in age, gender, weight, BMI, WHR, fasting and 2-hour levels of glucose and insulin in the OGTT, HOMA IR and HOMA IS at baseline according to the G–308A promoter polymorphism of the TNF-α gene or to the C-174G promoter polymorphism of the IL-6 gene.

During the 3-year follow up 69 genotyped subjects (19 subjects in the intervention group and 50 subjects in the control group) developed type 2 diabetes. We found that the -308A allele (G-308A and A-308A genotypes) of the TNF-α gene was associated with high incidence of type 2 diabetes in all subjects in the FDPS (p=0.032), as 12.6%

of subjects (44/348) with the G-308G genotype and 20.7% of subjects (25/121) with the -308A allele converted from IGT to type 2 diabetes. IL-6 polymorphism was not associated with the progression to type 2 diabetes (C-174C: 14.1%, C-174G: 15.3%, G-174G: 14.3%, p=0.796). When the intervention and control groups were analyzed separately, the presence of the -308A allele of the TNF-α gene was a predictor of type 2 diabetes only in the intervention group (p=0.011, Figure 9B). No significant difference in weight loss between subjects with the G-308G genotype and -308A allele carriers was found in either the intervention group or the control group (Figure 9A).

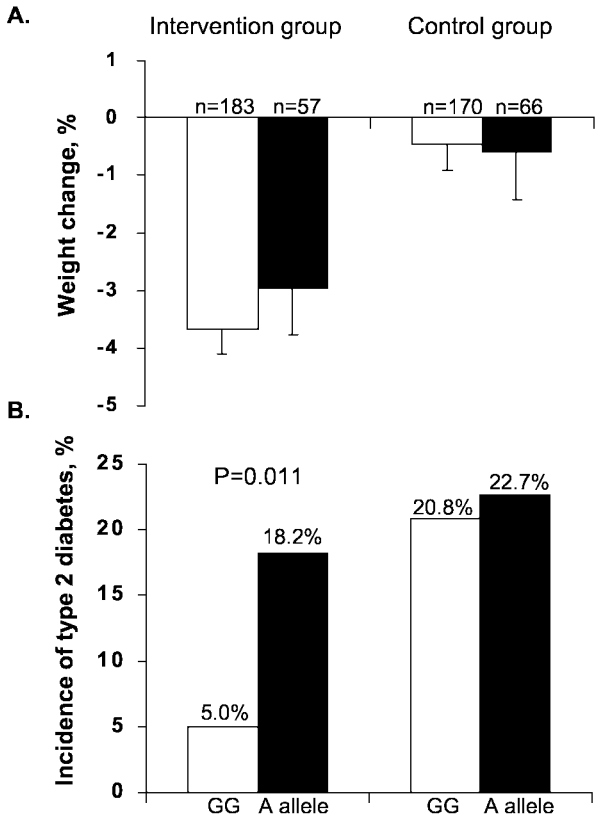

Figure 9. (A) 3-year weight change (%) (mean±SEM) and (B) 3-year incidence (%) of type 2 diabetes in the intervention group and in the control group according to the G-308A polymorphism of the TNF-α gene

In univariate logistic regression analyses (Table 8) the presence of the -308A allele of the TNF-α gene was associated with an almost 2-fold higher risk for type 2 diabetes than the G-308G genotype (odds ratio [OR]=1.80, confidence intervals [CI] 1.05-3.09, p=0.034). Because the study group and weight change during the follow up were significant predictors for the development of type 2 diabetes, we included these variables in the regression models. There was a significant interaction (p=0.027)

Table 8. TNF-α promoter polymorphism (G-308A) as a predictor for the development of type 2 diabetes (logistic regression analysis)

	Reg. Coef	P value	Odds ratio	95% CI
Model 1				
TNF-α A allele	0.587	0.034	1.80	1.05, 3.09
Model 2				
TNF-α A allele	1.439	0.003	4.22	1.62, 10.99
study group	1.608	<0.001	4.99	2.32, 10.74
TNF-α A allele × study group	-1.328	0.027	0.27	0.08, 0.86
Model 3				
TNF-α A allele	1.416	0.005	4.12	1.53, 11.08
study group	1.410	<0.001	4.10	1.84, 9.12
TNF-α A allele × study group	-1.472	0.020	0.23	0.07, 0.79
weight change	0.104	<0.001	1.11	1.05, 1.17
weight at baseline	0.034	<0.001	1.03	1.02, 1.05

Reg. Coef.= regression coefficient
95% CI= 95% confidence interval
TNF-α genotypes were encoded as 0=GG, and 1=A allele
Study groups were encoded as 0=intervention group and 1=control group
Weight at baseline in kilograms
Weight change was calculated as (weight [kg] 3 year– weight [kg] baseline)/weight [kg] baseline ×100%

between the TNF-α promoter polymorphism and the study group (Model 2). The risk for type 2 diabetes was significantly higher in subjects with the -308A allele, but its effect was only seen in the intervention group and not in the control group. When the study group, interaction between the genotype and study group, weight change, and weight at baseline were included into the model (Model 3), the OR for the development of type 2 diabetes for subjects carrying the -308A allele was 4.12 (CI 1.53-11.08, p=0.005).

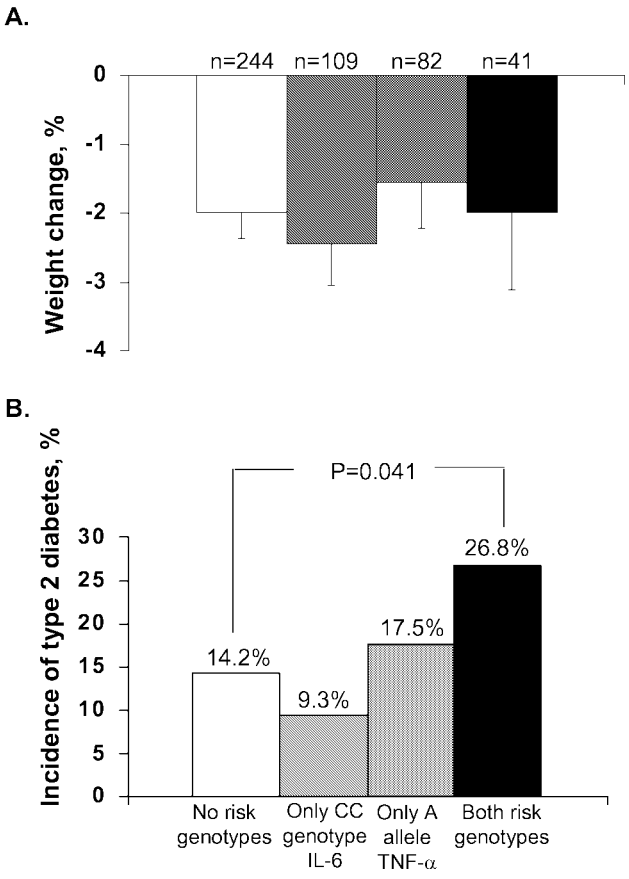

Figure 10. (A) 3-year weight change (%) (mean±SEM) and (B) 3-year incidence (%) of type 2 diabetes according to the G-308A polymorphism of the TNF-α gene and the C-174G polymorphism of the IL-6 gene in the whole study population

As the presence of the -308A allele of the TNF-α gene was a strong predictor for the development of type 2 diabetes in the intervention group only we investigated whether this association was modified by the degree of obesity. To investigate this we divided the intervention group into two groups on the basis of median BMI at baseline (30.35 kg/m^2). We performed logistic regression analysis separately in the group with high (mean BMI=34.7±4.0 kg/m^2) and low BMI group (mean BMI=27.7±1.6 kg/m^2).

We found that the -308A allele was associated with the progression from IGT to diabetes similarly in both groups (p=0.041 in high BMI group, and p=0.036 in low BMI group).

The C-174G polymorphism of the IL-6 gene was not associated with increased risk for type 2 diabetes (OR=0.93, CI 0.53-1.62), and there was no interaction between the IL-6 genotype and the study group. Subjects with the C-174C genotype lost weight similarly as did subjects with the C-174G and G-174G genotypes (C-174C: -2.08±5.78%, C-174G: -1.79±5.96%, G-174G: -2.31±6.64%).

Because the interaction between the C-174C genotype of the IL-6 gene and the -308A allele of the TNF-α was statistically significant (p=0.05) we also investigated whether there is an additive effect of the number of risk genotypes of the TNF-α and IL-6 genes on the risk of diabetes. Subjects simultaneously having the -308A allele of the TNF-α gene and the C-174C genotype of the IL-6 gene had the highest incidence of type 2 diabetes (26.8%) (Figure 10B). The weight reduction did not differ among the genotype groups (Figure 10A). In univariate logistic regression analysis, subjects having both the -308A allele of the TNF-α gene and the C-174C genotype of the IL-6 gene (n=41) had a 2.22-fold (CI 1.02-4.85, p=0.045) higher risk of developing type 2 diabetes compared with subjects having neither of these risk genotypes (n=240). The association remained statistically significant after the inclusion of the study group and weight change into the model (CI 1.05-5.48, p=0.039).

We also investigated the changes in insulin sensitivity (HOMA IR) and insulin secretion (HOMA IS) in subjects who converted and who did not convert to type 2 diabetes according to the TNF-α and IL-6 risk genotypes (Figure 11). There was a significant increase in HOMA IR from baseline to 3 years both in subjects having the

G-308G genotype of the TNF-α gene (p<0.001) and in those having the -308A allele (p=0.001) who converted to type 2 diabetes compared with those who did not (Figure 11A). There was no change in insulin secretion in subjects with the G-308G genotype, whereas in subjects having the -308A allele there was a decrease in insulin secretion among those who converted to diabetes (Figure 11B). Subjects having both the -308A allele of the TNF-α gene and the C-174C genotype of the IL-6 gene and who converted to type 2 diabetes had an increase in insulin resistance (Figure 11C), and a significant decrease (p=0.009) in insulin secretion (Figure 11D) compared with subjects who did

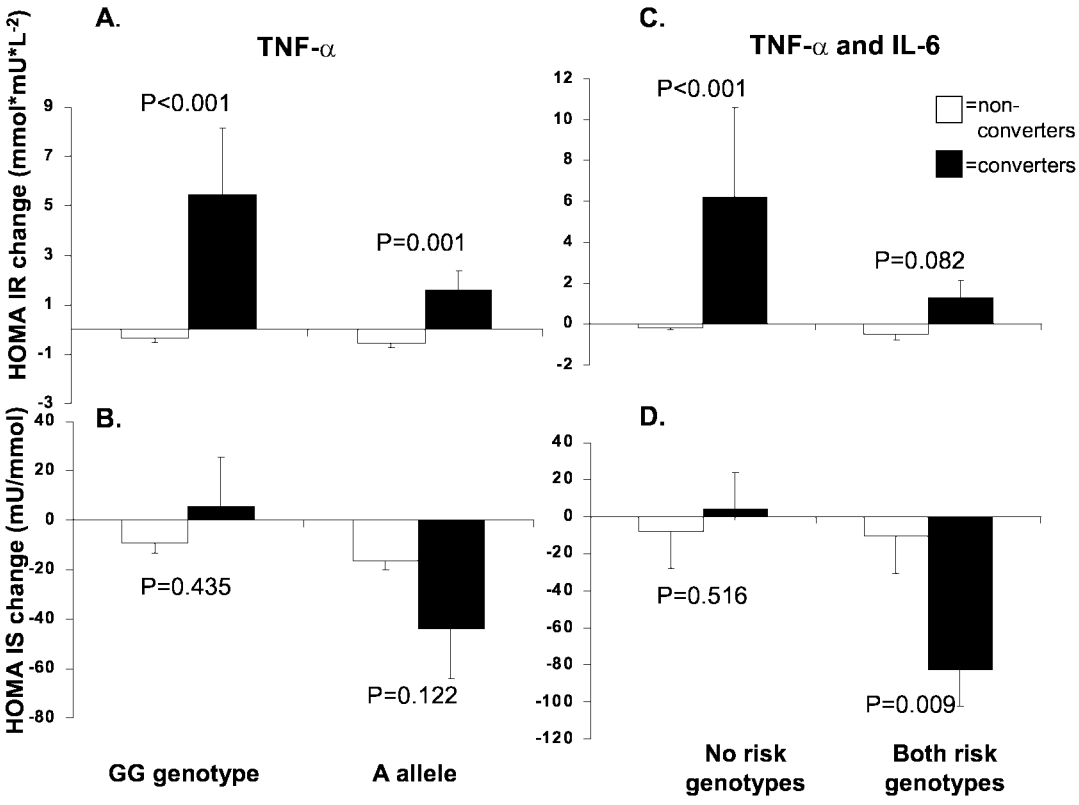

Figure 11. (A) 3-year change (mean±SEM) in HOMA insulin resistance index (HOMA IR) and (B) in HOMA insulin secretion index (HOMA IS) according to the G-308A polymorphism of the TNF-α gene in subjects who converted and who did not convert to type 2 diabetes. (C) 3-year change (mean±SEM) in HOMA IR index and (D) in HOMA IS index according to the G-308A polymorphism of the TNF-α gene and the C-174G polymorphism of the IL-6 gene in subjects who converted and who did not convert to type 2 diabetes

not develop type 2 diabetes. In subjects without these risk genotypes, there was no change in insulin secretion in either converters or non-converters.

6 DISCUSSION

6.1 STUDY SUBJECTS

Subjects included in Studies I and IV were healthy unrelated residents of Eastern Finland. They all had normal glucose tolerance, and no history of chronic diseases. Therefore, they should represent the distribution of insulin sensitivity and energy expenditure in the general Finnish population.

Subjects for Study II were selected from the Spanish Insulin Resistance Study, which was a large cross-sectional population-based study. Therefore, these unrelated subjects are a representative sample of the Spanish population.

Subjects for Study III represent a sample of subjects born in Helsinki during 1924-1933. Therefore, the sample can be potentially influenced by a survival bias (suggested by the larger number of women than men). However, the allele frequency of an autosomal polymorphism should be independent of gender, and therefore the results should not be seriously affected by a selection bias.

Subjects for Study V, who were the participants of the FDPS, were randomized into the control and intervention groups by center, sex and mean 2-h plasma glucose concentration. The size of the FDPS was large enough to detect a 35% reduction in the incidence of diabetes with 80% power, and it was estimated that the study would last approximately 6 years (244). However, a significant decrease in the rates of type 2 diabetes occurred already after 3 years in the intervention group (27). For genetic studies the number of subjects whose DNA was available (n=490) in the FDPS is quite small because the frequency of the rare allele is usually low, often about 10%. With respect to the genes that were screened in Study V, the frequencies of risk alleles/genotypes were quite common, making statistical analysis of the data reliable.

6.2 METHODS

6.2.1 Measurements of insulin sensitivity

In Studies I and IV we applied the hyperinsulinemic euglycemic clamp technique, which is the gold standard for measuring insulin sensitivity. In other studies (Studies II, III, V) we used the HOMA model, which correlates closely with results of the hyperinsulinemic euglycemic clamp (104).

6.2.2 Measurements of insulin secretion

We used AUC insulin during first 10 min of the IVGTT in Study I to evaluate first phase insulin secretion, which is precise method in quantifying insulin secretion. In Study V we used the HOMA IS index. Its main advantage is simplicity and ease of use in large cohorts and which has been shown to give a quite good estimation of insulin secretion (245).

6.2.3 Restriction fragment length polymorphism

RFPL analysis is a sensitive method for the detection of allelic variants in the candidate genes. The specificity of restriction enzymes is very high. Furthermore, the underdigestion of PCR products is an unlikely cause for genotyping errors in the RFLP analysis since all abnormal restriction patterns were always reanalyzed.

6.2.4 Single strand conformational polymorphism

SSCP is a commonly used method for screening of sequence variations (246). It is technically simple with a relatively high sensitivity (80-90%) and specificity (247-249). The sensitivity of SSCP depends on electrophoretic conditions, fragment length, type of substitution and a total number of substitutions. To increase the sensitivity of the method we applied standardized running conditions with two different temperatures. Our method has been previously validated against known variants in the lipoprotein lipase gene (250). These SSCP conditions have been successfully used in several of our studies.

6.2.5 Candidate gene approach

The candidate gene approach is a suitable method for the identification of genes with modest effects on a trait. However, a causal relationship between variants in a candidate gene and a trait is difficult to verify. To confirm that the observed association is true the result should be confirmed in an independent study sample or in another population.

6.3 PLASMA CELL GLYCOPROTEIN-1 GENE

6.3.1 Effects on glucose metabolism and characteristics of the metabolic syndrome

Subjects with the 121Q allele of the PC-1 gene had lower insulin sensitivity measured with the hyperinsulinemic euglycemic clamp compared with subjects with the K121K genotype (Study I). In addition, subjects with the 121Q allele had higher leptin and triglycerides levels (Study II).

Our findings in Study I confirm previous associations of the PC-1 gene polymorphism with insulin sensitivity. Furthermore, our results imply that although PC-1 impairs insulin signaling particularly at the receptor level, non-oxidative glucose disposal in subjects with the 121Q allele may be more severely affected than glucose oxidation. This may imply that PC-1 impairs insulin signaling also downstream of IR, leading to lower glycogen synthesis. In agreement with this interpretation are results showing that PC-1 inhibits p70S6 kinase (87), which may interact with glycogen synthesis.

The K121Q polymorphism of the PC-1 gene did not affect first-phase insulin secretion (Study I). No data on the expression of PC-1 in the human pancreas are available, but in mice no expression of PC-1 in the pancreatic tissue was observed (251). Therefore, PC-1 may not affect insulin signaling in β-cells. Although the role of the soluble form of PC-1 in the regulation of insulin secretion is unknown, it is unlikely that PC-1 affects insulin secretion directly.

In Study I the 121Q allele of the PC-1 gene was not associated with elevated serum triglycerides or low HDL cholesterol, which are typical components of the insulin resistance syndrome. The lack of the association could be related to different effects of PC-1 in skeletal muscle and adipose tissue. Indeed, PC-1 has been shown to regulate

insulin signaling in skeletal muscle (85), but no association of PC-1 with insulin receptor tyrosine kinase activity (86,252) or glucose uptake (252) in adipocytes has been observed. Thus, our results suggest that the main site of action of PC-1 is in skeletal muscle, which accounts for most of the rates of insulin-stimulated glucose disposal. However, Study II showed an association of the 121Q allele with leptin and triglycerides levels. PC-1 is also expressed in the liver, and PC-1 action on IR in the liver could be responsible for its association with triglycerides.

Leptin, the product of the *ob* gene, is closely correlating with insulin levels, and also with key variables of the metabolic syndrome. As insulin is known to be a main stimulator of leptin expression (253), PC-1 which is abundantly expressed in adipose tissue, could disrupt this regulation. Study II showed that the 121Q allele was associated with high leptin levels, which suggests that leptin synthesis is increased in subjects carrying the 121Q allele. Common obesity in humans is characterized by high circulating leptin levels, usually together with hyperinsulinemia and insulin resistance, suggesting that leptin may be a central component of the metabolic syndrome (254,255). The association of the Q121 allele with leptin levels remained significant even after adjustment for sex, BMI and glucose tolerance.

Studies in humans have suggested that leptin correlates with insulin resistance (256). Subjects with insulin resistance may be relatively resistant to the effects of leptin, which could lead to excessive leptin secretion. Leptin in mice increases glucose turnover and stimulates glucose uptake in skeletal muscle and brown adipose tissue and decreases hepatic glucose production (257). It is also possible that higher levels of leptin in subjects carrying the 121Q allele reflect a higher relative amount of body fat. However, we did not measure body composition of our subjects. The third possibility for the association between leptin and PC-1 is that PC-1 increases adenosine levels (258), which in adipose tissue stimulate leptin secretion (259).

Slightly different associations of the PC-1 gene polymorphism in Studies I and II could be due to different ethnicity. Therefore, it is possible that in different populations the K121Q polymorphism of the PC-1 gene can be associated with a different phenotype. Furthermore, methodological differences could explain different findings. In

Study II the HOMA model was used, whereas in Study I we applied the hyperinsulinemic clamp.

6.3.2 Effects on intrauterine development and long-term health consequences

In Study III we found an association of the 121Q allele with a low ponderal index, although there was no difference in birth length. This may suggest that PC-1 regulates intrauterine weight gain, as was previously shown for the IGF-1 and glucokinase genes (132,260). Subjects with the 121Q allele of the PC-1 gene may have an altered response to insulin during fetal growth, resulting in decreased fetal weight gain. Insulin is an important growth determinant (261,262), especially in the third trimester when fetal weight increases substantially. Indeed, severe insulin-resistant states (e.g. lepreuchanism) are associated with growth retardation (263). Thus, proper insulin sensitivity can be crucial for normal growth. Subjects carrying risk genotypes, including the 121Q allele of the PC-1 gene, may be poorly adapted to undernutrition. Additionally, the effects of PC-1 at the postreceptor site can particularly impair growth. However, no influence of PC-1 on DNA synthesis has been found (87). Birth length may be also determined by other genes that regulate fetal response to undernutrition and oxygen supply. Therefore, the interaction of the PC-1 gene with birth length may reflect an interaction with environment or with other genes.

In Study III, subjects with the 121Q allele of the PC-1 gene who were short for gestational age were both hyperinsulinemic and insulin resistant. This supports the influence of the K121Q polymorphism on insulin sensitivity. Subjects with the 121Q allele of the PC-1 gene who were short for gestational age also had the highest prevalence of diabetes combined with hypertension. Almost 90% of type 2 diabetic subjects below 49 cm at birth who had the 121Q allele also had hypertension, whereas only about 50% of type 2 diabetic subjects above 49 cm at birth with the K121K genotype had hypertension. The prevalence of hypertension in patients with type 2 diabetes is around 60%, which is about 1.5-2 times higher than in the general population (264,265). The causes for elevated blood pressure in patients with type 2 diabetes are unknown, but hypertension is often associated with hyperinsulinemia and insulin resistance (107,266).

We found that the association of the 121Q allele with type 2 diabetes and hypertension was restricted to subjects who had impaired fetal growth, suggesting an interaction with environmental (for example, nutrition and oxygen supply) and genetic factors affecting intrauterine growth. One possible mechanism explaining the association of the PC-1 gene polymorphism with elevated blood pressure is insulin resistance. Indeed, the most insulin resistant subjects in our study were subjects with the 121Q allele who were short at birth. These subjects also developed diabetes and hypertension. Moreover, in this group the prevalence of hypertension in subjects with diabetes was higher than in patients with diabetes in general. Insulin resistance in essential hypertension is located in skeletal muscle, and is limited to nonoxidative pathways of glucose disposal (glycogen synthesis), and correlates directly with the severity of hypertension. However, it is also possible that enzymatic role of PC-1 in the regulation of signaling by nucleotides through purinergic receptors may be important, because they play a role in the development of hypertension (267,268). PC-1 decreases ATP and increases adenosine levels. A diminished ATP level reduces autophosphorylation of IR. ATP also stimulates glucose transport in skeletal muscle cells (269). However, enzymatic activity of PC-1 and regulation of ATP level seems not to be the main mechanism of PC-1 action on insulin signaling, becuase mutated PC-1 with a single amino acid change that abolishes the phosphodiesterase and pyrophosphatase activities is still able to inhibit IR phosphorylation (258). There are also conflicting findings showing that the inhibition of the IR by PC-1 is not specific and results from the hydrolysis of ATP (270).

6.3.3 Summary

Subjects with the 121Q allele of the PC-1 gene were more insulin resistant than subjects with the K121K genotype, and they also had higher leptin and triglycerides levels. Moreover, there was an interaction between the K121Q polymorphism and the intrauterine environment. The impact of the K121Q polymorphism of the PC-1 gene on insulin sensitivity, estimated by the HOMA model, and the prevalence of diabetes and hypertension depended on size at birth. Subjects who were short at birth and who had the 121Q allele were most insulin resistant and had the highest prevalence of type 2

diabetes and hypertension. Subjects with the 121Q allele also had a lower ponderal index, suggesting that inhibitory action of PC-1 on insulin signaling may affect intrauterine growth. This interaction affects insulin sensitivity and increases susceptibility to type 2 diabetes and hypertension. This is consistent with the complex pathogenesis of type 2 diabetes, and supports the notion that insulin sensitivity is influenced by both genetic and environmental factors. PC-1 affects insulin signaling and is associated with insulin resistance and decreased fetal growth, both of which may lead to increased risk of insulin resistance, type 2 diabetes, and hypertension (Figure 12).

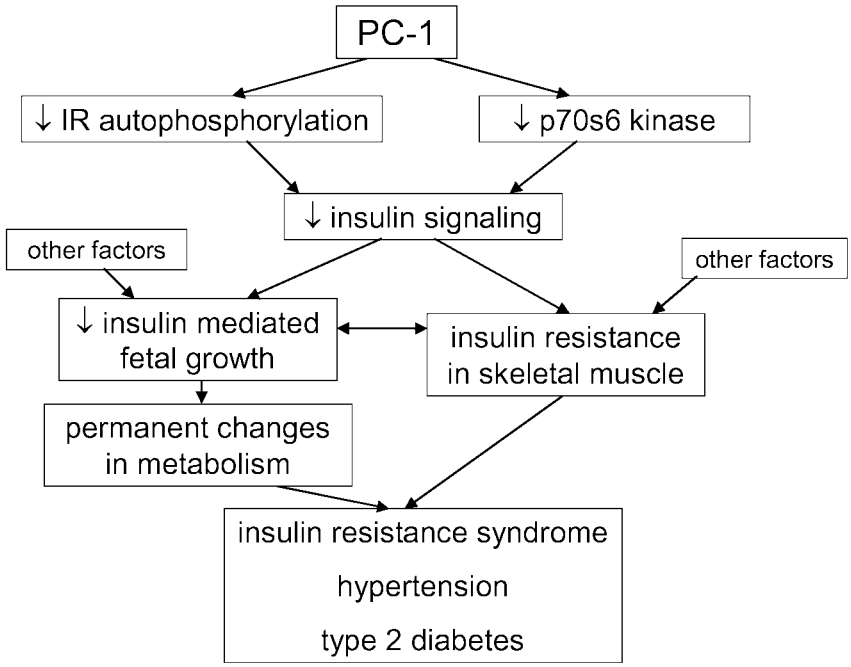

Figure 12. Mechanisms of the PC-1 gene effects in the pathophysiology of type 2 diabetes

6.4 INTERLEUKIN-6 AND TUMOR NECROSIS FACTOR-α

6.4.1 IL-6, insulin sensitivity and energy expenditure

In Study IV we showed that the C-174G promoter polymorphism of the IL-6 gene affects EE and BMR both in the fasting state and during hyperinsulinemia in healthy subjects. Moreover, the subjects with the C-174C genotype had lower insulin sensitivity measured by the hyperinsulinemic euglycemic clamp compared to the subjects with the C-174G or G-174G genotypes, and the rates of oxidative and non-oxidative glucose disposal were similarly affected by this polymorphism.

Many tissues, also adipose tissue, secrete IL-6, and the levels of IL-6 correlate positively with BMI (198). However, IL-6 tissue-specific expression may be more important, and thus the measurement of circulating IL-6 level may not reflect the biological significance of this cytokine at the tissue level. In agreement with this notion we did not find significant correlations between circulating IL-6 level and EE or insulin sensitivity measured with the euglycemic hyperinsulinemic clamp.

Several mechanisms can be considered to explain the association of the C-174G polymorphism with EE. First, IL-6 can regulate EE centrally, as it is expressed in hypothalamus. In IL-6 knockout mice a central injection of IL-6 caused a significant increase in EE, which was not mediated by peripheral injection (205). In humans, high cytokine levels (including IL-6) and cytokine brain synthesis were found to increase resting EE and induce cachexia (271). Additionally, a subcutaneous injection of IL-6 increased resting metabolic rate and hypothalamic-pituitary-adrenal axis activity in a dose-dependent fashion, suggesting that hypothalamic corticotrophin-releasing hormone may mediate both of these actions in humans (272).

A second possibility by which IL-6 may affect EE is enhanced adrenergic stimulation. In humans IL-6 has been shown to increase heart rate and norepinephrine levels (273), and to stimulate the sympathetic nervous system (43,274), which is the primary efferent pathway regulating EE. Moreover, sympathetic neurons have been shown to secrete IL-6, express IL-6 receptors and respond to IL-6 (275). In patients with renal cell carcinoma IL-6 infusion increased plasma norepinephrine levels and

resting EE (276). Adrenergic agonists in humans increase EE, and skeletal muscle seems to be the most important tissue in energy metabolism (277).

Third, IL-6 may exert its effects through the involvement of leptin. IL-6 knockout mice are obese, have high leptin levels and are leptin insensitive. Leptin action on EE is mediated by corticotrophin-releasing-factor, which increases EE and stimulates the sympathetic nervous system in rats (278). In humans EE has been shown to be increased during leptin administration (279). Most likely the effect of the C-174G variant on EE is mediated centrally, as suggested by IL-6 null mouse model.

BMR was decreased by approximately 8% in subjects with the C-174C genotype compared to subjects carrying the -174G allele. This means that caloric consumption was 134 kcal/day less in these subjects, and could lead to weight gain (280) and thus to increased risk of diabetes. Indeed, the -174G allele has been more commonly observed among lean subjects, with lower waist circumference and with lower insulin and glucose values (281). In Study V, however, we did not observe differences in weight loss during the 3-year follow up between subjects with different genotypes of the IL-6 gene.

We also found an association of the IL-6 polymorphism with insulin sensitivity. Because both oxidative and non-oxidative glucose metabolism were similarly affected the effect of the IL-6 promoter polymorphism could be located at the proximal site of insulin signaling. In fact, IL-6 exerts its inhibitory effect on early insulin signaling, because in a hepatic cell line it diminishes not only IRS-1 phosphorylation, but also glycogen synthesis, which is downstream. Our study subjects with the C-174C genotype had a lower EE than subjects with the -174G allele. Thus, it was not unexpected that they had a somewhat higher BMI. However, obesity can not explain the decrease in insulin sensitivity among subjects with the C-174C genotype because in these subjects the rates of WBGU were significantly lower than in subjects with the -174G allele even after adjustment for BMI, age and gender. It is also possible that increased insulin sensitivity could increase EE, since insulin stimulates hypothalamic neurons leading to the activation of the sympathetic nervous system and to an increase in EE (282). On the other hand, increased EE could cause an increase in peripheral glucose uptake. Finally, the effect of the IL-6 polymorphism on insulin sensitivity could be independent of its

effect on EE, but because insulin sensitivity and EE correlated very strongly, this possibility is not very likely.

Our results are in contrast with one previous report showing that the C-174C genotype was associated with high insulin sensitivity (210). However, the number of subjects in that study was only 32 and the evaluation of insulin sensitivity was based on a frequently sampled intravenous glucose tolerance test. Also the -174G allele of the IL-6 gene was associated with type 2 diabetes in one cross-sectional study (211).

6.4.2 Tumor necrosis factor-α, interleukin-6 and the risk for type 2 diabetes

Study V showed for the first time that the G-308A promoter variant of the TNF-α gene predicted the conversion from IGT to type 2 diabetes. Previously, in one study the -308A allele of the TNF-α gene was found to be associated with the risk of diabetes in an elderly population-based cohort (232). There is substantial evidence that TNF-α contributes to insulin resistance and thus to type 2 diabetes based on *in vitro* data, animal models and human studies. Long-term exposure of cultured cells to TNF-α induces insulin resistance (94). TNF-α inhibits IR signaling by decreasing autophosphorylation of IR and promoting serine phosphorylation of IRS proteins (19). Animals lacking TNF-α or TNF receptors have improved insulin sensitivity in genetic (ob/ob) and dietary (high-fat diet) models of rodent obesity (216,283). Infusion of animals with TNF-α can lead to insulin resistance in the liver and peripheral tissues, particularly in skeletal muscle (284). Increased FFA flux secondary to TNF-α's cellular actions has been hypothesized to contribute to insulin resistance (284). TNF-α is expressed in adipose tissue, and its mRNA and protein levels are increased in adipose tissue of insulin resistant humans (285). In our study the -308A allele of the TNF-α gene was associated with higher rates of glucose oxidation in normal weight subjects and lipid synthesis in overweight subjects (286). This underscores the interaction of this polymorphism with weight change.

The evidence that TNF-α impairs insulin secretion is much more limited. However, in pancreatic β-cell lines TNF-α decreased glucose-stimulated insulin secretion (99,126). No data are available to indicate that TNF-α induces impairment of insulin secretion in humans.

The G-308A promoter polymorphism of the TNF-α gene has been found to interfere with transcriptional activity. The -308A allele is a more potent inducer of TNF-α expression (217,218,287) and secretion (288), as synthesis of TNF-α is regulated partly at the transcriptional level. Thus, a high incidence of type 2 diabetes, particularly in obese subjects, may be due to increased production of TNF-α in subjects with the -308A allele. Although the expression of TNF-α has been found to correlate with obesity (92), we found that the -308A allele was associated with the progression from IGT to type 2 diabetes independently of baseline weight and weight change.

Also the C-174C genotype is likely to affect IL-6 expression and its physiological regulation, because the C-174C genotype has been shown to be a weaker inducer of IL-6 gene expression than is the -174G allele (206,207). However, in Study IV we did not observe any differences in IL-6 levels among the genotypes, but no data are available on site-specific expression. Interestingly, in some studies subjects carrying the C-174C genotype had higher circulating IL-6 levels.

We found an interaction between the TNF-α and IL-6 promoter polymorphisms. Subjects with the C-174C genotype of the IL-6 gene and the –308A allele of the TNF-α gene had a two times higher incidence of type 2 diabetes than subjects without these genotypes. There are several possibilities how TNF-α and IL-6 polymorphisms could interact with each other. The synthesis of IL-6 is tightly regulated, and a multiple response element of the IL-6 gene promoter (-173- -145) is controlled also by TNF-α. TNF-α stimulates transcription of the IL-6 gene (236,289), and induces the production of IL-6 and its receptor (275). On the other hand, IL-6 has been suggested to negatively control TNF-α production (237), and TNF-α infusion in humans increases plasma IL-6 levels in a dose-dependent fashion (290). Weight loss reduces both TNF-α expression and IL-6 levels (92,291). Insulin sensitivity correlates inversely both with adipose tissue TNF-α secretion and IL-6 levels (199).

The risk for type 2 diabetes is determined by impaired insulin sensitivity, impaired insulin secretion, or both (292). The TNF-α promoter polymorphism (G-308A) has been previously found to be associated with insulin resistance in some (219), but not in all studies (228,293). Our results based on the HOMA models during the trial indicated that HOMA IR, but not HOMA IS, predicted the conversion from IGT to type 2 diabetes independently of the TNF-α promoter polymorphism. HOMA IR was significantly higher among converters to diabetes than among non-converters independently of genotype. However, among the subjects with the G-308G genotype insulin secretion (HOMA IS) compensated for insulin resistance, whereas among subjects with the -308A allele insulin secretion was reduced. Interestingly, in subjects with both risk genotypes of the TNF-α and IL-6 genes the reduction in insulin secretion was clearly more pronounced than in subjects carrying only one risk genotype, suggesting that the effect on insulin secretion was additive. TNF-α has been reported to inhibit insulin secretion in pancreatic β-cells (126), whereas IL-6 has been reported to increase insulin secretion (203). Additionally, as insulin secretion is also regulated by insulin itself, possible impairment in insulin signaling mediated locally by TNF-α in β-cells may impair insulin secretion. It is worth noting that the TNF-α gene is located in the HLA region on chromosome 6. Genes in this region confer the major susceptibility to type 1 diabetes, and seem to play role in type 2 diabetes (294). Since the genes in this region are in very strong linkage disequilibrium, it is possible that some of the effect associated with TNF-α may be due to other HLA genes.

6.4.3 Summary

The promoter polymorphisms of the IL-6 (C-174G) and TNF-α (G-308A) genes affect insulin sensitivity, EE and progression from IGT to type 2 diabetes, which is consistent with a hypothesis that inflammation is an important mechanism in the pathophysiology of type 2 diabetes. IL-6 and TNF-α increase the risk for type 2 diabetes most likely affecting insulin signaling in several tissues (Figure 13). However, in Study V the C–174G polymorphism of the IL-6 gene alone did not predict the progression from IGT to type 2 diabetes, but had a significant gene-gene interaction with the TNF-α. The G–

308A polymorphism of the TNF-α gene predicted the progression to type 2 diabetes also alone. We also found that lifestyle intervention was successful only in subjects with the G-308G genotype of the TNF-α promoter polymorphism, but not in those having the -308A allele, suggesting a gene-lifestyle interaction.

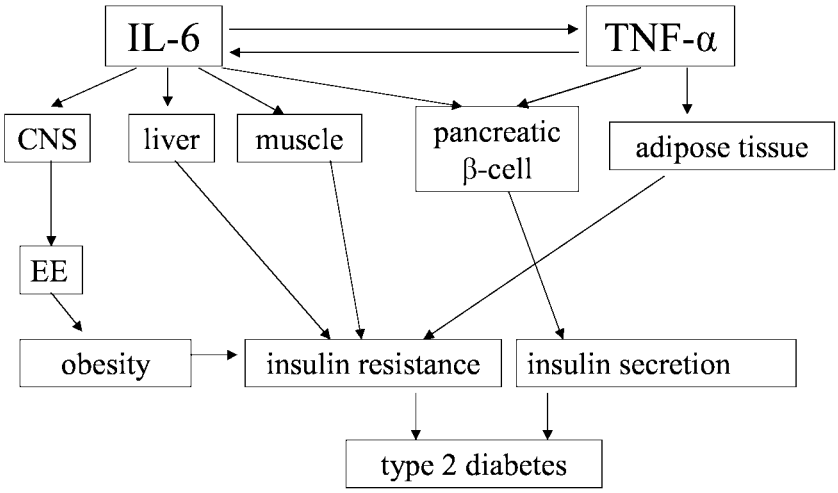

Figure 13. Cytokines action in the pathophysiology of insulin resistance

6.5 CONCLUDING REMARKS

During the last years there has been an increasing interest in the genetics of type 2 diabetes. Although several candidate genes have been extensively studied, we still do not know the genetic basis of 'common' type 2 diabetes. The search for candidate genes has been difficult, because type 2 diabetes is a complex, heterogeneous, multifactorial disease resulting from both genetic susceptibility and environmental risk factors.

In this thesis, genes regulating insulin signaling (the PC-1, IL-6 and TNF-α genes) have been investigated as candidate genes for type 2 diabetes. Our results support the role of these genes in insulin resistance and type 2 diabetes. Therefore, variants in the

genes regulating insulin signaling, particularly cytokines, are likely to play a significant role in the risk of developing type 2 diabetes.

Direct screening for mutations in candidate genes in patients with the appropriate pathophysiological abnormality can be a successful strategy, although the studies published so far have not been able to identify major genes for type 2 diabetes. New technologies, such as gene expression studies with microarrays (295), will lead to a better understanding of the pathophysiology of type 2 diabetes, and hopefully to better treatment options and eventually prevention.

6.5.1 Limitations of the candidate gene approach

During the last years genetic research has concentrated on studies of candidate genes or on the identification of new genetic loci predisposing to diseases. However, the association of a variant in a candidate gene with type 2 diabetes does not necessarily mean a causal relationship. The functionality of a variant of the gene of interest should be demonstrated *in vivo*.

Most likely, multiple genes are responsible for genetic susceptibility to type 2 diabetes. The contribution of these genes to the diabetic phenotype may be modest, variable among different populations, and dependent on interactions with other genes and the environment. Nevertheless, genes involved in insulin signaling, insulin secretion, insulin resistance, glucose metabolism and obesity have been associated with type 2 diabetes, and they will be suitable candidate genes also in future studies.

It seems evident that no single gene is responsible for the development of common type 2 diabetes. Several gene variants together with environmental and lifestyle factors are likely to be important. Recently the E1506K mutation in the SUR1 gene, which causes congenital hyperinsulinism, was found to lead to insulin deficiency and type 2 diabetes in adult life. Thus a new subtype of autosomal dominant diabetes has been identified and characterized genetically (296). Further effort is needed to find new genetic mechanisms for type 2 diabetes.

6.5.2 Future perspectives

For the candidate gene approach the discovery of new genes and pathways involved in the pathogenesis of type 2 diabetes is critical. However, the identification of these genes represents a significant challenge, as is the case in all complex diseases. Gene expression-based technologies may prove to be a more rewarding strategy to identify diabetes candidate genes. There are a number of RNA-based technologies available to identify genes that are differentially expressed in various tissues in type 2 diabetic patients, and these include differential display polymerase chain reaction, suppression subtractive hybridization (297), and cDNA microarrays (295). Combining DNA and RNA based technologies, we can focus our attention on differentially expressed genes located in chromosomal regions previously linked with diabetes. Also genome wide scans with a homogenous group of subjects and use of a denser map can reveal loci of interest. Additionally, the use of animal models is an essential component of genetic investigation in sorting out the importance of genes of interest (65).

The human genome has been completely sequenced (298). Knowledge of human genes and their functions may allow effective preventive measures and better treatment modalities for type 2 diabetes. Future progress will link the results of genomic approaches to data obtained by other methods, such as proteomics, and will allow a more detailed molecular characterization of the disease.

7 SUMMARY

The most important findings in Studies I-V were:

Study I: The K121Q polymorphism of the PC-1 gene was associated with insulin resistance, but not with impaired insulin secretion or dyslipidemia, in healthy normoglycemic Finnish subjects. In subjects with the 121Q allele compared with subjects with the K121K genotype non-oxidative glucose disposal may be more severely affected than glucose oxidation.

Study II: The K121Q polymorphism of the PC-1 gene was associated with high leptin and triglycerides levels independently of gender, BMI and degree of glucose tolerance in a Spanish population.

Study III: The effect of the K121Q polymorphism of the PC-1 gene on insulin levels and insulin sensitivity was dependent on birth length, since insulin resistance was highest in subjects carrying the 121Q allele who were small at birth. The interaction between the K121Q polymorphism of the PC-1 gene and birth length can increase susceptibility to type 2 diabetes and hypertension in adulthood.

Study IV: The C-174G promoter polymorphism of the IL-6 gene affected energy expenditure and basal metabolic rate in healthy Finnish subjects. Moreover, the subjects with the C-174C genotype had lower insulin sensitivity than the subjects with the C-174G and G-174G genotypes, and the rates of glucose oxidation and non-oxidative glucose disposal were similarly affected by this polymorphism.

Study V: The -308A allele of the promoter polymorphism of the TNF-α gene was a predictor for the conversion from impaired glucose tolerance to type 2 diabetes in the Finnish Diabetes Prevention Study. Furthermore, this polymorphism seems to have a gene-gene interaction with the C-174G polymorphism of the IL-6 gene, and a gene-environmental interaction with lifestyle factors.

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