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EIJA RUOTSALAINEN

Low-grade Inflammation and Markers of Endothelial Dysfunction in Subjects with High Risk of Type 2 Diabetes

Doctoral dissertation

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Series Editors: Professor Raimo Sulkava, M.D., Ph.D.
School of Public Health and Clinical Nutrition

Professor Markku Tammi, M.D., Ph.D.
Institute of Biomedicine, Department of Anatomy

Author`s address: Department of Medicine
Kuopio University Hospital
P.O. Box 1777
FI-70211 KUOPIO
FINLAND
Tel. +358 17 173311
Fax +358 17 173931

Supervisors: Professor Markku Laakso, M.D., Ph.D.
Department of Medicine
University of Kuopio and Kuopio University Hospital

Docent Jussi Pihlajamäki, M.D., Ph.D.
Department of Medicine
University of Kuopio and Kuopio University Hospital

Reviewers: Docent Jorma Lahtela, M.D., Ph.D.
Department of Medicine
University of Tampere and Tampere University Hospital

Dr. Jussi Sutinen, M.D., Ph.D.
Department of Medicine
University of Helsinki and Helsinki University Hospital

Opponent: Professor Ville Valtonen, M.D., Ph.D.
Department of Medicine
University of Helsinki and Helsinki University Hospital

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ABSTRACT

Defects in glucose and energy metabolism and abnormalities in cardiovascular risk factors in subjects with the metabolic syndrome have not been fully elucidated. Furthermore, little is known whether low-grade inflammation and endothelial dysfunction are evident in the healthy offspring of type 2 diabetic patients, who are at high risk of developing type 2 diabetes.

The aim of this study was to investigate metabolic defects and early changes in levels of cytokines and adhesion molecules in subjects at high risk of type 2 diabetes.

Altogether 129 non-diabetic offspring of type 2 diabetic patients were studied. Insulin sensitivity was assessed by the euglycemic hyperinsulinemic clamp, insulin secretion with an intravenous glucose tolerance test and energy expenditure with indirect calorimetry. Body composition and abdominal fat distribution were determined with CT. Levels of C-reactive protein (CRP), inflammatory cytokines, and adhesion molecules were measured in plasma.

Of the study subjects those with the metabolic syndrome were characterized by insulin resistance, an excess of intra-abdominal fat, lower energy expenditure and higher lipid oxidation during hyperinsulinemia, lower levels of adiponectin and higher levels of pro-inflammatory cytokines and adhesion molecules as compared to subjects without the metabolic syndrome. Offspring of type 2 diabetic patients were found to have abnormally high levels of hs-CRP, interleukin-1β (IL-1β), and interleukin-1 receptor antagonist (IL-1Ra), whereas levels of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were not elevated. Offspring of type 2 diabetic subjects were insulin-resistant with regard to the suppression of insulin-induced cytokine responses. The levels of adhesion molecules were not increased, but levels of the inflammatory markers correlated with the levels of adhesion molecules.

In conclusion, the metabolic syndrome leads to multiple defects in glucose and energy metabolism, hypoadiponectinemia, and elevated levels of pro-inflammatory cytokines and adhesion molecules. The level of anti-inflammatory IL-1Ra seems to be the most sensitive marker of cytokine response in subjects with high risk of type 2 diabetes. The cytokine response is disturbed during hyperinsulinemia in insulin-resistant offspring of type 2 diabetic patients, and is especially linked to fat-derived cytokines, highlighting the crucial role of adipose tissue in the disease process.

National Library of Medicine Classification: WK 810, WK 820, QZ 150, QW 568, WG 500, QU 55.7
Medical Subject Headings: Diabetes Mellitus, Type 2; Risk Factors; Inflammation; Biological Markers; Endothelium, vascular/physiopathology; Cytokines; C-Reactive Protein; Cell Adhesion Molecules; Glucose Clamp Technique; Glucose Tolerance Test; Calorimetry, Indirect; Energy Metabolism; Body Composition; Abdominal Fat; Metabolic Syndrome X; Lipid Metabolism; Hyperinsulinism; Adiponectin; Interleukin-1beta; Interleukin-6; Interleukin 1 Receptor Antagonist Protein; Tumor Necrosis Factor-alpha; Glucose Metabolism Disorders

It is good to have an end to journey toward,
but it is the journey that matters, in the end.
(Ernest Hemingway)

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Kuopio, September 2009

Eija Ruotsalainen

LIST OF ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
CVD	cardiovascular disease
hs-CRP	high sensitivity C-reactive protein
CT	computed tomography
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
HDL	high-density lipoprotein
ICAM-1	intercellular adhesion molecule-1
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
IVGTT	intravenous glucose tolerance test
LBM	lean body mass
LDL	low-density lipoprotein
MCP-1	macrophage chemoattractant-1
NGT	normal glucose tolerance
NO	nitric oxide
OGTT	oral glucose tolerance test
RRd	diastolic blood pressure
RRs	systolic blood pressure
TNF-α	tumor necrosis factor-α
VAP-1	vascular adhesion protein-1
VCAM-1	vascular cell adhesion molecule-1
WBGU	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 Salmenniemi U, Ruotsalainen E, Pihlajamäki J, Vauhkonen I, Kainulainen S, Punnonen K, Vanninen E, Laakso M. Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome. Circulation 2004; 110:3842-8

II Ruotsalainen E, Salmenniemi U, Vauhkonen I, Pihlajamäki J, Punnonen K, Kainulainen S, Laakso M. Changes in inflammatory cytokines are related to impaired glucose tolerance in offspring of type 2 diabetic subjects. Diabetes Care 2006:29:2714-20

III Ruotsalainen E, Vauhkonen I, Salmenniemi U, Pihlajamäki J, Punnonen K, Kainulainen S, Jalkanen S, Salmi M, Laakso M. Markers of endothelial dysfunction and low-grade inflammation are associated in the offspring of type 2 diabetic subjects. Atherosclerosis 2008:197:271-7

IV Ruotsalainen E, Vauhkonen I, Salmenniemi U, Pihlajamäki J, Punnonen K, Laakso M. Changes in cytokine levels during acute hyperinsulinemia in offspring of type 2 diabetic subjects. Submitted

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CONTENTS

1. INTRODUCTION

The incidence of type 2 diabetes has been increasing worldwide, mostly due to the increasing prevalence of obesity, sedentary lifestyle and longer life expectancy (1,2). The clustering of cardiovascular risk factors associated with insulin resistance and abdominal obesity is known as the metabolic syndrome. Individuals with the metabolic syndrome are at risk for the development of both type 2 diabetes and cardiovascular disease. At present, various definitions for metabolic syndrome exist. Information on metabolic defects in glucose and energy metabolism in subjects with the metabolic syndrome is limited.

Low-grade inflammation has been suggested to contribute to the pathogenesis of type 2 diabetes (3,4). Inflammation can be seen in individuals who progress to type 2 diabetes years in advance of disease onset. The offspring of type 2 diabetic patients are ideal subjects for studies of early defects in the pathogenesis of type 2 diabetes. These individuals are at high risk for developing type 2 diabetes, but only three previous studies have reported the levels of pro-inflammatory cytokines in this population. In these studies, levels of only one cytokine, TNF- α, were investigated and the results were contradictory (5-7).

In this study, we performed a detailed metabolic characterization of the offspring of type 2 diabetic subjects to investigate the metabolic abnormalities related to the metabolic syndrome. Furthermore, we measured multiple inflammatory and anti-inflammatory cytokines to investigate whether the innate immune system is activated in the early pre-diabetic state. We also determined the levels of adhesion molecules and their association with inflammatory markers to evaluate, whether endothelial function is impaired in subjects at high risk of type 2 diabetes.

2. REVIEW OF THE LITERATURE

2.1. Type 2 diabetes

Type 2 diabetes is the most common metabolic disease in the world and it accounts for 90-95% of all cases with diabetes (8). The prevalence of type 2 diabetes is increasing at an exponential rate with the obesity epidemic (8) and is seen in ever-younger age groups (8,9). Type 2 diabetes develops because pancreatic ß-cells eventually fail to produce enough insulin to compensate for insulin resistance (10).

Type 2 diabetes is associated with aging, obesity and physical inactivity. However, due to increasing incidence of obesity and physical inactivity among young people, the age of onset of type 2 diabetes is substantially lower than previously. Insulin resistance precedes the onset of type 2 diabetes by years or decades (11,12). Type 2 diabetic patients are exposed mainly to macrovascular complications. The risk for coronary heart disease is two- to fourfold higher compared to nondiabetic populations (13). Furthermore, cardiovascular disease accounts for 58% of all deaths attributable to diabetes (14,15). In type 2 diabetic patients, the most common cerebrovascular disease is ischaemic stroke. In the UKPDS Study, stroke occurred in 6 % of patients in the 10 years after diagnosis of diabetes (16). The long-term microvascular complications of type 2 diabetes include retinopathy, nephropathy, peripheral neuropathy and autonomic neuropathy.

2.2. Risk factors of type 2 diabetes

2.2.1. Obesity and fat distribution

Obesity is a major risk factor for type 2 diabetes, and 60-90 % of type 2 diabetic patients are obese (17,18). In a large U.S. cohort of 84,941 middle-aged women, the presence of overweight or obesity was the single most important predictor of type 2 diabetes (19). In a cohort of 51,529 middle-aged

men, the risk of type 2 diabetes was associated strongly with overall adiposity (18). Men with body mass index (BMI) of ≥ 35 kg / m² had a 42.1 fold greater risk for type 2 diabetes than men with a BMI < 23 kg / m².

Although overall obesity increases the risk of type 2 diabetes, the accumulation of fat in the abdominal region may be an even more powerful risk factor (20). In particular, intra-abdominal fat is detrimental to glucose metabolism and insulin sensitivity (21). Fat distribution is partially genetically determined (22). First-degree relatives of type 2 diabetic subjects have an increased waist-to-hip ratio (WHR) compared to their spouses without a family history of type 2 diabetes (23).

2.2.2. Lifestyle factors

Physical inactivity increases insulin-mediated glucose uptake (24), improves insulin sensitivity (25) and decreases the amount of visceral fat (26-28). In persons with impaired glucose tolerance (IGT), lifestyle interventions including regular physical activity have been shown to reduce the subsequent development of type 2 diabetes by more than half (29,30). In a systematic review by Jeon et al (31) moderate-intensity physical activity was shown to reduce the risk of type 2 diabetes even in those who did not achieve weight loss.

Moderate physical activity has been shown to reduce the risk of type 2 diabetes independently of age and BMI in a cohort of 7735 men (32). Also, The Nurse's Health Study showed that greater leisure-time physical activity, in terms of both duration and intensity, was associated with a reduced risk of type 2 diabetes (33). In addition, approximately half the cases of type 2 diabetes in this study could have been prevented by combining healthy diet, regular exercise, abstinence from smoking, and moderate alcohol consumption (19). Even among obese women (BMI $\geq$ 30), the combination of healthy diet and regular exercise was associated with a 24 percent reduction in the risk of developing type 2 diabetes (19). Thus, healthy diet and adequate exercise decrease the

risk of developing type 2 diabetes, independently of the effect on body weight, and regardless of the presence or absence of obesity.

Active smoking is associated with an increased risk of type 2 diabetes (19,34-37). The number of cigarettes smoked daily and the number of pack-years of exposure were closely associated with impaired fasting glucose and type 2 diabetes in a cohort of middle-aged Japanese men (36). Cigarette smoking causes insulin resistance in peripheral tissues, whereas insulin secretion may be unimpaired or over-stimulated (38-40).

Dietary patterns. In the KANWU study, insulin sensitivity was improved by a diet that was high in monounsaturated fatty acids and low in saturated fatty acids (41). In the Iowa Women's Health Study, the amount of dietary vegetable fat was inversely related to the incidence of diabetes (42). Moreover, substituting polyunsaturated fatty acids for saturated fatty acids reduced the rate of developing type 2 diabetes.

Several epidemiological studies have suggested that diets rich in whole grains (43-45) or cereal fiber (19,44,46,47) may protect against diabetes. This effect may be mediated by positive effects on body weight and also by slowing gastrointestinal absorption. Schulze et al. (48) suggested that a diet high in sugar-sweetened soft drinks, refined grains, diet soft drinks, and processed meat and low in wine, coffee, cruciferous vegetables, and yellow vegetables may increase the risk of type 2 diabetes, possibly by exacerbating inflammatory processes.

2.2.3. Other risk factors

Low-grade inflammation. Pickup et al. (49,50) were the first to suggest that type 2 diabetes is an inflammatory condition characterized by elevated concentrations of acute phase inflammatory reactants in the plasma. Elevated circulating inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) predict the development of type 2 diabetes (51-53). Pickup et al. (54) hypothesized that many of the abnormalities seen in type 2 diabetes and impaired glucose tolerance are mal-adaptations of the normal innate

immune system response to environmental threats. Although the markers of low-grade inflammation are increased in type 2 diabetes, the degree of immune activation in this disease is far below that seen in acute infections (3).

Endothelial dysfunction. The innermost layer of blood vessels, called the "endothelium", is biologically active and responsible for the regulation of several important functions (55), including vascular tone, platelet adhesion, coagulation and leukocyte adherence. The term "endothelial dysfunction" refers specifically to the impairment of endothelium-dependent relaxation, and is caused by a loss of the normal nitric oxide (NO) bioactivity within the vessel wall (56). Endothelial function deteriorates with age, as well as in the presence of diabetes, obesity, hypertension, smoking and hypercholesterolemia (55), which are all major risk factors for the development of atherosclerosis.

Depression. The meta-analysis by Knol et al. identified depression as a risk factor for type 2 diabetes, comparable in significance to smoking and lack of physical activity. The pathophysiological mechanisms responsible for this association remain unclear (57).

Birth weight. In 1993, Barker et al. reported a relationship between low birth weight and an increased risk of developing type 2 diabetes in adulthood (58). However, a meta-analysis of 14 articles by Harder et al. (59) demonstrated a U-shaped correlation between birth weight and later risk of type 2 diabetes. Thus, high birth weight seems to increase the risk of type 2 diabetes to the same extent as low birth weight.

Infection. Several studies have demonstrated the association of Chlamydia pneumoniae infection and metabolic syndrome, insulin resistance, and coronary artery disease (60-63). However, the association was not confirmed in some studies or disappeared after adjusting for body weight (62,64). Moreover, antibiotic prevention treatment failed to reduce the prevalence of secondary coronary events in a large clinical trial (65,66). Wang et al. (67), demonstrated in their recent study, that Chlamydia pneumoniae infection plays a causal role on the development of insulin resistance and type 2 diabetes in obese C57BL/6 mice. This finding may be useful in the study of Chlamydia pneumoniae vaccination for type 2 diabetes control (67).

Hyperglycemia is common in critically ill patients (68) and it may lead to complications such as severe infections, polyneuropathy, multiple organ failure and death (69). In a study by Jacob et al (69), sepsis-induced inflammatory responses were exacerbated in a non-obese rat model of type 2 diabetes, suggesting that the observed increase in inflammatory response is due to the diabetic phenotype. Type 2 diabetes is more prevalent among patients with HIV infection especially among patients receiving protease inhibitors (70). Furthermore, chronic hepatitis C infection has been associated with type 2 diabetes in several observational studies (71).

2.3. Genetics of type 2 diabetes

Type 2 diabetes has a strong heritability. First, type 2 diabetes clusters in families (72). Second, the concordance rate of type 2 diabetes in monozygotic twins (50 – 96%) is higher than that in dizygotic twins (10-37%) (73-76). Third, the prevalence of type 2 diabetes varies among ethnic populations, being highest in American Indian tribes (~40%) and lowest in Colombian Amerindians (0%) and traditional Brazilian Amerindians (0%) (77). The risk for type 2 diabetes increases approximately two- to fourfold when one or both parents have type 2 diabetes (78-80).

The inheritance of type 2 diabetes does not follow the Mendelian pattern. Type 2 diabetes is a multi-factorial disease in which individual risk is defined by the complex interplay between genetic and environmental factors (81). Although causal genes have been identified for many monogenic forms of diabetes (82), elucidation of the genetic background of type 2 diabetes proceeded slowly until 2007. Five genome-wide studies have now been published, increasing the number of confirmed type 2 diabetes susceptibility loci from three (*PPRAG*, *KCNJ11*, T*CF7L2*) to nine with the addition of *CDKAL1*, *CDKN2A/B, IGF2BP2*, *HHEX/IDE, FTO and SLC30A8*) (83). In a recent meta-analysis six new loci were detected, including *JAZF*, *CDC123*/*CAMK1D, TSPAN8*/*LGR5, THADA*, *ADAMTS9* and *NOTCH2 gene regions* (84). The majority of type 2 diabetes gene variants have been implicated in decreased β-cell insulin secretion,

supporting the crucial role of β-cell dys-regulation in the pathogenesis of type 2 diabetes (85). All of the recently identified candidate genes regulate insulin secretion, whereas genes regulating insulin sensitivity have not been found (86)

2.4. Pathophysiology of type 2 diabetes

Type 2 diabetes is a progressive, heterogeneous disease characterized by varying degrees of insulin resistance and relative insulin deficiency. Although sedentary lifestyle and obesity seem to be the triggering pathogenic factors, both genetic and environmental elements are essential to the development of this disease. Furthermore, hyperglycemia itself can impair and destroy ß-cells**,** and thus eventually stop insulin production (87).

Insulin is the key hormone in blood glucose regulation. It has diverse functions including stimulation of nutrient transport into cells, regulation of gene expression, modification of enzymatic activity and regulation of energy homeostasis via actions in the arcuate nucleus (88). Normoglycaemia is maintained by a balanced interplay between insulin action and insulin secretion (89). Normally, the pancreatic ß-cell can adapt to changes in insulin action. A decrease in insulin action is followed by an up-regulation of insulin secretion, and vice versa (89).

A continued decline in pancreatic ß-cell function is critical in defining the risk and development of type 2 diabetes (90). The Pima Indians have a higher prevalence of type 2 diabetes than any other population in the world. These individuals are often insulin-resistant, and progress to type 2 diabetes through excessive loss of their ß-cell mass (91). β-cell failure progresses even when the glucose level is within the normal range (92).

The offspring of type 2 diabetic subjects are also at increased risk of developing type 2 diabetes, and have been shown to possess impaired ß-cell function even in the presence of normal glucose tolerance (93). In these individuals, the decline in glucose tolerance over time is strongly correlated to the loss of ß-cell function (94). Impaired ß-cell function is reversible to a certain degree (95). Even in the presence of initial severe hyperglycemia, ß-cell

function can be restored when euglycemia is attained pharmacologically, by bariatric surgery or by life-style changes (96). Pancreatic ß-cell regeneration does occur in adults through a combination of replication from existing ß-cells, plus ß-cell neogenesis from precursor cells within the adult pancreatic ducts (97).

Over the past few decades there has been much debate regarding the relative importance of insulin resistance and ß-cell dysfunction in the pathophysiology of type 2 diabetes. Several studies have suggested that insulin resistance is the primary abnormality, and that ß-cell dysfunction is a late event arising from the increased secretory demand placed on the ß-cells by prolonged insulin resistance (98). In contrast, others have suggested that impaired β-cell function is a prerequisite for the progression from NGT to hyperglycemia (99-101). For example, the UKPDS (102) and the longitudinal study in Pima Indians (91) suggest that the major determinant of glucose intolerance is a progressive loss of β-cell function. Kahn (103), however, concludes that both insulin resistance and β-cell dysfunction are present very early in the natural history of diabetes. In any case, type 2 diabetes occurs when the β-cells can no longer sustain insulin secretion in the setting of insulin resistance (104).

Several mechanisms have been proposed to induce ß-cell loss in type 2 diabetes (105). These include glucose toxicity (106), reactive oxygen species (107) and inflammatory cytokines such as IL-1ß (108). Normoglycaemia can be maintained until approximately 60% of the ß-cell mass is lost (109). Interleukin-1ß contributes to ß-cell glucotoxicity in the pathogenesis of type 2 diabetes (108). Long-term exposure of cultured human islets to elevated glucose levels leads to ß-cell production and release of IL-1ß (108). In turn, IL-1ß acts back on the ß-cells to induce impaired function and apoptosis (110). This effect is mediated by closure of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels, which are key regulators of ß-cell function and survival (110). ß-cells ATP-sensitive K^+ (K_{ATP}) channels are octamers composed of four inwardly rectifying K^+ channels and four sulfonylurea receptors (111). Sulfonylureas block K^+ (K_{ATP}) channels, stimulating the effect of glucose in eliciting insulin release (112). Maedler et al (111) showed that the sulfonylurea glibenclamide induces

ß-cell apoptosis in human islets. Therefore, sulfonylureas, may have adverse effects on ß-cell mass (111). Interestingly, leptin has been shown to modulate IL-1ß-induced apoptosis in human ß-cells (113)

2.5. Low-grade inflammation and type 2 diabetes

2.5.1. C-reactive protein and cytokines as markers of low-grade inflammation

Innate immunity. Immunity is divided into two systems determined by the speed and specificity of the reaction (114). The innate immune system is a non-specific primary defence mechanism against environmental threats such as microbial infection and physical or chemical injury. It does not exhibit a memory response, and it reacts similarly to a variety of organisms and threats. In contrast, the adaptive or so-called *acquired immune system* acts as a second line of defence, and also protects in the event of re-exposure to a previously encountered pathogen.

All protective mechanisms of the innate immune system are encoded in the germline of the host (115). These include passive physical (e.g. epithelial cell layers, mucociliary blanket), chemical and microbiological barriers (114). However, in most cases the immediate host defence is provided by the active elements of the immune system (neutrophils, macrophages, monocytes, complement, cytokines and acute phase proteins).

Although the innate and adaptive immune systems have distinct functions, they usually act together. The innate response represents the initial, rapid line of host defence, whereas the adaptive response becomes prominent after several days, when antigen-specific T and B cells have undergone clonal expansion (116). An important function of the innate immune system is the control of the adaptive immune response (117).

Inflammation is a consequence of the activation of innate immunity. Inflammation causes local effects, whereas a systemic reaction is known as an acute-phase response. This response is designed to restore homeostasis after environmental threats (118) and is characterized by changes in the

concentrations of acute-phase reactants (119,120). The concentrations of some of these proteins increase for example, C-reactive protein (CRP), fibrinogen and serum amyloid A, while the concentrations of others decrease for example, albumin and transferrin (118). Acute phase proteins are mostly synthesized in the liver, and their production is stimulated by cytokines of the innate immune response – mainly interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α (121).

Cytokines. Cytokines are low molecular weight messenger molecules secreted by virtually all cells and have a variety of functions (114). Cytokines bind to specific receptors on target cells and mediate intracellular signals. Typically these molecules affect cell activation, division, apoptosis or movement. They act either in an autocrine (on the producer cells), paracrine (on cells near-by) or endocrine fashion (via bloodstream). Cytokines are generally classified as interleukins, growth factors, chemokines, interferons or colony-stimulating factors. They can also be divided by their inflammatory activity into pro- and anti-inflammatory subgroups. Cytokines allow an organism to respond rapidly to an immune challenge by coordinating an appropriate immune response. A balance between the inflammatory and anti-inflammatory responses is essential for normal cellular function. Unbalanced cytokine production is associated with many diseases.

Cytokines are often produced in cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can act synergistically or antagonistically. As cytokines have an effect on the expression of other inflammatory factors and on each other, the question of a possible causal relationship of cytokines and diseases is very complicated (122).

The measurement of cytokine levels is useful for investigating disease pathogenesis, and cytokine levels serve as diagnostic and prognostic indicators in many diseases (123). Immunoassays are the most widely used techniques (124). The concentrations of cytokines in biological fluids are often near the lower limit of detection. Thus, the sensitivity of assays should be defined appropriately, taking the variability of the assay into account (65). There is also the possibility that cytokines are transported to target organs by circulating monocytes, resulting in undetectable cytokine levels in the plasma (65).

Furthermore, the measurement of IL-1 agonists (IL-1α and IL-1ß) without measurement of IL-1 receptor antagonist (IL-1Ra) cannot give a complete picture of the biological role of IL-1 in pathological or physiological processes (65). Cytokine levels reflect a sum of the production, removal and retention of these molecules. Frozen stored samples are preferred (123).

C-reactive protein (CRP). CRP is an acute-phase reactant produced by hepatocytes primarily in response to IL-6, although its production is also regulated by other cytokines, including IL-1 and TNF-α (119). Serum levels of CRP rise dramatically in response to infection, inflammation and injury (125). CRP is widely used as part of the diagnostic workup, to monitor disease status, and to monitor treatment results (126). About 90% of apparently healthy individuals have CRP concentrations < 3 mg/l and 99% have concentrations < 10 mg /l (127). Chronically elevated CRP is a strong risk factor for cardiovascular events (128), suggesting that inflammation is an important contributor to atherosclerosis. High-sensitivity CRP (hs-CRP) identifies patients with unstable coronary lesions who have previously gone unrecognized by traditional coronary heart disease markers (129). C-reactive protein itself may also contribute to the pathogenesis of atherothrombosis by having a direct effect on human endothelial cells (130). Parental injection of human CRP enhanced markedly tissue damage via a complement-dependent mechanism, in experimental acute myocardial infarction produced by coronary artery ligation (131). On the contrary, CRP plays an important role in host defense by complement activation, opsonization and by inducing phagocytosis (132). There are data indicating that elevated hs-CRP levels predict the development of the metabolic syndrome (133) and type 2 diabetes (134).

2.5.2. Low-grade inflammation, insulin resistance, metabolic syndrome and type 2 diabetes

The concept that activated innate immunity may be the common antecedent of type 2 diabetes provides an exciting and novel approach to the understanding of the pathogenesis of type 2 diabetes (121). The first data to give rise to the

inflammation hypothesis came from cross-sectional studies in the 1960s demonstrating that systemic concentrations of many immune mediators appear to be chronically up-regulated in type 2 diabetes (135,136). A simplified model for the role of low-grade inflammation in insulin resistance, type 2 diabetes and endothelial dysfunction is given in Figure 1.

In 1993 Hotamisligil et al. (137) demonstrated that TNF-α is over-expressed in obese mice and rats, thus providing the first link between insulin resistance and a pro-inflammatory cytokine. TNF-α is also expressed in human adipocytes and its concentration is decreased by weight loss (138). Dandona et al. (139) demonstrated that obesity is associated with increased plasma concentrations of TNF-α, which fall with weight loss. Further studies showed that obesity is a state of chronic inflammation, as indicated by increased plasma concentrations of CRP (140), IL-6 (141) and plasminogen activator inhibitor-1 (PAI-1) (142).

In 1993 Crook et al. (143) showed that circulating concentrations of CRP, serum amyloid A, α1-acid glycoprotein and sialic acid were increased in type 2 diabetic patients but not in type 1 diabetic patients, thus linking for the first time type 2 diabetes with an activated acute phase response. Pickup et al. (50) hypothesized that the similar dyslipidemia seen in both type 2 diabetes and the acute phase response might be cytokine-mediated and might provide a unifying mechanism for these conditions. Pickup et al. (50) also observed significant increases of serum sialic acid, α-1 acid glycoprotein, and IL-6 levels and urinary albumin excretion rates in non-diabetic subjects, type 2 diabetic patients without syndrome X [hyperinsulinemia, impaired glucose tolerance, hypertension, increased triglyceride, decreased HDL-cholesterol (144)] and type 2 diabetic patients with syndrome X, with the highest levels occurring in this last group. They concluded that type 2 diabetes is associated with an elevated acute-phase response, which is closely involved in the pathogenesis of this disease. Furthermore, abnormalities of the innate immune system could contribute to hypertriglyceridemia, low HDL cholesterol, hypertension, glucose intolerance, insulin resistance and accelerated atherosclerosis in type 2 diabetes (50). This disorder of innate immunity also has wide-ranging effects on psychological behaviour, sleeping patterns, reproductive hormones, haemostasis, metal ion

metabolism and capillary permeability (54). All these abnormalities are often observed in type 2 diabetes. Thus, based on these findings, the evident mechanism for the development of type 2 diabetes is the long-term activation of the innate immune system, resulting in chronic inflammation and eliciting disease instead of repair in individuals who subsequently develop type 2 diabetes (54).

Several cross-sectional studies have confirmed that levels of acute-phase reactants (such as CRP and sometimes IL-6, TNF-α and fibrinogen) are positively correlated with measures of insulin resistance (140,145-148). In a study by Temelkova-Kurktschiev, inflammatory markers were related to insulin resistance but not to insulin secretion (149). In one study, levels of IL-6 but not levels of TNF- α were increased in subjects with IGT or IFG compared with levels in individuals with normal glucose tolerance (150). The association of low-grade inflammation with newly diagnosed (151) or established type 2 diabetes (152-155) was also confirmed by an observation of elevated concentrations of acute phase reactants such as CRP and IL-6.

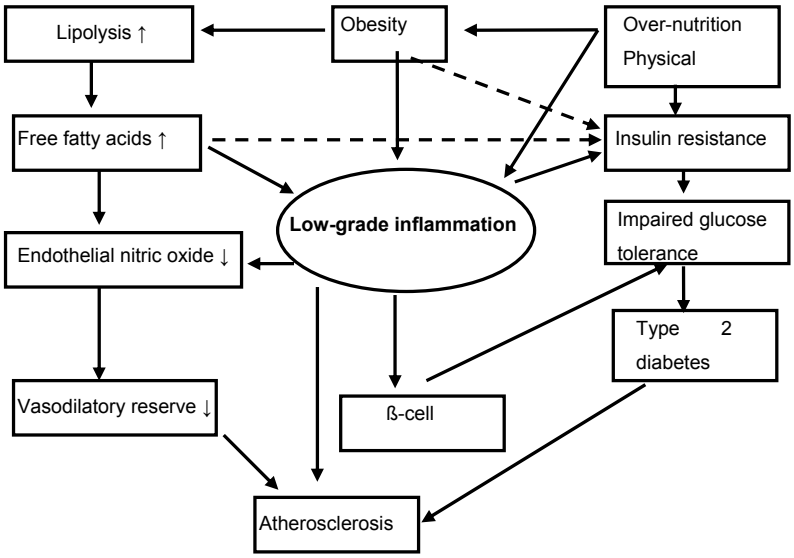

Figure 1. Simplified model for the role of low-grade inflammation in the aetiology of type 2 diabetes and endothelial dysfunction. Several factors such as over-nutrition, physical inactivity and obesity activate inflammatory signalling pathways and cause insulin resistance. Obesity leads to an inflammatory response by itself and by inducing increased lipolysis and release of free fatty acids (FFA). As a consequence of FFA and cytokine release the synthesis of adhesion molecules is up-regulated leading to impaired endothelial nitric oxide production and endothelial dysfunction. Cytokines act directly on pancreatic ß-cells by impairing insulin secretion and inducing ß-cell apoptosis. Defective insulin secretion leads to impaired glucose tolerance. Modified from ref (156).

2.5.3. Markers of inflammation as risk factor for type 2 diabetes

The Atherosclerosis Risk in Communities-study was the first to show that several inflammatory markers, including white blood cell count, low serum albumin, α1-acid glycoprotein, fibrinogen and sialic acid, are predictive of later type 2 diabetes in a middle-aged population (51,157). Recently, this hypothesis has been strongly supported by several studies including the Women's Health Study showing that elevated CRP and IL-6 levels were associated with the development of type 2 diabetes on healthy middle-aged women (53). In elderly subjects in the U.S. Cardiovascular Health Study baseline CRP was particularly high in those older individuals who later developed type 2 diabetes (158).

Similar findings have been reported in Pima Indians (white blood count) (159), in multiethnic subjects in the U.S. Insulin Resistance and Atherosclerosis Study (CRP, fibrinogen, and PAI-1) (160), in women in the Nurse's Health Study (CRP) (161), in Scottish men in the West of Scotland Coronary Prevention Study (CRP) (162), in the U.S. National Health and Nutrition Examination Survey (white blood count, erythrocyte sedimentation rate) (163), in Japanese men (white blood count) (164), in participants in the Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study in Germany (IL-6, with additional risk of IL-6 and IL-1β combined) (165), and in middle-aged men in the MONICA Augsburg Study in Germany (CRP) (166). In the Mexico City Diabetes Study, elevated CRP levels were significant predictors of diabetes in women but not in men. The authors suggested that low-grade inflammation may have a greater effect in perturbing the actions of insulin in females than in males (133). In a population-based study of 923 middle-aged subjects in Pieksämäki, East-Finland, women with metabolic syndrome had higher levels of hs-CRP and IL-1Ra than did men with metabolic syndrome (167). Low-grade inflammation in women may thus explain, why the metabolic syndrome is a stronger predictor of cardiovascular disease in women than in men (167).

2.5.4. Possible mechanisms of activated innate immunity in type 2 diabetes

Type 2 diabetes is associated with a general activation of the innate immune system, in which there is a chronic, cytokine-mediated state of low-grade inflammation. These changes are adaptive mechanisms designed to restore homeostasis during and after external threats. How chronic inflammation can cause type 2 diabetes is not clear. The possibility that the inflammatory changes might be a consequence of type 2 diabetes rather than a contributor to its development has been debated (3). Prospective studies have reported subtle pro-inflammatory changes many years before the onset of the disease (51,53,157).

Obesity. The link between obesity and inflammation has raised the question of whether obesity-induced inflammation plays a pathogenic role in the

development and progression of type 2 diabetes. In obese subjects, hypertrophied adipocytes secrete large amounts of the macrophage chemoattractant MCP-1, perhaps contributing to macrophage infiltration into adipose tissue (168). Macrophage recruitment results in a pro-inflammatory state in obese adipose tissue. Infiltrating macrophages secrete large amounts of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1ß. The excess of circulating triglycerides and free fatty acids results in the accumulation of activated lipids in skeletal muscle, disrupting functions such as mitochondrial oxidative phosphorylation and insulin-stimulated glucose transport, thus triggering insulin resistance (168).

Genetic factors. Genetic influence on innate immunity is suggested from studies showing that subjects with the highest transcription rates of genes encoding TNF-α and IL-6 are prone to develop obesity, insulin resistance and type 2 diabetes (169).

In the meta-analysis of more than 20,000 subjects by Huth et al.(170), the GC and CC genotypes of IL-6-174G>C were associated with a decreased risk of type 2 diabetes providing further evidence that immune mediators are causally related to type 2 diabetes. In a study by Fernandez-Real et al (171), a polymorphism of IL-6 gene was shown to influence the relationship among insulin sensitivity and postload glucose levels. Pannacciulli et al (172) showed that a family history of type 2 diabetes was associated with increased levels of C-reactive protein in non-smoking healthy women.

Diet. Dietary habits may contribute to the activation of innate immunity in genetically or metabolically predisposed individuals. Whole-grain diets with a low glycemic index probably decrease the risk of type 2 diabetes through induction of improved insulin resistance and ß-cell function (173), but modulation of inflammation may be another mechanism (174). The intake of foods with a high glycemic index is associated with hyperglycemia, and is thus a major stimulus for inflammation (175). Several cross-sectional studies have shown that omega-3 fatty acids have anti-inflammatory properties (176-178). A growing amount of evidence suggests an anti-inflammatory effect of fruit and vegetable consumption (179,180). Likewise, clinical trials of nut consumption

have reported decreases in inflammatory markers (181) and improvements in endothelial function (182).

Aging. Increased inflammatory activity accompanies aging. Several factors are likely to contribute to increased low-grade inflammatory activity in the elderly, including decreased production of sex steroids, smoking, atherosclerosis and higher relative or absolute amount of adipose tissue (183). Similarly, the incidence of type 2 diabetes is increased in the elderly. A low-grade systemic inflammatory response is also evident in smokers, as confirmed by numerous population-based studies (184-187). In the Hoorn Study of a city population in the Netherlands aged 50-74 years and without a history of diabetes, the number of stressful life events in the previous 5 years was positively associated with the prevalence of newly detected type 2 diabetes (188).

Bacterial and viral infections. Fernandez-Real et al. (189) hypothesized that burden of infection could be associated with chronic low-grade inflammation, resulting in insulin resistance before established atherosclerosis develops. Among apparently healthy men, herpes simplex virus (HSV)-2 seropositivity was modestly linked to insulin resistance, whereas total pathogen burden (based on herpes simplex virus (HSV)-1, HSV-2, enteroviruses, and Chlamydia pneumoniae IgG serostatus) showed the strongest association with insulin resistance, especially when these two last pathogens caused seropositivity (63). In fact, the reduction of lifetime exposure to infectious diseases and other sources of inflammation has made an important contribution to the decline in old-age mortality (190).

2.6. Endothelial dysfunction and type 2 diabetes

The endothelium is involved in the regulation of multiple functions, such as regulation of vascular tone, platelet adhesion, coagulation, fibrinolysis and leukocyte adherence (56). A key feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to an appropriate stimulus. Dysfunctional endothelial cells are unable to produce nitric oxide (NO)

and prostacyclin to the same extent as healthy endothelial cells and therefore vasodilatation is reduced. The release of vasoconstricting factors, such as endothelin-1 and angiotensin-II, is also changed. Thus, endothelial dysfunction refers to an imbalance in the release of vasodilating and vasoconstricting factors (191,192). The molecular basis of this condition is complicated and far from understood (192).

Several methods to measuring endothelial dysfunction have been developed but no single method has been proven superior. Instead, different techniques seem to be complementary to one another (193). The reference method for assessing endothelial dysfunction is the quantitative coronary angiography with an intra-coronary ultrasound using a Doppler transducer. However, this technique is complicated and invasive. Therefore, simple non-invasive methods have been developed, e.g. flow-mediated vasodilatation and plethysmography.

2.6.1. Biomarkers of endothelial dysfunction

Measurement of endothelial biochemical markers may be the simplest method to monitoring endothelial function indirectly (193). A number of circulating markers are linked to endothelial dysfunction, including adhesion molecules, selectins, integrins, cytokines and fibrinolytic molecules. These all promote the adherence of monocytes and hence accelerate atherogenesis (194,195).

Sub-clinical tissue injury and adiposity induce the release of pro-inflammatory cytokines, especially TNF-α and IL-6, which stimulate an acute-phase response marked by elevated levels of CRP (140). When endothelial cells are activated by inflammatory cytokines, the increased expression of vascular cellular adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 promote the adherence of monocytes. E-Selectin is absent in inactive cells but is rapidly induced by inflammatory cytokines. ICAM-1 and VCAM-1 are expressed by endothelial cells and leukocytes not only in response to inflammatory cytokines but also in response to elevated levels of free fatty acids, oxidized low-density lipoprotein cholesterol, and advanced glycosylation end products occurring in diabetes (196). Adhesion molecules play a key role

in the early formation of atherosclerotic plaque by facilitating leukocyte rolling, adhesion and transmigration into the endothelial space (197). Thus, elevated plasma levels of adhesion molecules are an early marker of endothelial dysfunction and can be used as an indirect measure of endothelial dysfunction.

2.6.2. Endothelial dysfunction, insulin resistance and type 2 diabetes

Endothelial dysfunction is an early abnormality in insulin-resistant states (156,198). In addition, systemic inflammation is associated with insulin resistance, incipient coronary vascular disease and diabetes (51,53,160,199). Large amounts of cytokines are released from adipose tissue (121) in an inflammatory process, which is driven by caloric excess and might be regulated by genetic factors. Cytokines exert a toxic effect on endothelial cells and cause increased capillary permeability (200) further aggravating the atherosclerotic process (121). Similarly, CRP promotes atherosclerosis and endothelial cell inflammation (201,202).

Endothelial dysfunction is a consistent finding in type 2 diabetes (203-205). There is also growing evidence supporting the hypothesis that endothelial dysfunction precedes the development of fullblown type 2 diabetic state. In the MONICA/ KORA Study, E-Selectin was predictive of type 2 diabetes (206). Elevated levels of plasminogen activator inhibitor (PAI-1) predicted the development of fullblown type 2 diabetes in the Insulin Resistance Atherosclerosis Study (160).

Furthermore, in the Framingham Offspring Study, PAI-1 and von Willebrand factor increased the risk of incident diabetes independent of other diabetes risk factors (207). Based on these findings, endothelial dysfunction seems to be a unifying link between cardiovascular disease and type 2 diabetes supporting the theory of common soil.

Insulin can also promote atherosclerosis by direct action on the arterial wall. It causes in-vitro proliferation of smooth muscle cells in animal models and in human beings (208). Several prospective epidemiological studies have confirmed that circulating insulin concentration is a cardiovascular risk factor

(209). Another possibility is that insulin resistance itself, by production of inflammatory cytokines, induces atherogenesis and that hyperinsulinemia could be body's compensatory attempt to suppress the inflammation and overcome insulin resistance (55,121,156,189,200). Moreover, glucose has pro-inflammatory effects, since it increases synthesis of reactive oxygen species and accentuates several inflammatory markers in vitro (121,189,210).

3. THE AIMS OF THE STUDY

This study was undertaken to investigate metabolic defects and early changes in levels of cytokines and adhesion molecules in offspring of type 2 diabetic patients. The specific aims were:

1. To investigate the metabolic defects in glucose and energy metabolism as well as the abnormalities in a variety of cardiovascular risk factors in subjects with the metabolic syndrome (Study I).
2. To investigate the early changes in inflammatory markers in the offspring of type 2 diabetic patients (Study II).
3. To characterize the role of various biomarkers of endothelial activation in a cohort of offspring of type 2 diabetic patients and to assess the association of adhesion molecules with inflammatory markers and metabolic parameters (Study III).
4. To investigate the changes in the levels of cytokines and adhesion molecules in response to acute hyperinsulinemia in the offspring of type 2 diabetic subjects (Study IV).

4. SUBJECTS AND METHODS

4.1. Subjects

Healthy non-diabetic offspring of patients with type 2 diabetes were included in this study in 2000-2003. The probands were chosen from the North Savo area, which has a population of 250,000, of whom 4% carry a diagnosis of diabetes. Families for our study were sought from earlier diabetes studies, from among outpatient clinic and hospital ward patients, as well as through newspaper advertisements. Of the 130 suitable families that were identified, 50 had to be excluded (43 because of IGT and 7 because of type 2 diabetes in spouse of type 2 diabetic proband). This left a total of 80 families consisting of 130 offspring (one to three offspring from each family). The exclusion criteria for the offspring were: 1) diabetes mellitus or any other disease that could potentially disturb carbohydrate metabolism; 2) diabetes mellitus in both parents; 3) pregnancy; 4) any acute ongoing infection; 5) age under 25 or over 50 years.The clamp study did not succeed in one subject, whose results were excluded from all analyses. The final study population consisted of 129 subjects, and their characteristics are listed in Table 1.

The control group consisted of 19 healthy nondiabetic subjects, who were either medical students studying in the University of Kuopio or staff working in the Kuopio University Hospital. The demographics of the control group are given in Table 1.

In the first study, 119 non-diabetic offspring of diabetic probands and 19 controls were included. The second and the third study consisted of 129 offspring and 19 controls, whereas in the fourth study, 40 offspring and 19 controls were studied.

4.2. Study design

The studies were conducted on the metabolic ward of the Department of Medicine at the Kuopio University Hospital on three different occasions 1-2 months apart. On day 1, the subjects were interviewed regarding their medical history, smoking, alcohol consumption and physical activity. Blood pressure was measured in sitting position after a 5-min rest with a mercury shygmomanometer. The average of three measurements was used to calculate systolic and diastolic blood pressure as well as the mean blood pressure ([2 x diastolic blood pressure + systolic blood pressure]/ 3). Weight and height were measured to the nearest 0.1 cm and 0.5 kg, respectively. BMI was calculated as weight in kilograms divided by height in meters squared. Waist (at the midpoint between the lateral iliac crest and lowest rib) and hip circumference (at he level of trochanter major) were measured to the nearest 0.5 cm. Fasting blood samples were drawn after 12 hours fasting followed by an OGTT. Glucose tolerance status was evaluated according to the World Health Organization Criteria.

On day 2, body composition was determined by bioelectrical impedance. Thereafter, an intravenous glucose tolerance test (IVGTT) and euglycemic hyperinsulinemic clamp were performed after an overnight fast. Indirect calorimetry was performed during the last 30-min of the euglycemic clamp. On day 3, abdominal fat distribution was evaluated by CT and exercise test was performed to determine maximum oxygen uptake.

4.3. Metabolic studies

4.3.1. Oral glucose tolerance test

In a 2-hour OGTT (75 g of glucose) blood samples for plasma glucose and insulin determinations were drawn at 0, 30, 60, 90 and 120 min. Those with normal or impaired glucose tolerance according to the WHO criteria (211) were

included in the study. The subjects were advised to avoid vigorous exercise two days before the OGTT.

4.3.2. Intravenous glucose tolerance test

An IVGTT was performed to determine the first phase insulin secretion capacity (212). After an overnight fast an intravenous catheter was placed into the left antecubital vein for the infusion of glucose. Another cannula for blood sampling was inserted into a vein in the dorsum of the right hand, which was placed in a heated (50°C) box for arterialization of venous blood. After baseline blood collection and indirect calorimetry a bolus of glucose (300 mg/kg in a 50% solution) was given within 30 seconds into the antecubital vein in order to acutely raise the blood glucose level. Samples for the measurement of blood glucose and plasma insulin were drawn at –5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 min.

4.3.3. Euglycemic clamp

The degree of insulin sensitivity was evaluated with the euglycemic hyperinsulinemic clamp technique (213). After an IVGTT, a priming dose of insulin (Actrapid 100 IU/ml, Novo Nordisk, Gentofte, Denmark) was administered during the initial 10 minutes to acutely raise plasma insulin to the desired level, where it was maintained by a continuous infusion rate of 40 mU/min/m² body surface area. The resulting average plasma insulin concentration was 66.8 ± 14.91 mU/l and 59.49 ± 7.24 mU/l in offspring and controls, respectively. Blood glucose was clamped at 5.0 mmol/l for the next 120 min by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. The mean amount of glucose given was calculated for each 20-min interval and the mean value for the last 20-min interval (the last 60 min interval in study I) was used to define the rates of whole body glucose uptake (WBGU). The resulting mean glucose concentration at 100-120 min was 5.07 ± 0.23 mmol/l and 5.03 ± 0.193 mmol/l

in offspring and controls, respectively. The mean coefficient for variation of blood glucose was < 4 %. Samples for plasma lactate, insulin and serum FFA measurements were drawn at 0 and 120 min.

4.4. Indirect calorimetry

Indirect calorimetry was performed with a computerized flow-through canopy gas analyzer system (DELTATRAC®, TM Datex, Helsinki, Finland). Gas exchange was measured for 30 minutes in the fasting state (before an IVGTT) and during the last 30 minutes of the euglycemic clamp. The values obtained during the first 10 minutes were discarded and the mean value of the remaining 20-min data was used for calculations of glucose and lipid oxidation. Protein oxidation was calculated on the basis of the urinary non-protein nitrogen excretion rate (214). The fraction of carbohydrate non-oxidation during the euglycemic clamp was estimated by subtracting the carbohydrate oxidation rate from the glucose infusion rate.

4.5. Body composition and fat distribution

Body composition was determined by bioelectrical impedance (RJL Systems®, Detroit, US) in the supine position after a 12-hour fast. Abdominal fat distribution was evaluated by CT (Siemens Volume Zoom, Forchheim, Germany) at the level of fourth lumbal vertebra. Subcutaneous and IAF were calculated as previously described (215).

4.6. Cardiopulmonary exercise test

The cardiopulmonary test was performed with bicycle ergometer (Siemens Elema 380) until exhaustion. Respiratory gas exchange was analyzed continuously during the test with a computer-based system (Sensor Medics 2900, Metabolic Measurement Cart / System, Yorba Linda, CA, USA). The

average values of oxygen uptake measured during the last 20 seconds of the exercise were used to calculate $V0^{2}\text{-}_{max.}$

4.7. Biochemical assays and calculations

Blood and plasma glucose levels were measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus, Yellow Springs Instrument Co., Inc, Ohio, US). Plasma insulin and C-peptide were determined by radioimmunoassay (Phasedeph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden). Serum lipid and lipoprotein concentrations were determined from fresh serum samples drawn after a 12-hour overnight fast. Cholesterol and triglyceride levels from the whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Roche Diagnostics, Mannheim, Germany). Plasma concentrations of TNF-α, IL-1β, IL-1Ra, IL-6, IL-10, IL-18 and serum levels of soluble adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], E-Selectin and P-Selectin were measured with high-sensitivity assay kits from R&D Systems. IL-8 was measured using a kit from Biosource International (Camarillo, CA, USA). CRP was measured using an Immulite analyzer and a DPC High Sensitivity CRP assay (Diagnostic Products Corporation, Los Angeles, CA). Soluble vascular adhesion protein-1 (VAP-1) was measured using in-house sandwich ELISA. Plasma for determination of CRP, cytokines and adhesion molecules was stored at -70° C until analysis within 3.5 years.

4.8. DNA analyses

Genotyping was performed either by direct sequencing (ABI prism genetic analyzator) (IL-1Ra gene: G114C), by restriction length polymorphism (IL-6 gene: C-174G, IL-10 gene: A-592C, TNF-receptor 2 gene: M196R) or by TaqMan assays (CRP gene: G942C, G1059C, IL-1β gene: T511C, C3954T, IL-10 gene: A1082G, TNF-α gene: G-308A).

4.9. Statistical analysis

All data analyses were performed with the SPSS 10.0, 11.0 or 14.0 for Windows programs (SPSS Inc, Chicago, Illinois, USA). The results for continuous variables are shown as mean ± SD or mean ± SEM as indicated. Variables with skewed distribution were logarithmically transformed for statistical analyses. The differences between the three groups were assessed by the analysis of variance (ANOVA) for continuous variables and by the χ^2 test for categorical variables. ANCOVA (Study I) and linear mixed model analysis (Studies II, III and IV) were applied to adjust for family relationship and other confounding factors. Correlation between continuous variables was tested using linear regression analysis. In factor analyses (Study I) the principal component method was used for extraction of the initial components. Factors with eigenvalues $\geq$ 1 were retained and varimax rotation was applied for the elucidation of factors. Variable loadings $\geq$ 0.4 were considered statistically significant in the interpretation of the factors. The incremental insulin areas under the curve were calculated by the trapezoidal method.

4.10. Approval of the Ethics Committee

The Ethics Committee of Kuopio University Hospital approved the study protocol. All study subjects gave informed consent.

5. RESULTS

5.1. Characteristics of the study subjects

The baseline clinical and laboratory characteristics of the study subjects are shown in Table 1. Of 129 subjects (Studies II and III), 20 (15.5%) had impaired glucose tolerance (IGT). In Study I, the subjects were grouped according to their metabolic syndrome (MetS) factor score.

Table 1. Clinical and laboratory characteristics of the study subjects

	Offspring of type 2 diabetic patients			Controls
	Study I	Studies II and III	Study IV	
	N=119	N=129	N=40	N=19
Men/Women	55/64	59/70	19/21	8/11
Age (years)	35.5 ± 6.0	35.5 ± 6.3	36.5 ± 6.6	34.5 ± 4.5
Body mass index (kg/m²)	26.1 ± 4.7	26.1 ± 4.6	28.1 ± 6.0	24.6 ± 2.6
Waist (cm)	88 ± 12	88 ± 12	93 ± 15	82 ± 8
Systolic blood pressure (mmHg)	126 ± 11	127 ± 12	133 ± 15	124 ± 10
Diastolic blood pressure (mmHg)	84 ± 9	84 ± 10	89 ± 12	82 ± 10
Fasting plasma glucose (mmol/L)	5.1 ± 0.4	5.2 ± 0.4	5.2 ± 0.5	5.1 ± 0.6
120 min plasma glucose (mmol/L)	6.2 ± 1.4	6.3 ± 1.4	7.5 ± 1.4	5.6 ± 1.1
Fasting plasma insulin (pmol/L)	46.2 ± 22.5	46.8 ± 22.8	55.2 ± 29.4	47.9 ± 23.0
120 min plasma insulin (pmol/L)	245.6 ± 195.2	247.2 ± 189.6	367.2 ± 249.6	194.0 ± 107.2
WBGU µmol/kg/min	57.34 ± 16.9	56.54 ± 16.87	50.0 ± 13.5	70.0 ± 27.9
Total cholesterol (mmol/L)	4.90 ± 0.87	4.90 ± 0.87	4.9± 0.74	4.73 ± 0.96
HDL cholesterol (mmol/L)	1.27 ± 0.28	1.27 ± 0.28	1.16 ± 0.29	1.37 ± 0.33
Total triglycerides (mmol/L)	1.13 ± 0.60	1.14 ± 0.61	1.37 ± 0.69	1.24 ± 0.84

HDL= high density lipoprotein

5.2. Factor analysis on the components of the metabolic syndrome (Study I)

The metabolic syndrome was characterized by applying factor analysis in 119 non-diabetic offspring of diabetic probands. A single factor, the metabolic syndrome factor, was identified using the following variables: 2-hour glucose, fasting insulin, body mass index, waist, HDL cholesterol, triglycerides, and mean blood pressure. Subjects with the highest factor score tertile were defined as having the metabolic syndrome. During hyperinsulinemia, the highest factor score tertile was associated with decreased rates of glucose oxidation ($p<0.001$, adjusted for gender, Figure 2A) and non-oxidative glucose disposal ($P<0.001$, adjusted for gender, Figure 2A), high rates of lipid oxidation ($P=0.001$, adjusted for gender, Figure 3C), low energy expenditure ($P=0.031$, adjusted for gender, Figure 3A), and impaired suppression of free fatty acids ($P=0.003$, adjusted for gender, Figure 3B).

The amount of intra-abdominal (Figure 2C) and subcutaneous fat (Figure 2D) increased with increasing metabolic syndrome factor score. In contrast, adiponectin level decreased significantly ($P=0.001$, adjusted for gender and intra- abdominal fat, Figure 2B) and maximum oxygen uptake was lower in high metabolic syndrome score subjects ($P=0.012$, Figure 3D). Furthermore, the metabolic syndrome was associated with high levels of C-reactive protein ($P<0.001$, adjusted for gender and intra-abdominal fat, Figure 4A), IL-1ß ($P=0.015$, Figure 4E), IL-1Ra ($P=0.002$, Figure 4D), IL-6 ($P=0.042$, Figure 4C) and IL-8 ($P=0.014$, Figure 4F), whereas TNF-α (Figure 4B) did not differ among the factor score tertiles. Levels of P-Selectin ($P=0.056$, Figure 5A) and ICAM-1 ($P=0.006$, Figure 5C) increased with increasing metabolic syndrome score, whereas no change was observed in E-Selectin (Figure 5B) and VCAM-1 (Figure 5D).

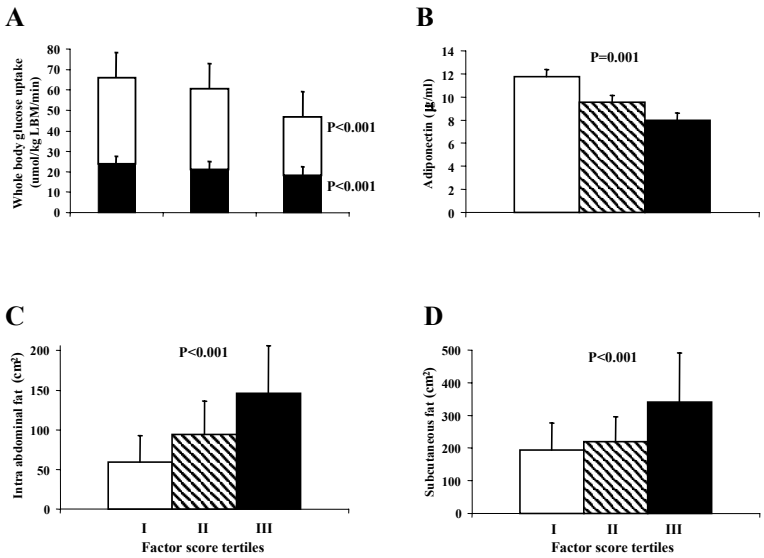

Figure 2. Rates of whole body glucose uptake (■ glucose oxidation, □ non-oxidative glucose disposal, P values given respectively (A), adiponectin concentration (B), intra-abdominal fat mass (C), and subcutaneous fat mass (D) according to the factor score tertile (I = lowest, II = middle, III = highest tertile) derived from factor analysis. P values are unadjusted.

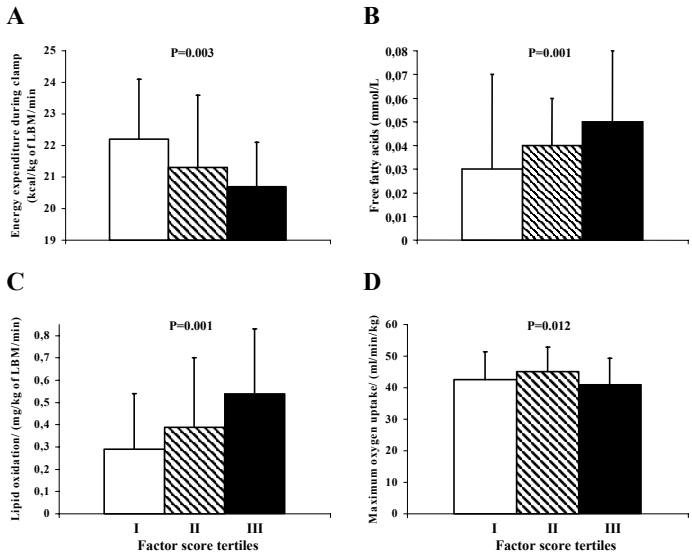

Figure 3. Energy expenditure during the hyperinsulinemic clamp (A), free fatty acid levels during the hyperinsulinemic clamp (B), lipid oxidation during the hyperinsulinemic clamp (C), and maximal oxygen uptake during the exercise (D) according to the factor score tertiles (I = lowest, II = middle, III = highest tertile) derived from factor analysis. P values are unadjusted

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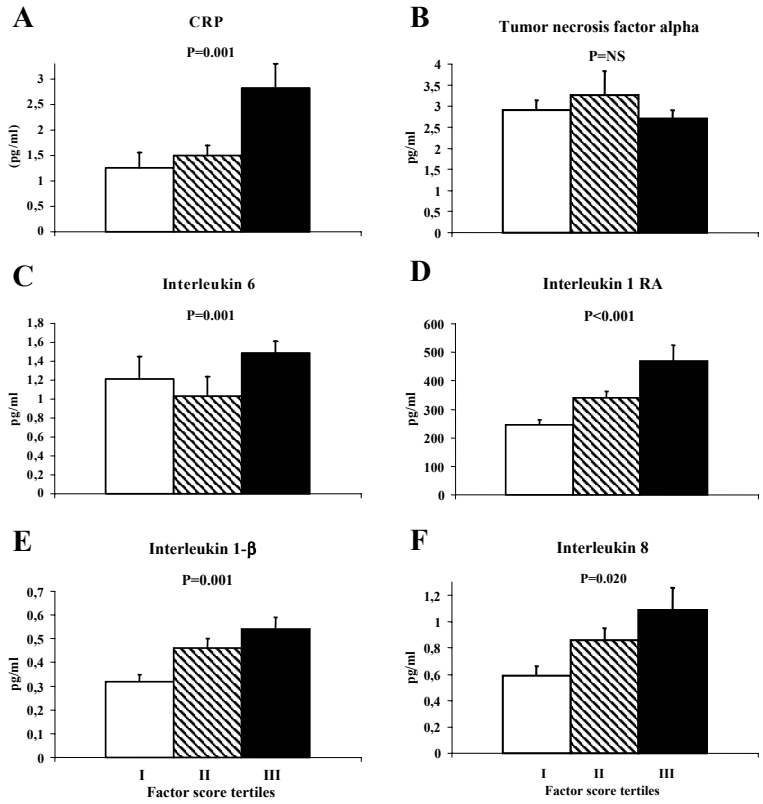

Figure 4. Fasting hs-C-reactive protein (A), and cytokine levels (B-F) according to factor score tertiles (I = lowest, II = middle, III = highest) derived from factor analysis. P values are unadjusted.

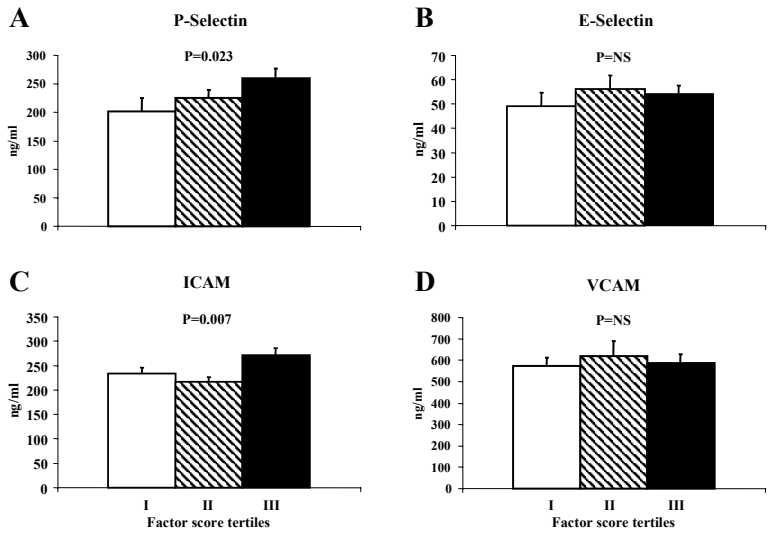

Figure 5. Fasting adhesion molecule levels (A-D) according to factor score tertiles (I = lowest, II = middle, III = highest) derived from factor analysis. P values are unadjusted.

5.3. Inflammatory cytokines in the offspring of type 2 diabetic subjects (Study II)

A total of 19 control subjects and 129 offspring (109 with NGT and 20 with IGT) were included in the study. The rates of WBGU was lower in the IGT group than in controls (P<0.01, Figure 6A) and a similar, but not a statistically significant trend was observed in the NGT group compared to the control group. The first-phase insulin release did not compensatorily increase the IGT group (Figure 6B). The areas of both visceral (P<0.01, Figure 6C) and subcutaneous fat (P<0.05, Figure 6D) were greater in the IGT group compared to the control group. The differences persisted after adjustment for age, gender, BMI and familiality. The ratio of subcutaneous fat to visceral fat did not differ between the groups (data not shown).

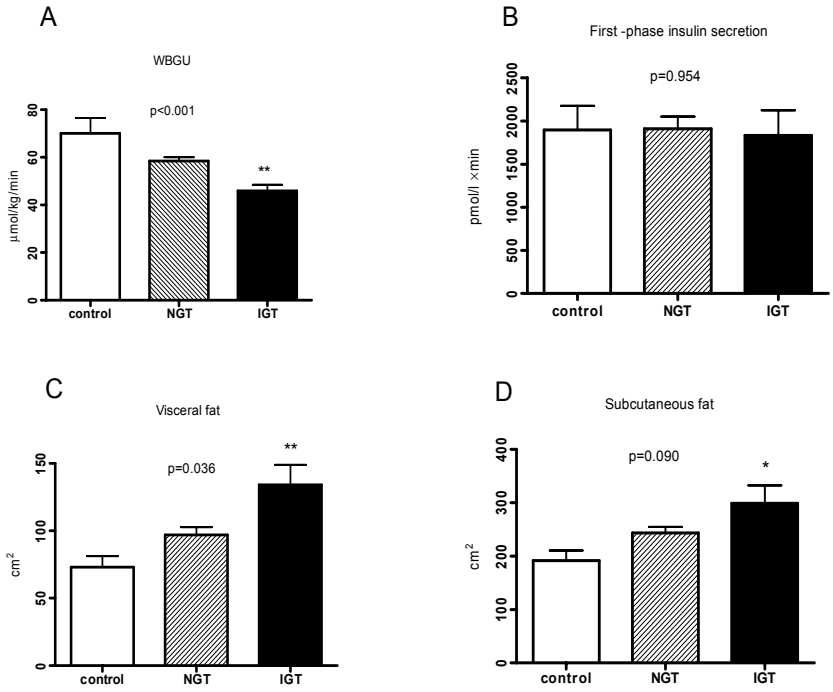

Figure 6. Rates of WBGU (A), first-phase insulin secretion (B), visceral fat (C), and subcutaneous fat (D) in offspring of type 2 diabetic patients. □, control group; striped bar, NGT group; ■, IGT group. P value after adjustment for age, sex, BMI, and family relationship (mixed linear model). Data are means ± SEM. *P<0.01, **P<0.01, ***P<0.001 for IGT vs. control group

Levels of fasting cytokines are shown in Figure 7. CRP levels were significantly higher in the IGT group than in the control group. Levels of TNF-α did not differ significantly among the three groups, but after adjustment for age, sex, BMI, and familiality, a statistically significant difference was observed among the three groups. There were no significant differences in fasting levels of IL-6, IL-8, IL-10 and IL-18 (data not shown) among the three groups. Compared with the control group, levels of IL-1β were significantly higher in the NGT group, whereas there was a significant decrease in IL-1β levels in the IGT group. Levels of IL-1Ra increased linearly in the NGT and IGT groups compared with the control group.

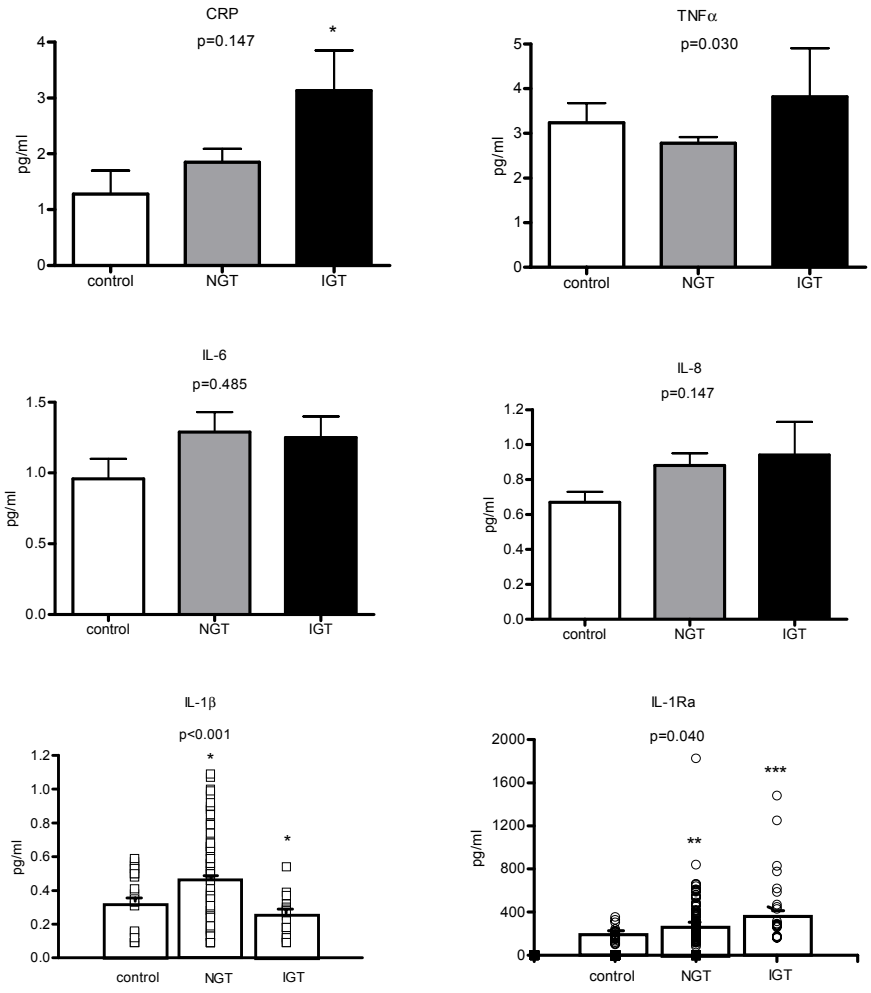

Figure 7. Fasting cytokines in offspring of type 2 diabetic patients. White bars = control group, hatched bars = NGT group, black bars = IGT group. The individual data for IL-1β and IL-1Ra is shown in scattergrams (white bars in all respective groups). P-value after the adjustment for gender, body mass index and familiality (mixed linear model). Mean ± SEM. * P<0.05, **P<0.01, *** P<0.001, NGT or IGT vs. control group.

5.4. Markers of endothelial dysfunction and low-grade inflammation in the offspring of type 2 diabetic patients (Study III)

Fasting levels of VCAM-1, ICAM-1, E-Selectin and VAP-1 did not differ significantly among control, NGT and IGT groups (Figure 8). The subjects with IGT tended to have higher E-Selectin levels than control subjects (63.4 ± 6.8 ng/ml versus 46.6 ± 5.1 ng/ml, P=0.052).

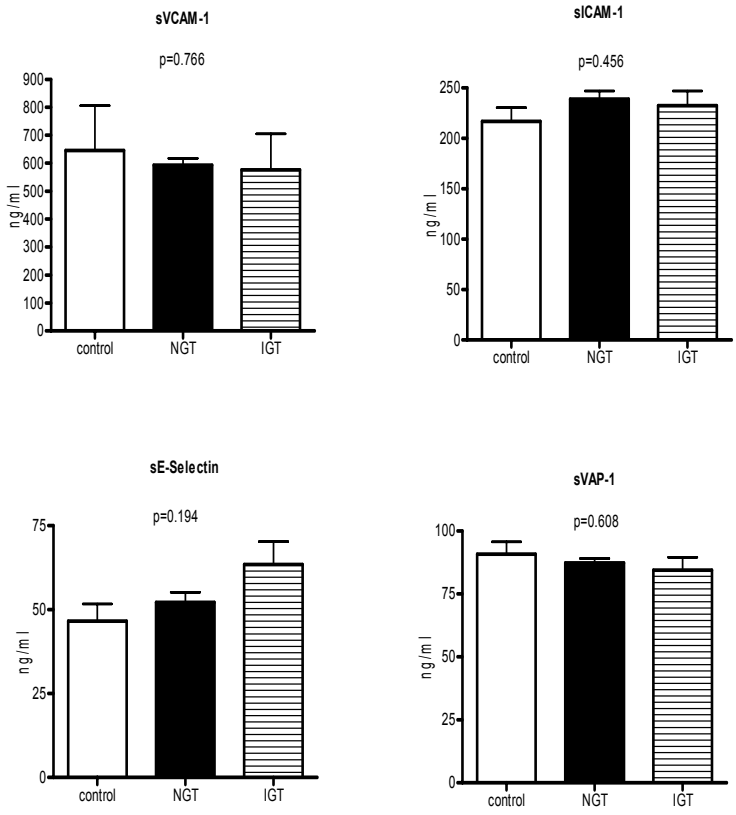

Fig. 8. The levels of fasting adhesion molecules by glucose tolerance status. P value after adjustment for sex, BMI, smoking, antihypertensive medication and family relationship (mixed linear model). Data are means ± SEM.

No statistically significant correlations among fasting adhesion molecule levels were found in any of the three groups. In control group, VCAM-1 correlated with levels of TNF-α (0.482, P<0.05) and with the rates of whole body glucose uptake (WBGU) (0.732, P<0.01). An inverse correlation was observed between VCAM-1 level and fasting glucose (-0.549, P<0.05), 120 min glucose (-0.528, P<0.05) and amount of visceral fat (-0.639, P<0.01). ICAM-1 levels correlated significantly with IL-1Ra levels (0.526, P<0.05), BMI (0.633, P<0.01) and the amount of subcutaneous fat (0.695, P<0.01).The only significant correlation of E-Selectin was observed between the first- phase insulin secretion (-0.586, p<0.01). VAP-1 levels correlated inversely with levels of 120 min plasma glucose (-0.461, P<0.05).

Table 2 shows correlations of fasting cytokines, clinical and metabolic parameters with adhesion molecules in normoglycemic offspring of type 2 diabetic patients. VCAM-1 levels correlated significantly with levels of TNF-α (0.214, P<0.05) and IL-1β (0.224, P<0.05), whereas ICAM levels correlated with levels of CRP (0.271, P<0.01) and IL-6 (0.292, P<0.01). Levels of E-Selectin correlated significantly with levels of IL-8 (0.282, P<0.01) and IL-18 (0.268, P<0.01), and VAP-1 with IL-18 (0.222, P<0.05). Among the metabolic parameters, VCAM-1 levels correlated with 120 min plasma glucose (0.218, P<0.05), fasting plasma insulin (0.229, P<0.05) and 120 min plasma insulin (0.227, P<0.05). ICAM-1 levels correlated significantly with BMI (0.278, p<0.01), (p<0.05), amount of visceral fat (0.243, p<0.05) and subcutaneous fat (0.212, p<0.05), and with fasting plasma insulin levels (0.220, P<0.05). A significant correlation was observed between E-Selectin and fasting plasma glucose levels (0.307, P<0.01), and amount of visceral fat (0.281, P<0.01). E-Selectin levels were inversely correlated with the rates of WBGU (-0.191, P<0.05). In contrast, levels of VAP-1 showed no significant correlations with any of the metabolic or inflammatory factors measured in the NGT group.

Table 2. Spearman correlations among fasting cytokines, clinical and metabolic parameters with adhesion molecules in normoglycemic offspring of type 2 diabetic patients. RRd = diastolic blood pressure, RRs= systolic blood pressure.

	s-VCAM-1	s-ICAM-1	sE-Selectin	s-VAP-1
hs-CRP	0.121	0.271**	-0.037	-0.163
TNF-α	0.214*	0.016	-0.018	0.043
IL-6	0.054	0.292**	-0.035	-0.073
IL-1β	0.224*	0.023	0.128	-0.062
IL-1Ra	0.049	0.048	0.095	0.028
IL-8	-0.022	-0.026	0.282**	-0.071
IL-10	0.180	0.093	0.173	0.088
IL-18	-0.046	0.130	0.268**	0.222*
RRs	-0.007	-0.031	0.005	0.078
RRd	0.044	-0.038	0.024	-0.063
BMI	0.022	0.278**	0.047	0.039
Fasting plasma glucose	0.074	0.095	0.307**	0.045
120 min plasma glucose	0.218*	0.101	0.096	-0.007
Fasting plasma insulin	0.229*	0.220*	0.129	0.081
120 min plasma insulin	0.227*	0.168	-0.023	0.062
LDL cholesterol	-0.193*	0.076	0.221*	0.021
HDL cholesterol	-0.103	-0.186	-0.130	-0.139
Total triglycerides	-0.095	0.192*	0.158	-0.004
Visceral fat	-0.013	0.243*	0.281**	0.106
Subcutaneous fat	0.114	0.212*	-0.013	0.012
First-phase insulin secretion	0.053	0.190	-0.036	0.077
Whole body glucose uptake 40mU/L clamp /LBM	-0.134	0.176	-0.191*	0.128

* P<0.05, ** P<0.01

Table 3 shows that among offspring with IGT, ICAM-1 levels correlated with IL-6 levels (0.605, P<0.01), and E-Selectin levels with IL-1β levels (0.565, P<0.01). ICAM-1 levels also showed a significant positive correlation with BMI (0.565, P<0.01), amount of subcutaneous fat (0.570, P<0.05), fasting insulin levels (0.652, P<0.01), and 120 min insulin levels (0.620, P<0.01), but an

inverse correlation with the rates of whole body glucose uptake (-0.569, P<0.01). VCAM-1 and VAP-1 levels did not correlate significantly with cytokines or metabolic parameters in the IGT group.

Table 3. Spearman correlations among fasting cytokines, clinical and metabolic parameters with adhesion molecules in offspring of type 2 diabetic patients having impaired glucose tolerance

	s-VCAM-1	s-ICAM-1	sE-Selectin	s-VAP-1
CRP	-0.083	0.351	0.024	-0.138
TNF-α	0.101	-0.127	0.438	0.245
IL-6	-0.199	0.605**	-0.329	0.042
IL-1β	0.214	0.153	0.565**	0.187
IL-1Ra	0.087	0.343	0.086	-0.077
IL- 8	0.033	-0.152	0.288	-0.295
IL-10	-0.031	-0.297	0.316	-0.182
IL-18	0.229	-0.087	0.405	0.071
RRs	0.073	-0.135	0.471*	-0.239
RRd	-0.160	-0.063	0.305	-0.172
Fat percent	-0.062	0.416	0.044	0.110
BMI	-0.068	0.565**	0.141	0.018
Fasting plasma glucose	0.194	-0.036	0.348	0.010
120 min plasma glucose	0.022	0.202	-0.216	-0.249
Fasting plasma insulin	0.080	0.652**	0.194	-0.028
120 min plasma insulin	-0.096	0.620**	0.092	-0.106
LDL cholesterol	-0.138	0.019	-0.152	0.117
HDL cholesterol	0.263	-0.508*	0.154	0.010
Total triglycerides	-0.052	0.133	-0.022	-0.365
Visceral fat	-0.125	0.419	0.010	-0.018
Subcutaneous fat	-0.151	0.570*	0.041	-0.144
First-phase insulin secretion	-0.325	0.246	-0.133	-0.180
Whole body glucose uptake 40mU/L clamp /LBM	0.296	-0.569**	-0.177	0.121

* P<0.05 , ** P< 0.01.

5.5. Changes in cytokine levels during acute hyperinsulinemia in offspring of type 2 diabetic subjects (Study IV)

Study IV included subjects 40 offspring of type 2 diabetic patients: 20 with normal glucose tolerance (NGT) and 20 subjects with impaired glucose tolerance (IGT), as assessed by an OGTT, and 19 healthy controls with no family history of type 2 diabetes. The groups were comparable with respect to gender, but differed significantly with respect to age (Control 34.5 ± 4.5, NGT 34.6 ± 6.1, IGT 38.6 ± 6.6 years, P< 0.05), and tended to differ with respect to BMI (24.6 ± 2.6, 28.2 ± 6.1, 28.0 ± 6.2 kg/m², P=0.064). In addition, the offspring in the NGT and IGT groups were markedly insulin resistant, with significantly higher plasma insulin levels at 120 min in the OGTT as compared to controls. The NGT and IGT groups also had higher systolic blood pressure levels than did the control group (Control 124 ± 10, NGT 133 ± 13, IGT 133 ± 18 mmHg, P<0.05). No difference in the first-phase insulin release between the study groups was found.

The changes in cytokine levels between the fasting state and the hyperinsulinemic state are shown in Figure 9. Levels of hs-CRP decreased significantly during hyperinsulinemia compared to the levels in the fasting state in all study groups (P<0.001). In contrast, levels of IL-6 increased significantly during hyperinsulinemia (P<0.001) in all groups, and the increase in the NGT and IGT groups was similar to that in the control group (P=0.294). The fasting and hyperinsulinemic levels of IL-1ß or IL-1Ra did not differ in any of the groups. In contrast, TNF-α and IL-8 levels decreased significantly (P<0.05) in the control group during hyperinsulinemia, but remained unchanged in the offspring with NGT or IGT. Hyperinsulinemia also significantly decreased the levels of IL-10 and IL-18 in the control group (P<0.05 and P<0.001, respectively), but not in the NGT and IGT groups.

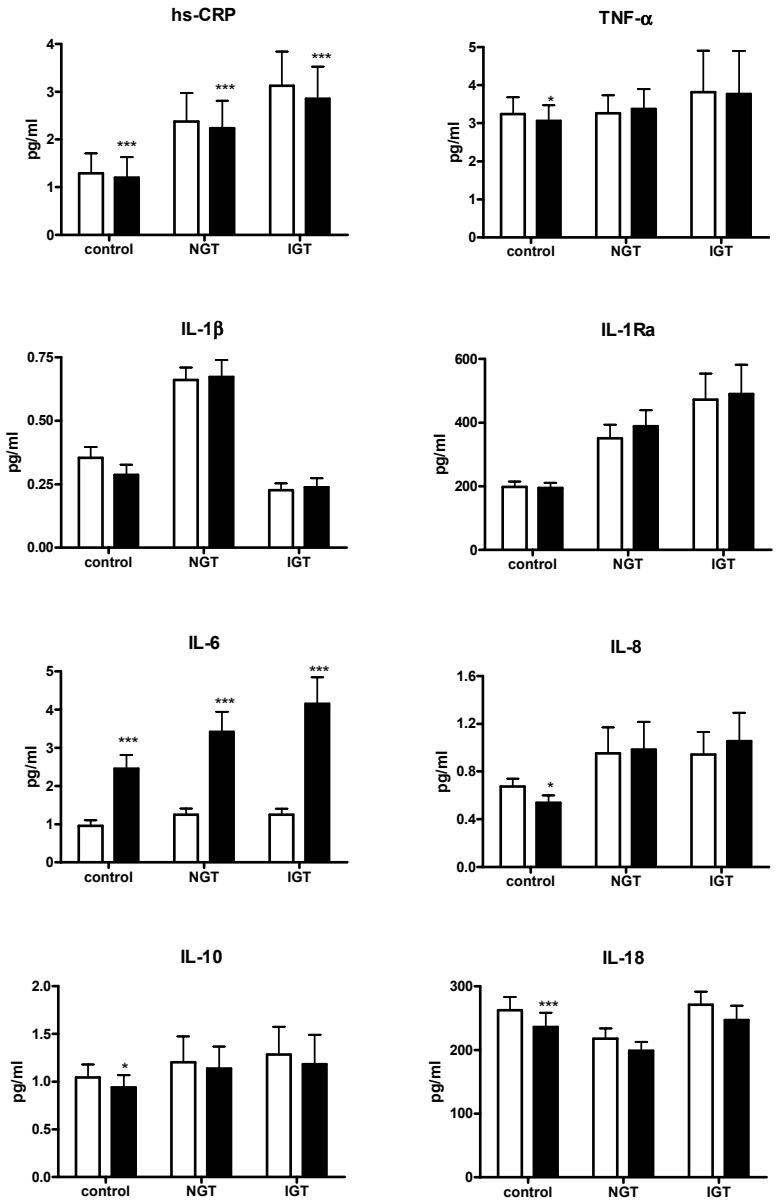

Figure 9. Changes in cytokine levels during the hyperinsulinemic euglycemic clamp. White bars = fasting levels of cytokines in control, NGT and IGT groups. Black bars = levels of cytokines during the hyperinsulinemic euglycemic clamp in the control, NGT and IGT groups. P-value after the adjustment for body mass index, smoking and family relationship (mixed model). Mean ± SEM. *P < 0.05, *** P < 0.001.

To investigate the effect of obesity on our results we performed further statistical analyses between non-obese and obese (cut-off point of BMI of 27.0 kg/ m²) subjects in all study groups. As shown in Table 4, changes in cytokine levels did not systematically differ between non-obese and obese subjects in any glucose tolerance category. Insulin did not have significant effects on the levels of any of the adhesion molecules in any group.

Table 4. Cytokine levels (pg/ml) in the fasting state and during hyperinsulinemia. BMI cut-off point of 27 kg/m² for non-obese and obese subjects. Data are mean ± SD.

		Control	NGT	IGT	P
Fasting hs-CRP:	Non-Obese	1.10 ±1.60	2.83 ± 3.54	2.75 ± 3.43	0.257
	Obese	2.21 ± 2.82	2.00 ± 1.77	3.51 ± 3.15	0.365
Clamp hs-CRP:	Non-obese	1.01 ± 1.62	2.72 ± 3.46	2.44 ± 3.12	0.225
	Obese	2.14 ± 2.87	1.85 ± 1.66	3.28 ± 2.97	0.324
Fasting TNF-α:	Non-obese	3.41 ± 2.04	4.41 ± 2.78	5.12 ± 6.80	0.702
	Obese	2.38 ± 0.38	2.32 ± 0.26 a)	2.51 ± 0.51	0.610
Clamp TNF-α:	Non-obese	3.22 ± 1.90	4.57 ± 3.17	5.14 ± 7.07	0.559
	Obese	2.21 ± 0.56	2.40 ± 0.28	2.40 ± 0.64	0.755
Fasting IL-1-ß:	Non-obese	0.34 ± 0.19	0.65 ± 0.22	0.22 ± 0.15	0.000
	Obese	0.45 ± 0.87	0.67 ± 0.23	0.23 ± 0.09	0.000
Clamp IL-1-ß:	Non-obese	0.26 ± 0.16	0.67 ± 0.34	0.19 ± 0.12	0.000
	Obese	0.45 ± 0.17	0.67 ± 0.28	0.28 ± 0.18	0.004
Fasting IL-1Ra:	Non-obese	187.53 ± 63.86	358.56 ± 112.00	370.47 ± 342.53	0.003
	Obese	256.43 ± 92.43	345.01 ± 242.31	574.57 ± 380.02* a)	0.087
Clamp IL-1Ra:	Non-obese	182.47 ± 56.44	436.12 ± 251.47	350.38 ± 380.97	0.004
	Obese	259.10 ± 132.62	349.63 ± 208.05	630.32 ± 415.77* b)	0.047
Fasting IL-6:	Non-obese	0.86 ± 0.86	1.31 ± 0.64	0.82 ± 0.39	0.058
	Obese	1.53 ± 1.45	1.21 ± 0.82	1.68 ± 0.66**c)	0.235
Clamp IL-6 clamp:	Non-obese	2.57 ± 1.65	3.63 ± 2.85	4.11± 3.94	0.338
	Obese	1.82 ± 0.99	3.24 ± 1.99	4.20 ± 2.17	0.112
Fasting IL-8:	Non-obese	0.65 ± 0.27	1.18 ± 1.29	1.06 ± 1.09	0.951
	Obese	0.79 ± 0.40	0.76 ± 0.64	0.82 ± 0.54	0.692
Clamp IL-8:	Non-obese	0.53 ± 0.27	1,46 ± 1.40	0.93 ± 0.48	0.249
	Obese	0.58 ± 0.33	0.60 ± 0.38	1.18 ± 1.46	0.282
Fasting IL-10:	Non-obese	1.04 ± 0.61	1.47 ± 1.57	1.51 ± 1.66	0.825
	Obese	1.06 ± 0.55	0.98 ± 0.82	1.05 ± 0.86	0.929
Clamp IL-10:	Non-obese	0.97 ± 0.60	1.28 ± 1.23	1.39 ± 1.83	0.963
	Obese	0.79 ± 0.41	1.02 ± 0.88	0.97 ± 0.76	0.907
Fasting IL-18:	Non-obese	259.76 ± 89.14	224.03 ± 94.29	243.20 ± 0.67	0.602
	Obese	278.43 ± 122.85	213.05 ± 50.54	298.93 ± 109.46	0.099
Clamp IL -18:	Non-obese	234.63 ± 97.90	204.86 ± 75.32	201.66 ± 75.12	0.568
	Obese	246.20 ± 112.24	194.55 ± 51.95	292.73 ± 106.75*d)	0.051

*P<0.05, compared to control subjects within the same obesity status (non-obese or obese)
a) P= 0.040, b) P= 0.028, c) P= 0.001, d) P=0.042 comparing non-obese vs. obese subjects within the same glucose tolerance group.

6. DISCUSSION

6.1. Study population

The diabetic patients (probands) were selected from type 2 diabetic subjects living in the region of Kuopio University Hospital. The probands included participants in some of our previous studies, as well as individuals obtained through advertisements in local newspapers and health centers. Participation of probands required confirmation of type 2 diabetes according to the WHO criteria (216). Spouses of the probands were required to have normal glucose tolerance in an oral glucose tolerance test. Altogether 129 subjects from 78 families (1-3 offspring from each family) were included in the study. The study group was limited to non-diabetic subjects without chronic diseases or medication that could potentially disturb glucose metabolism. The control subjects were healthy non-diabetic (based on an OGTT) volunteers without a family history of type 2 diabetes. The study groups were representative samples either from the population of Kuopio University Hospital (controls) or from offspring of type 2 diabetic subjects living in the same region.

6.2. Study design

Given that the metabolic studies were thorough and complicated, the sample size was large. The study subjects underwent a detailed physiological and metabolic characterization.

6.3. Study methods

We used the euglycemic hyperinsulinemic clamp to measure insulin sensitivity, because this method is widely recognized to be the “gold standard” (213). An insulin infusion rate of 40 mU/m² was used to produce hyperinsulinemia during the clamp which leads to a complete suppression of hepatic glucose output in moderately obese and healthy subjects (217).

A clamp duration of 120 min was used to achieve a steady-state of glucose disposal in non-diabetic subjects. The steady state of glucose disposal was adequately achieved indicating that the clamp study was well performed. The first detectable finding of defective β-cell function has been considered to be an impairment of the first phase insulin secretion during an IVGTT, although the hyperbolic association between insulin secretion and insulin sensitivity may lead to overestimation of insulin secretion capacity in insulin-resistant subjects (218). Carrying out the euglycemic clamp immediately after an IVGTT has been previously shown to have no significant effect on subsequently measured metabolic indices (219). CT and MRI are the most accurate methods available for the assessment of abdominal fat; CT was chosen because of better availability. Laboratory and genetic analyses were performed using standardized methods in the research laboratories of clinical chemistry and medicine.

A linear mixed model was used to take into account the varying number of subjects among the families, and to adjust for familiality and other confounding factors. Factor analysis has many advantages compared to conventional statistical methods when studying the metabolic syndrome. Factor analysis is preferable when there are complex inter-correlations between the variables included, as is clearly the case with the metabolic syndrome. Instead of using distinct cut-off points, this method permits evaluation of each of the multiple variables comprising the metabolic syndrome as a continuum. Furthermore, instead of scoring subjects only as having or not having the syndrome, the use of factor scoring allowed us to analyze the metabolic syndrome itself as a continuous variable. Factor analysis has some limitations. The formation of factors is sensitive to the selection of variables, as demonstrated also by our data (e.g. the inclusion of both systolic and diastolic blood pressure led to a two-factor solution). Furthermore, the number of variables often affects the number of factors. However, despite these limitations, factor analysis is a powerful method for examining the clustering of cardiovascular risk factors belonging to the metabolic syndrome.

6.4. Metabolic abnormalities in offspring of type 2 diabetic patients (Study I)

Factor analysis yielded important information about metabolic abnormalities associated with the metabolic syndrome in the offspring of our diabetic probands. Subjects with the metabolic syndrome belonged to the highest factor score tertile. Our study showed that subjects with the metabolic syndrome have an excess of intra-abdominal fat, hypoadiponectinemia, multiple defects in glucose and energy metabolism, as well as elevated levels of cytokines and adhesion molecules.

The basis of the metabolic syndrome seems to be a tight link between insulin resistance and an excess of visceral fat, although the primary abnormality remains to be elucidated. We observed that hyperinsulinemia was not able to suppress free fatty acid levels in subjects with the metabolic syndrome. This novel finding indicates that insulin resistance in people with the metabolic syndrome occurs not only in skeletal muscle, but also in adipose tissue. People with the metabolic syndrome have a well-known tendency to gain weight. In this study, we observed significantly lower energy expenditures in those of our subjects with the metabolic syndrome compared to those without; this could indicate both central insulin resistance and a lower increase in meal-induced thermogenesis.

We also found that levels of hs-CRP, inflammatory cytokines and adhesion molecules increased with increasing metabolic syndrome factor score. The most marked elevations were found in IL-1Ra and IL-1β levels, whereas TNF-α levels did not differ among the factor score tertiles. In addition, levels of P-Selectin and ICAM-1 were associated with presence of the metabolic syndrome, whereas levels of E-Selectin and VCAM-1 were not.

The metabolic syndrome was associated with a high amount of intra-abdominal fat, a low adiponectin level and elevated levels of cytokines and adhesion molecules. Adiponectin inhibits the expression of ICAM-1, VCAM-1, and E-Selectin (220) and has several anti-atherogenic (221,222) and anti-inflammatory properties. Therefore, hypoadiponectinemia could be responsible for endothelial damage and maintenance of a low-grade inflammatory state.

Our findings confirm the roles of low-grade inflammation and endothelial dysfunction in the metabolic syndrome. Previous studies have shown that elevated levels of CRP, IL-6 and TNF-α predict type 2 diabetes (51) and coronary heart disease (223). In our study, TNF- α levels did not significantly differ among the factor score tertiles. Instead, the most marked elevations were found in IL-1Ra and IL-1β levels**,** suggesting that these cytokines may be better markers for the metabolic syndrome than TNF- α.

6.5. Changes in inflammatory cytokines in the offspring of type 2 diabetic patients (Studies II and IV).

The offspring of type 2 diabetic patients are at increased risk of developing type 2 diabetes. Therefore, the offspring of type 2 diabetic patients are ideal study subjects for investigating early findings in the pathogenesis of this disease. CRP and pro-inflammatory cytokine levels are elevated in both IGT and overt type 2 diabetes, and they predict the conversion to type 2 diabetes (54,161,165,224). However, only three previous studies have investigated whether low-grade inflammation is detectable in the offspring of type 2 diabetic patients. These studies measured levels of TNF-α, but not those of other pro-inflammatory cytokines (5-7). In addition, the results of these studies are controversial. Kellerer et al. (5) reported that circulating TNF-α levels did not correlate with obesity-induced insulin resistance, whereas Costa et al. (7) showed that the TNF-α pathway could predispose to the development of type 2 diabetes in the first-degree relatives of type 2 diabetic patients. Moreover, Maltezos et al. (6) observed significantly elevated concentrations of TNF-α in healthy non-diabetic offspring of type 2 diabetic subjects.

In this study, we observed increased levels of IL-1Ra in the normoglycemic offspring of type 2 diabetic patients, and even higher levels of IL-1Ra in offspring with IGT. These findings expand observations from previous studies that reported decreased concentrations of IL-1Ra in type 2 diabetes (225) and IL-1Ra overproduction in insulin resistance (226). Thus, individuals at risk for developing type 2 diabetes are characterized not only by an up-regulation of

pro-inflammatory immune mediators, but also by the up-regulation of the anti-inflammatory cytokine IL-1Ra.

The function of IL-1Ra is to protect against the inflammatory effects of IL-1 (227). Normally, enough IL-1Ra is produced to counteract IL-1-mediated inflammation (227). In the case of inflammation, there is an insufficient amount of IL-1Ra to control the activity of IL-1 (227). The imbalance between IL-1 and IL-1Ra has been extensively studied in experimental animal models of autoimmune diseases. In several diseases, either local overproduction of IL-1 and /or underproduction of IL-1Ra predisposes to the development of disease, and the therapeutic administration of IL-1Ra is efficacious in preventing tissue damage (228).

Adipose tissue plays an important role in cytokine secretion, and may be a major source of pro-inflammatory cytokines (4). However, it is also an important source of IL-1Ra (229). Consistent with these findings is a marked elevation of IL-1Ra levels observed in human obesity (230). In our study, offspring with IGT offspring had a significantly higher amount of visceral and subcutaneous fat than did control subjects. Together with increased IL-1Ra levels observed in these subjects, this supports previous findings of an association between obesity and elevated IL-1Ra levels. Somm et al. (231) have concluded that the overall effects of IL-1Ra promote weight gain and insulin resistance, thus favoring the development of type 2 diabetes.

Although IL-1Ra is considered a protective cytokine, we hypothesized that in the offspring at high risk of developing diabetes, increased levels of IL-1Ra might predispose them to insulin resistance, instead of protecting them from it. However, a recent clinical trial showed that blocking IL-1β signaling with IL-1Ra improved β-cell secretory function in patients with type 2 diabetes (232). Furthermore, in a study by Sauter et al (233), IL-1Ra was shown to improve β-cell survival and function, thus supporting a role for IL-1Ra in the treatment of type 2 diabetes. In the Whitehall II cohort study, elevated levels of IL-1Ra were associated with an increased risk of developing type 2 diabetes (234). Herder et al. (234) speculated that the elevation of IL-1Ra levels in individuals at risk of developing type 2 diabetes may represent an attempt to counteract the pro-

inflammatory effects of IL-1β and to preserve insulin secretion and insulin sensitivity - an effort that eventually fails.

The role of low-grade inflammation in insulin resistance and type 2 diabetes has been well established. Little is known about whether non-diabetic offspring of type 2 diabetic patients shows evidence of inflammation. We demonstrated that glucose-intolerant offspring of type 2 diabetic patients had elevated hs-CRP-levels, which is in line with the findings of previous studies (3-8, 25). Interestingly, TNF-α and IL-6 levels did not differ among our study groups, suggesting that these conventionally determined cytokines may not be the best markers for low-grade inflammation in the pre-diabetic state.

We found that the levels of pro-inflammatory IL-1β were increased in normoglycemic offspring of type 2 diabetic patients, but decreased in glucose-intolerant offspring. In contrast, levels of IL-1Ra were increased in normoglycemic offspring, and were even higher in glucose-intolerant offspring. To determine the biologic activity of IL-1β, we calculated the ratio of levels of IL-1Ra to IL-1β. Eizirik et al. (235) have shown that a 10- to 100-fold excess of IL-1Ra over IL-1β suffices to block the effects of IL-1β on pancreatic islets. We found a greater than 100-fold excess of IL-1Ra to IL-1β, indicating decreased biological activity of IL-1β in the normoglycemic offspring, and even more markedly decreased activity in the glucose-intolerant offspring. This degree of IL-1Ra excess would be expected to block the biological activity of IL-1β in human islets. Consistent with previous studies, we suggested that it is unlikely that IL-1β would mediate β-cell failure during progression to type 2 diabetes.

IL-1β has been shown to mediate both impaired function and destruction of pancreatic β-cells during the development of autoimmune type 1 diabetes (236). The potential role of impaired β-cell function in the deterioration of glucose tolerance has been debated (237-239). Maedler et al. (108) have shown that IL-1β is induced by elevated glucose concentrations when islets from non-diabetic organ donors are exposed to high glucose levels. Recently, Cnop et al. (94)observed that the decline in glucose tolerance over time in first-degree relatives of type 2 diabetic individuals is strongly related to the loss of β-cell function (94). However**,** the mechanisms underlying this progressive decline in

β-cell function are not fully understood. Cnop et al. (94) concluded that insulin resistance is likely to be involved in the pathogenesis of type 2 diabetes, but the progressive loss of β-cell function appears to be the critical determinant for disease progression from NGT to IGT and then to type 2 diabetes. Although type 1 and type 2 diabetes have fundamental etiological differences, the induction of IL-1β by elevated glucose concentrations may connect type 2 and type 1 diabetes (108).

Insulin resistance and / or hyperinsulinemia predict the development of type 2 diabetes (240) and cardiovascular disease (241) independently of other risk factors. There is only one previous study reporting the effects of acute hyperinsulinemia on levels of IL-8, and information regarding other cytokines remains unclear (242). We showed for the first time that acute hyperinsulinemia induced by the euglycemic hyperinsulinemic clamp significantly lowered the levels of TNF-α, IL-8, IL-10 and IL-18 in healthy control subjects. However, in both normoglycemic and glucose-intolerant offspring of type 2 diabetic subjects, hyperinsulinemia was unable to suppress cytokine levels. Therefore, we suggest that the offspring of type 2 diabetic patients are insulin resistant not only with regard to glucose metabolism, but also with regard to the inhibition of cytokine responses during hyperinsulinemia. The disturbed cytokine response was particularly linked with fat-derived cytokines, highlighting the crucial role of adipose tissue in this disease process.

6.6. Changes of adhesion molecule levels in the offspring of type 2 diabetic patients (Studies III and IV)

Low-grade inflammation and endothelial dysfunction precede the development of both type 2 diabetes and cardiovascular disease (CVD) (197). The presence of inflammation in the vascular endothelium links these diseases, and may contribute to the disease process itself. Levels of adhesion molecules have been previously shown to be elevated in insulin resistance (243,244), type 2 diabetes (245) and CVD (246). Furthermore, the offspring of type 2 diabetic patients show evidence of endothelial dysfunction, as estimated by non-invasive

methods, blood levels of adhesion molecules, or both (204,247-249). Adhesion molecule expression is known to be induced by pro-inflammatory cytokines such as IL-1β, TNF-α and CRP, and therefore adhesion molecules are closely associated with the inflammatory process.

In our study, the levels of multiple endothelial biomarkers were measured in a large cohort (n= 129) of non-diabetic offspring of type 2 diabetic subjects. No increase in adhesion molecule levels (s-ICAM-1, s-VCAM-1, sE-Selectin) was observed in any of our subgroups. Our findings contradict those of earlier studies which reported elevated levels of adhesion molecules in the offspring of type 2 diabetic patients (248,249). The reason for these opposite findings is unclear, but might be related to the use of different protocols and / or the small sample sizes of previous studies.

We also studied the levels of vascular adhesion protein-1 (VAP-1) in the offspring of type 2 diabetic patients. Elevated levels of VAP-1 have been previously reported in patients with type 1 diabetes (250), but we observed no increase in the levels of VAP-1 in the offspring at high risk for type 2 diabetes.

In young first-degree relatives of type 2 diabetic individuals, an association between non-invasively measured endothelial dysfunction and clamp-derived insulin resistance has been demonstrated (247). We observed an inverse correlation between rates of WBGU and E-Selectin levels in normoglycemic offspring and levels of ICAM-1 in the offspring of type 2 diabetic patients with IGT. Our findings confirm previous reports of a link between adhesion molecule levels and insulin resistance, and extend this data to healthy individuals at high risk of developing diabetes and CVD.

Our study demonstrated an association between levels of inflammatory markers and adhesion molecules, which is in line with previous studies showing a strong correlation between markers of endothelial dysfunction and inflammatory activity. Endothelial dysfunction is induced by cytokine-mediated low-grade inflammation, which in turn is an early finding in the process leading to type 2 diabetes. Therefore, we hypothesize that low-grade inflammation might be the primary abnormality preceding the elevation of adhesion molecule levels in incipient type 2 diabetes.

We also investigated the effect of acute hyperinsulinemia on levels of adhesion molecules. Previous reports regarding the effect of hyperinsulinemia on adhesion molecule levels have been contradictory. In two studies, hyperinsulinemia increased levels of sE-Selectin in IGT subjects and in type 2 diabetic subjects, whereas levels of sICAM-1 and sVCAM-1 remained unchanged (243,251). In our study, hyperinsulinemia did not alter adhesion molecule levels in any of the study groups. Our finding is in keeping with previous reports that the levels of sICAM-1, sVCAM-1 and sE-Selectin remain unaffected during hyperinsulinemia, and extend this data to healthy individuals who are genetically predisposed to develop type 2 diabetes.

6.7. Concluding remarks

The present study adds information to the understanding of the metabolic abnormalities in offspring of type 2 diabetic patients. We showed that insulin resistance in people with the metabolic syndrome occurs not only in skeletal muscle, but also in adipose tissue, leading to multiple defects in glucose and energy metabolism, hypoadiponectinemia, and elevated levels of pro-inflammatory cytokines and adhesion molecules.

The offspring of type 2 diabetic patients are insulin-resistant, and are characterized by an immune activation that includes up-regulation of pro-inflammatory cytokines and of the anti-inflammatory cytokine IL-1Ra. Moreover, IL-1Ra seems to be the most sensitive marker for cytokine response in incipient type 2 diabetes. Further studies have confirmed our findings by showing that an elevation in levels of IL-1Ra precedes the onset of type 2 diabetes (234). In a recent clinical trial, the administration of IL-1Ra to patients with type 2 diabetes improved their β-cell function and glycemic control (232). It is therefore possible that in incipient type 2 diabetes, the increase in IL-1Ra levels reflects an attempt by the body to prevent the deleterious effects of IL-1β. The decreased levels of IL-1Ra seen in patients with type 2 diabetes may indicate that the body's attempt to antagonise the effects of IL-1β by initially increasing IL-1Ra production has failed. Thus, interventions to block the deleterious effects of IL-

1β by the administration of IL-1Ra may provide new insight into the therapy and prevention of type 2 diabetes.

The offspring of type 2 diabetic patients are at high risk of developing type 2 diabetes. In these individuals, continuous low-grade inflammation may contribute to type 2 diabetes development both by inducing insulin resistance and by reducing insulin secretion. Efforts to prevent development of the hyperglycemia that is the result of both insulin resistance and a progressive loss of β-cell function need to be intensified in order to prevent even greater numbers at-risk individuals from progressing to the type 2 diabetic state.

7. SUMMARY

Study I: Insulin resistance in people with the metabolic syndrome is seen not only in skeletal muscle but also in adipose tissue, leading to multiple defects in glucose and energy metabolism, hypoadiponectinemia, and elevated levels of pro-inflammatory molecules and adhesion molecules.

Study II: The offspring of type 2 diabetic patients have changes in levels of CRP, IL-1β, and IL-1Ra. The level of IL-1Ra is the most sensitive marker of cytokine response in incident type 2 diabetes.

Study III: The levels of adhesion molecules are not increased in the offspring of type 2 diabetic subjects and are not the best markers of endothelial dysfunction in the pre-diabetic state. Inflammatory markers and adhesion molecules are related suggesting that low-grade inflammation may precede the elevation of levels of adhesion molecules.

Study IV: The offspring of type 2 diabetic subjects are not only insulin resistant with regard to glucose metabolism but also with regard to the suppression of cytokine responses. The disturbed cytokine response is especially linked to fat-derived cytokines highlighting the crucial role of adipose tissue in this process.

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Multiple Abnormalities in Glucose and Energy Metabolism and Coordinated Changes in Levels of Adiponectin, Cytokines, and Adhesion Molecules in Subjects With Metabolic Syndrome

Urpu Salmenniemi, Eija Ruotsalainen, Jussi Pihlajamäki, Ilkka Vauhkonen, Sakari Kainulainen, Kari Punnonen, Esko Vanninen and Markku Laakso

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Urpu Salmenniemi, MD; Eija Ruotsalainen, MD; Jussi Pihlajamäki, MD; Ilkka Vauhkonen, MD; Sakari Kainulainen, MD; Kari Punnonen, MD; Esko Vanninen, MD; Markku Laakso, MD

Background—Detailed metabolic defects in glucose and energy metabolism and abnormalities in a variety of cardiovascular risk factors are largely unknown in subjects with the metabolic syndrome.

Methods and Results—We characterized the metabolic syndrome in 119 nondiabetic offspring of diabetic probands. Cardiovascular risk factors, including cytokines and adhesion molecules, were measured. Insulin sensitivity was assessed by the euglycemic hyperinsulinemic clamp and indirect calorimetry; intra-abdominal fat and subcutaneous fat were assessed by CT; and maximal oxygen consumption was measured with a bicycle ergometer test. By applying factor analysis, we identified a single factor, the metabolic syndrome factor, from the following variables: 2-hour glucose, fasting insulin, body mass index, waist, HDL cholesterol, triglycerides, and mean blood pressure. Subjects with the highest factor score were defined as having the metabolic syndrome. During hyperinsulinemia, the highest factor score was associated with decreased rates of glucose oxidation and nonoxidative glucose disposal, high rates of lipid oxidation, low energy expenditure, and impaired suppression of free fatty acids during hyperinsulinemia. Furthermore, the metabolic syndrome was associated with a high amount of visceral fat, hypoadiponectinemia, a low maximum oxygen uptake, and high levels of C-reactive protein, proinflammatory cytokines, and adhesion molecules.

Conclusions—The metabolic syndrome is characterized by an excess of intra-abdominal fat, hypoadiponectinemia, insulin resistance in skeletal muscle and adipose tissue, multiple defects in glucose and energy metabolism, and elevated levels of cytokines and adhesion molecules. **(*Circulation*. 2004;110:3842-3848.)**

Key Words: adiponectin ■ cell adhesion molecules ■ cytokines ■ insulin resistance ■ metabolic syndrome X

The Metabolic Syndrome (MetS), a clustering of cardiovascular risk factors, is a powerful predictor of cardiovascular disease.[1,2] When Reaven[3] introduced this concept ("syndrome X"), he included in this constellation a clustering of abnormal glucose tolerance, dyslipidemia (low HDL cholesterol, high total triglycerides), and elevated blood pressure (BP). According to his interpretation, the underlying cause of the syndrome was insulin resistance. Recently, several other candidates for this syndrome—obesity, central obesity, microalbuminuria, high levels of proinflammatory cytokines, prothrombotic and fibrinolytic factors, and oxidative stress—have been proposed.[1,2]

The importance of risk factor clustering with hyperinsulinemia as a predictor of type 2 diabetes[4] and cardiovascular disease[5,6] has been shown in many prospective studies. However, the pathophysiology of the MetS has remained unknown, although insulin resistance[2,3] and visceral obesity[7] have been proposed as underlying causes for this syndrome.

For clinical purposes, the MetS has been defined on the basis of different cutoff points of cardiovascular risk factors,[8] a method that does not take into account the fact that cardiovascular risk factors are continuous variables. Furthermore, the components of the MetS are highly intercorrelated, and conventional statistical methods cannot be used to investigate this syndrome. Recently, factor analysis, allowing the analysis of interrelated continuous variables, has been applied in studies of the MetS.[4–6,9–17]

In the present study, we characterized the MetS in the offspring of diabetic probands by applying factor analysis. Detailed metabolic and other measurements allowed us to quantify for the first time defects in glucose and energy metabolism and abnormalities in a variety of cardiovascular

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From the Departments of Medicine (U.S., E.R., J.P., I.V., M.L.), Radiology (S.K.), Clinical Chemistry (K.P.), and Clinical Physiology and Nuclear Medicine (E.V.), University of Kuopio, Kuopio, Finland.

The online-only Data Supplement, which contains Tables I and II, is available with this article at http://www.circulationaha.org.

Correspondence to Markku Laakso, MD, Professor and Chair, Department of Medicine, University of Kuopio, 70210 Kuopio, Finland. E-mail markku.laakso@kuh.fi

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risk factors in subjects with the MetS. We also analyzed whether simple clinical and laboratory measurements (waist, insulin) are accurate enough to be used as surrogate markers for "gold standard" measurements (visceral fat evaluated by CT, insulin sensitivity evaluated by the euglycemic hyperinsulinemic clamp) to define the MetS for clinical practice.

Methods

Subjects

The subjects were healthy nondiabetic offspring of patients with type 2 diabetes. The diabetic patients (probands) were randomly selected from type 2 diabetic subjects living in the region of the Kuopio (Finland) University Hospital. Spouses of the probands had to have a normal glucose tolerance in an oral glucose tolerance test. A total of 119 offspring (1 to 3 from each family) were studied. The Ethics Committee of the University of Kuopio approved the study protocol. All study subjects gave informed consent.

Study Design

On the first day, BP was measured in subjects a sitting position after a 5-minute rest with a mercury sphygmomanometer. The average of 3 measurements was used to calculate systolic and diastolic BPs, as well as the mean BP [(2 × diastolic BP + systolic BP) / 3]. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Waist (at the midpoint between the lateral iliac crest and lowest rib) and hip circumference (at the level of the trochanter major) were measured to the nearest 0.5 cm. Fasting blood samples were drawn after 12 hours of fasting, followed by an oral glucose tolerance test (75 g glucose). Subjects with nondiabetic glucose tolerance[18] were included in further studies. On the second day, after a 12-hour fast, an intravenous glucose tolerance test (IVGTT) and the hyperinsulinemic euglycemic clamp, including indirect calorimetry, were performed. A CT scan was performed to evaluate the amount of abdominal fat, and an exercise test was done to determine maximum oxygen uptake.

Metabolic Studies

An IVGTT was performed to determine the first-phase insulin secretion capacity after an overnight fast. After baseline blood collection, a bolus of glucose (300 mg/kg in a 50% solution) was given within 30 seconds into the antecubital vein. Samples for the measurement of blood glucose and plasma insulin (arterialized venous blood) were drawn at −5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 minutes.

After an IVGTT, the degree of insulin sensitivity was evaluated with the euglycemic hyperinsulinemic clamp technique (insulin infusion rate of 40 mU · min^{-1} · m^{-2} body surface area) as previously described.[19] Blood glucose was clamped at 5.0 mmol/L for the next 120 minutes by infusion of 20% glucose at various rates according to blood glucose measurements performed at 5-minute intervals. The mean amount of glucose infused during the last hour was used to calculate the rates of whole-body glucose uptake (WBGU).

Indirect calorimetry was performed with a computerized flow-through canopy gas analyzer system (DELTATRAC, TM Datex) as previously described.[19] The mean value of the data during the last 20 minutes of the clamp was used to calculate glucose and lipid oxidation.[20] The rates of nonoxidative glucose disposal during the clamp were estimated by subtracting the rates of glucose oxidation from the glucose infusion rate.

Body Composition, Fat Distribution, and Cardiopulmonary Exercise Test

Body composition was determined by bioelectrical impedance (RJL Systems) in subjects in the supine position after a 12-hour fast. Abdominal fat distribution was evaluated by CT (Siemens Volume Zoom) at the level of fourth lumbal vertebra. Subcutaneous and intra-abdominal fat (IAF) areas were calculated as previously described.[21] The cardiopulmonary test was performed with a bicycle ergometer (Siemens Elema 380) until exhaustion. Respiratory gas exchange was analyzed continuously during the test with a computer-based system (Sensor Medics 2900, Metabolic Measument Cart/System). The average values of oxygen uptake measured during the last 20 seconds of the exercise were used to calculate maximum oxygen uptake.

Laboratory Determinations

Blood and plasma glucose were measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus, Yellow Springs Instrument Co, Inc), and plasma insulin and C-peptide were determined by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB; 125J RIA Kit, Incstar Co, respectively). Cholesterol and triglyceride levels from whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Roche Diagnostics).[8] Serum free-fatty acids (FFAs) were determined by an enzymatic method from Wako Chemicals GmbH. Serum adiponectin was measured with an enzyme immunoassay (Human Adiponectin ELISA Kit, B-Bridge International Inc). Plasma concentrations of tumor necrosis factor-α (TNF-α) and cytokines (interleukin [IL]-1β, IL-1 receptor antagonist [IL-1RA], IL-6, IL-10) and serum levels of soluble adhesion molecules (intercellular adhesion molecule [ICAM-1], vascular cell adhesion molecule [VCAM-1], E-selectin, and P-selectin) were measured with high-sensitivity assay kits from R&D Systems. IL-8 was measured with a kit from Biosource International. C-reactive protein (CRP) was measured with an Immulite analyzer and a DPC high-sensitivity CRP assay. Nonprotein urinary nitrogen was measured by automated Kjeldahl method.[22]

Statistical Analysis

All data analyses were performed with SPSS 11.0 for Windows programs. The results for continuous variables are given as mean±SD and for cytokine levels and insulin response in an IVGTT as mean±SEM in the figures. The differences between the 3 groups were assessed by ANOVA for continuous variables and by the χ^2 test for noncontinuous variables. ANCOVA was used to adjust for family relationship (all comparisons) and other confounding factors. Variables with skewed distribution were logarithmically transformed for statistical analyses. Factor analysis was used to reduce a large set of intercorrelating variables into a smaller set of latent underlying factors as previously described.[4,5,9] We used the principal components method for extraction of the initial components. Factors with eigenvalues ≥1 were retained, and varimax rotation was applied. Variable loadings ≥0.40 were considered significant in the interpretation of factors. The factor score from the analysis was categorized into the factor tertiles. The incremental insulin area under the insulin curve in an IVGTT was calculated by the trapezoidal method.

Results

Table 1 presents anthropometric and metabolic characteristics of the study population. Of the 119 participants, slightly more than half were women (102 study subjects had a normal glucose tolerance, 15 had impaired glucose tolerance, and 2 had impaired fasting glucose).

Cardiovascular risk factors (120-minute glucose, fasting insulin, BMI, waist, HDL cholesterol, total triglycerides, mean BP, rates of WBGU, IAF) correlated significantly, and the highest correlations were among the parameters measuring obesity (waist and BMI, r=0.523, P<0.001), whereas mean BP correlated only weakly with other components of the MetS (<0.40). Pearson correlation coefficient between fasting plasma insulin and the rates of WBGU during the clamp was −0.572 (P<0.01) and between waist and IAF area was 0.700 (P<0.01).

TABLE 1. Characteristics of the Study Subjects

Men/women, n	55/64
NGT/IFG/IGT, n	102/2/15
Age, y	35.5±6.0 (25–50)
BMI, kg/m^2	26.1±4.7 (17.6–44.2)
Waist, cm	88±12 (60–134)
Body fat, %	30±8 (14–51)
Systolic BP, mm Hg	126±11 (108–160)
Diastolic BP, mm Hg	84±9 (60–106)
Oral glucose tolerance test	
Fasting plasma glucose, mmol/L	5.1±0.4 (4.1–6.4)
120-min Plasma glucose, mmol/L	6.2±1.4 (3.5–10.4)
Fasting insulin, pmol/L	46.2±22.5 (18.0–175.8)
120-min Insulin, pmol/L	245.6±195.2 (24.0–1345.2)
Total cholesterol, mmol/L	4.90±0.87 (3.0–7.04)
LDL cholesterol, mmol/L	3.19±0.78 (1.40–5.34)
HDL cholesterol, mmol/L	1.27±0.28 (0.67–2.17)
Total triglycerides, mmol/L	1.13±0.60 (0.34–3.91)
Current smoker, %	33

NGT indicates normal glucose tolerance; IFG, impaired fasting glucose; and IGT, impaired glucose tolerance. Data are mean±SD (range). n=119.

Table 2 presents the results of factor analyses. Model 1, including, among other cardiovascular risk factors, simple clinical measures of insulin resistance (fasting insulin) and abdominal fat (waist), resulted in one factor, the MetS factor, that explained 46.2% of the total variance. Waist (0.830) and fasting insulin (0.760) had the highest loadings. Substituting waist by IAF area assessed by CT and fasting insulin by the rates of WBGU during the clamp also resulted in one factor solution having the highest loading for IAF (0.802) (Model 2). Percentage of variance explained was quite similar to that in model 1 (43.3%). However, when IAF was replaced by subcutaneous fat, a 2-factor solution was obtained. When BMI was not included in the analysis, the results remained essentially unchanged. When both systolic and diastolic BPs instead of mean BP were included in the model, factor analysis yielded a 2-factor solution, with only systolic and diastolic BPs having significant loadings (>0.4) on the second factor. We also performed factor analysis including fasting glucose, 120-minute insulin, systolic BP, and diastolic BP, in addition to variables in model 1, in the analysis. This model resulted in 4 separate factors (factor 1, glucose/insulin factor; factor 2, obesity/insulin factor; factor 3, lipid factor; factor 4, BP factor) (see Data Supplement Table I for details). When adiponectin, CRP, ICAM, and maximal oxygen uptake also were included in the model, the 4-factor solution was obtained (adiponectin loaded with fasting glucose and insulin and lipids; CRP and ICAM with obesity, and maximal oxygen uptake with glucose and insulin) (see Data Supplement Table II for details).

TABLE 2. Results of Factor Analyses Using Different Measurements of Insulin Sensitivity (Fasting Insulin or Rates of WBGU) and Visceral Obesity (Waist Circumference or IAF in CT)

	Model 1, Factor 1	Model 2, Factor 1
120-min Plasma blood	0.532	0.574
Fasting insulin (log)	0.760	
BMI	0.781	0.666
Waist	0.830	
HDL cholesterol	−0.637	−0.654
Total triglycerides (log)	0.646	0.719
Mean BP	0.502	0.460
WBGU		−0.678
IAF in CT (log)		0.802
Variance explained, %	46.2	43.3

Subjects were divided into the tertiles of factor scores based on model 1 (Table 2); the highest factor score tertile represented subjects having the MetS. Glucose oxidation ($P<0.001$, adjusted for gender) and nonoxidative glucose disposal during the clamp ($P<0.001$ adjusted for gender; Figure 1A) decreased and compensatory hyperinsulinemia in an IVGTT ($P=0.003$) increased with increasing factor score. The amount of IAF and subcutaneous fat also increased with increasing MetS factor score (Figure 1C and 1D). In contrast, adiponectin level decreased significantly ($P=0.001$, adjusted for gender and IAF; Figure 1B). Energy expenditure during the clamp decreased linearly among the MetS factor score tertiles ($P=0.031$ adjusted for gender; Figure 2), as well as maximum oxygen uptake ($P<0.001$ adjusted for gender). In contrast, the rates of lipid oxidation increased ($P=0.001$ adjusted for gender), which was also seen as a decrease in respiratory quotient ($P<0.001$ adjusted for gender). FFA levels during the clamp increased over the MetS factor score tertiles ($P=0.003$, adjusted for gender).

The associations between fasting cytokine levels and the MetS factor score tertiles are shown in Figure 3. A statistically significant increase in high-sensitivity CRP level ($P<0.001$, adjusted for gender and IAF) was found with increasing MetS factor score. In addition, cytokines increased (all probability values adjusted for gender and IAF) with increasing MetS factor score (IL-1β, $P=0.015$; IL-1RA, $P=0.002$; IL-6, $P=0.042$; IL-8, $P=0.014$). There were no significant differences in TNF-α and IL-10 levels after the adjustment for gender and IAF. P-selectin ($P=0.056$) and ICAM-1 ($P=0.006$) increased with increasing MetS score, whereas no change was observed in E-selectin and VCAM-1 (Figure 4).

We also compared all gender-adjusted parameters measured in those with and without MetS according to the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (NCEP ATPIII) criteria.[8] Compared with subjects without the MetS, those with the MetS according to this definition had higher amount of IAF ($P<0.001$), lower rates of WBGU ($P=0.001$) and oxidative ($P=0.002$) and nonoxidative ($P=0.001$) glucose disposal, lower energy expenditure ($P=0.040$), and higher FFA levels ($P=0.001$) and lipid oxidation ($P=0.006$) during hyperinsulinemia, as well as lower adiponectin levels ($P=0.002$) and maximum oxygen uptake ($P=0.001$).

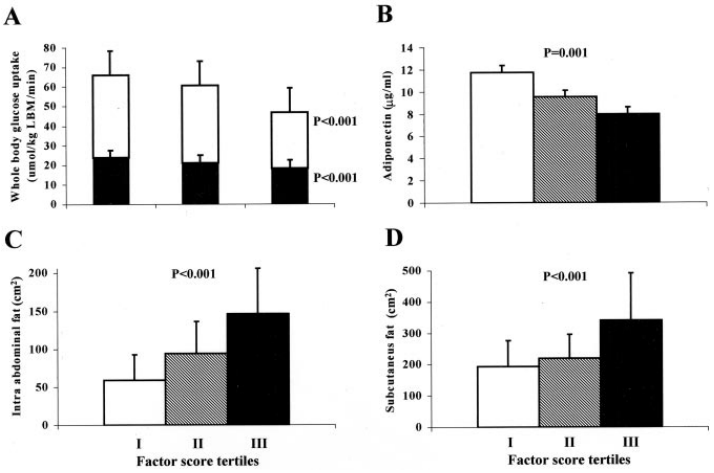

Figure 1. Rates of WBGU (■, glucose oxidation; □, nonoxidative glucose disposal) (A), adiponectin concentration (B), IAF mass (C), and subcutaneous fat mass (D) according to factor score tertiles (I=lowest, II=middle, III=highest tertile) derived from factor analysis. Probability values are unadjusted.

Discussion

Our study demonstrated that subjects with the MetS have multiple defects in glucose and energy metabolism, an excess of IAF, and hypoadiponectinemia. Furthermore, high levels of cytokines and adhesion molecules were associated with the MetS, indicating that low-grade inflammation and endothelial dysfunction are essential findings in subjects with the MetS.

Factor analysis is a particularly useful statistical method in studies of highly intercorrelating variables, as is the case with the putative components of the MetS. Our study showed that one factor, the MetS factor, explained almost half of the total variance among the variables included in statistical analysis. Furthermore, we demonstrated for the first time that fasting insulin and waist circumference gave results similar to insulin sensitivity measured directly by the hyperinsulinemic euglycemic clamp and IAF assessed by CT. These results indicate that fasting insulin level and waist can reliably be used to define the MetS for clinical practice.

Results of factor analysis, yielding only one factor for the MetS, differ somewhat from previous studies. Most studies have yielded 2 to 4 factors.[6,9,10,12,13–15,17] However, the finding of an obesity-hyperinsulinemia factor is rather consistent throughout different studies,[3–6,9–11,13,15] and in most cases, this factor has also included dyslipidemia (HDL cholesterol and triglycerides).[3–6,9–11,14,17] A separate BP factor having high loadings for systolic and diastolic BPs has been a rather consistent finding.[4,6,9,11–15] However, almost all these analyses have included both systolic and diastolic BPs. When we

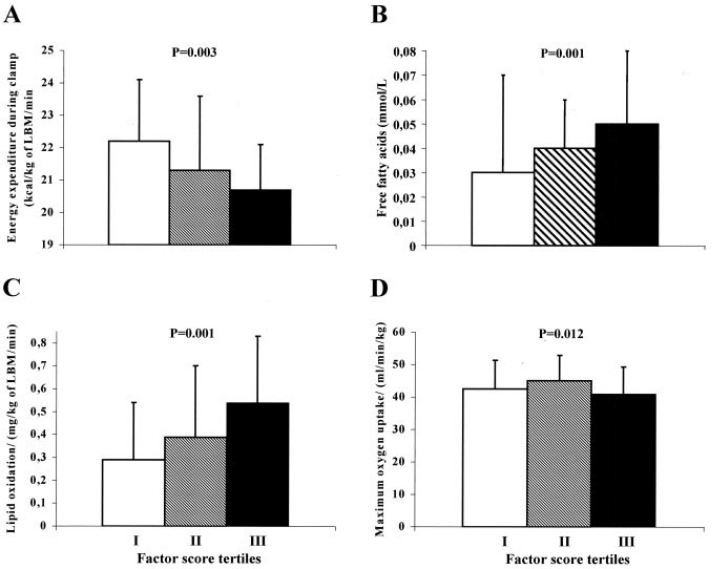

Figure 2. Energy expenditure during hyperinsulinemic clamp (A), FFA levels during hyperinsulinemic clamp (B), lipid oxidation during hyperinsulinemic clamp (C), and maximal oxygen uptake during exercise (D) according to factor score tertiles (I=lowest, II=middle, III=highest) derived from factor analysis. Probability values are unadjusted.

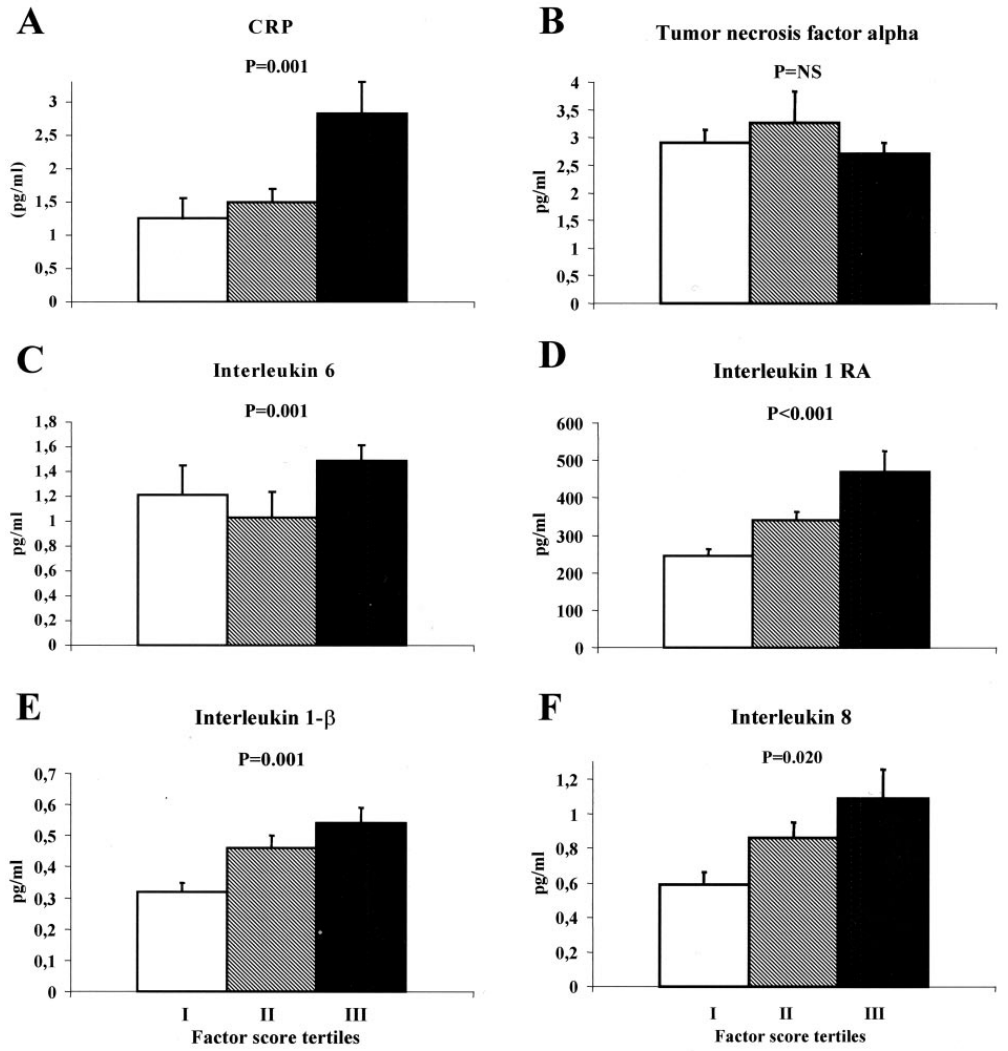

Figure 3. Fasting CRP (A) and cytokine levels (B–F) according to factor score tertiles (I=lowest, II=middle, III=highest) derived from factor analysis. Probability values are unadjusted.

repeated statistical analyses similarly, we also ended up with 2 separate factors. Furthermore, when we included 0- and 120-minute glucose and 120-minute insulin levels in the model, we obtained 4 separate factors often reported in previous studies.

Presenting the results of factor analysis as factor scores gave us an opportunity to obtain important information on metabolic abnormalities associated with the MetS defined as the highest tertile of the factor score. According to the NCEP ATPIII criteria[8] the prevalence of the MetS in our study was 10.9% in men and 9.2% in women. All 13 men and 9 of 11 women who had the MetS according to the NCET ATPIII criteria belonged in the highest MetS factor score tertile, indicating that the NCEP ATPIII criteria are quite specific for the MetS but that their sensitivity is likely to be rather low.

Our novel findings were that during hyperinsulinemia the MetS was associated with reduced rates of glucose oxidation and nonoxidative disposal, high rates of lipid oxidation, low energy expenditure, and impaired suppression of FFAs. Furthermore, the MetS was associated with a low adiponectin level, a high amount of IAF and subcutaneous fat, low maximum oxygen uptake, and high levels of CRP, proinflammatory cytokines, and adhesion molecules.

The tight link between insulin resistance and visceral fat in the MetS seems to be the basis of this syndrome, although we cannot conclude which is the primary abnormality. In addi-

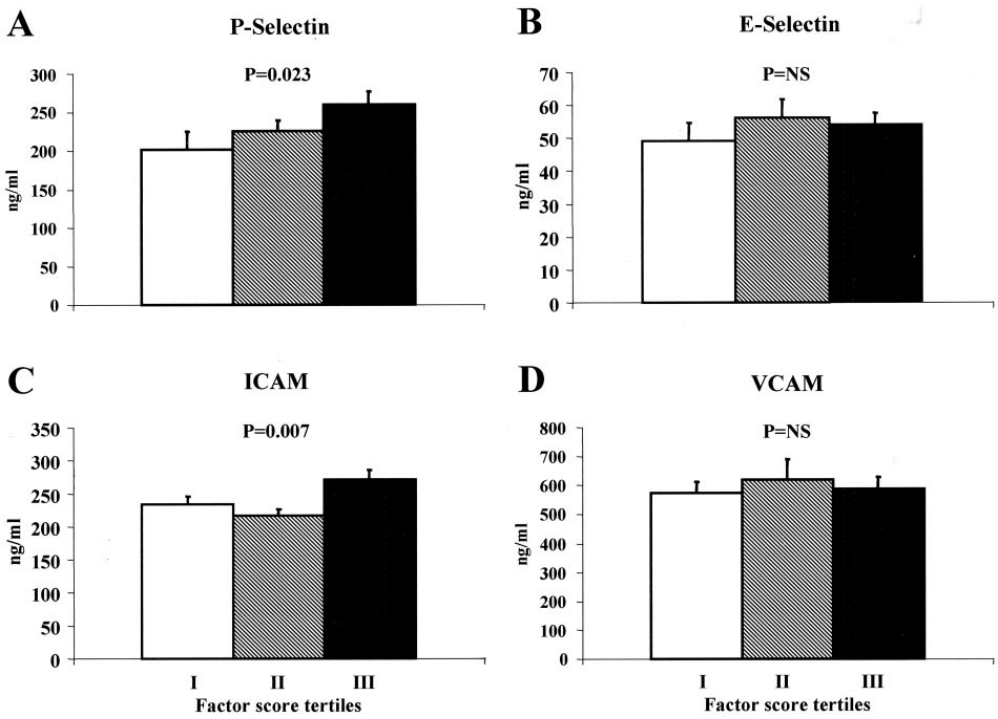

Figure 4. Fasting adhesion molecule levels (A–D) according to factor score tertiles (I=lowest, II=middle, III=highest) derived from factor analysis. Probability values are unadjusted.

tion to skeletal muscle, we observed insulin resistance in adipose tissue, because hyperinsulinemia was not able to suppress FFAs among subjects with MetS. Consequently, lipid oxidation was significantly elevated, which could, at least in part, be responsible for low rates of glucose oxidation during hyperinsulinemia. Impaired suppression of FFAs during hyperinsulinemia in subjects with the MetS contributes to elevated production of VLDL particles in the liver and thus hypertriglyceridemia.[23] However, causes of hypertriglyceridemia in the MetS are likely to be multifactorial, and other factors, in addition to the FFA flux into the liver, probably contribute to the dyslipidemia observed in these subjects.

Lower energy expenditure during the hyperinsulinemic clamp in subjects with the MetS is a novel finding. This finding may indicate that subjects with this syndrome have a lower increase in meal-induced thermogenesis and thus a tendency to gain weight. In addition, low energy expenditure during hyperinsulinemia could indicate central insulin resistance.

Adipose tissue secretes a variety of molecules and adipocytokines, including TNF-α, IL-6, and adiponectin. Adiponectin is produced abundantly in adipocytes, and in subjects with an excess of IAF, adiponectin levels are low.[24] High adiponectin level correlates with high insulin sensitivity.[25] We found that the MetS was associated with a high amount of IAF, a low adiponectin level, and elevated levels of cytokines and adhesion molecules. Adiponectin inhibits the expression of ICAM-1, VCAM-1, and E-selectin[26] and has several antiatherogenic[27,28] and antiinflammatory properties. Thus, hypoadiponectinemia can be responsible for endothelial damage and a low-grade systemic chronic inflammatory state.

Previous studies have shown that CRP, IL-6, and TNF-α predict type 2 diabetes[29] and coronary heart disease.[30] In our study, the most marked elevations were found in IL-1RA and IL-1β, whereas TNF-α did not differ between the factor score tertiles. Therefore, conventionally determined cytokines, TNF-α and IL-6, may not be the best markers for the MetS. P-selectin and ICAM-1 were also associated with the MetS, whereas E-selectin and VCAM-1 were not. The association of adhesion molecules with the MetS is logical because they have a close interaction with proinflammatory cytokines. Adhesion molecule expression is induced by proinflammatory cytokines such as IL-1β, TNF-α, and CRP produced by the liver in response to IL-6.[31]

In conclusion, our findings add new information for the understanding of metabolic abnormalities in the MetS. Our results show for the first time that insulin resistance in people with the MetS is seen not only in skeletal muscle but also in adipose tissue, leading to multiple defects in glucose and energy metabolism, hypoadiponectinemia, and elevated levels of proinflammatory cytokines and adhesion molecules. These results give further evidence that the MetS is an important risk factor for cardiovascular disease, but follow-up studies are needed to confirm this hypothesis.

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II

Changes in Inflammatory Cytokines Are Related to Impaired Glucose Tolerance in Offspring of Type 2 Diabetic Subjects

EIJA RUOTSALAINEN, MD[1]
URPU SALMENNIEMI, MD[1]
ILKKA VAUHKONEN, MD[1]
JUSSI PIHLAJAMÄKI, MD[1]
KARI PUNNONEN, MD[2]
SAKARI KAINULAINEN, MD[3]
MARKKU LAAKSO, MD[1]

OBJECTIVE — We sought to determine whether levels of inflammatory markers and different cytokines are abnormal in nondiabetic offspring of type 2 diabetic subjects.

RESEARCH DESIGN AND METHODS — Cytokine levels were measured in 19 healthy control subjects and 129 offspring of patients with type 2 diabetes (109 with normal glucose tolerance [NGT] and 20 with impaired glucose tolerance [IGT]). Insulin sensitivity was determined with the hyperinsulinemic-euglycemic clamp, insulin secretion with the intravenous glucose tolerance test, and abdominal fat distribution with computed tomography.

RESULTS — Levels of C-reactive protein and inflammatory cytokines were elevated in nondiabetic offspring of type 2 diabetic subjects. Interleukin (IL)-1β was increased in the NGT group and decreased in the IGT group. In contrast, levels of IL-1 receptor antagonist (IL-1Ra) were increased in both groups. IL-1β and -Ra levels correlated inversely ($P < 0.05$) with rates of whole-body glucose uptake and IL-1β positively with visceral fat mass ($P < 0.05$) in normoglycemic offspring.

CONCLUSIONS — Nondiabetic offspring of type 2 diabetic subjects have changes in the levels of inflammatory cytokines. The level of IL-1Ra seems to be the most sensitive marker of cytokine response in the pre-diabetic state.

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Impaired glucose tolerance (IGT) precedes type 2 diabetes and is attributable to either insulin resistance or decreased insulin secretion, or both. Proinflammatory cytokines deleteriously influence insulin sensitivity and β-cell function (1). Tumor necrosis factor (TNF)-α blocks insulin action by inducing serine phosphorylation of insulin receptor substrate 1 (2). Furthermore, long-term cytokinemia impairs insulin secretion in the β-cells (3). Thus, accumulating evidence supports the hypothesis that type 2 diabetes is a disease of the innate immune system (3,4).

C-reactive protein (CRP) and proinflammatory cytokine levels are elevated in both IGT and overt type 2 diabetes, and they predict the conversion to type 2 diabetes (3–8). However, Krakoff et al. (9) failed to show that CRP and interleukin (IL)-6 levels predict diabetes in Pima Indians. Although TNF-α causes insulin resistance at the cellular level, circulating TNF-α levels are neither associated with type 2 diabetes nor with the future risk of diabetes (4,10). The elevation of both IL-6 and -1β increases the risk of type 2 diabetes more strongly than elevated levels of IL-6 alone (4).

Inflammation in autoimmune diseases is characterized by a balance between pro- and anti-inflammatory cytokines. The members of the IL-1 cytokine superfamily, IL-1α and -1β, are strong inducers of inflammation (11–13). IL-1 receptor antagonist (IL-1Ra) acts in an antagonistic manner and serves as a natural compensatory mechanism for the IL-1–induced disease process (11,14). In healthy individuals, IL-1Ra is detectable in plasma, in contrast to usually undetectable levels of IL-1β (12). White adipose tissue is an important source of IL-1Ra (15). IL-1Ra levels are increased in human obesity (16) and may contribute to the development of insulin resistance (17).

In various disease states, the levels of IL-1β are increased. IL-1β has been shown to mediate impaired β-cell function and apoptosis in both type 1 and type 2 diabetes (18–20). The apoptotic pathway has been suggested to link both forms of diabetes. However, recent studies have shown that high glucose in vitro and in the diabetic milieu does not induce IL-1β production or nuclear factor-κB activation in human islets, which argues against the notion that the IL-1β–nuclear factor-κB–Fas pathway is a common mediator of β-cell death in type 2 diabetes (21,22).

IL-18, a member of the IL-1 cytokine superfamily, is an important regulator of innate and acquired immune response. Elevated levels of IL-18 have been observed in type 2 diabetic subjects (23,24). Low levels of IL-10, a cytokine with strong anti-inflammatory properties, have been associated with the metabolic syndrome and type 2 diabetes (25).

The offspring of patients with type 2 diabetes are at increased risk of developing diabetes, but only three previous studies have measured the levels of one proinflammatory cytokine, TNF-α, in offspring of type 2 diabetic patients (26–28). Therefore, we investigated whether levels of CRP and different cytokines are

From the [1]Department of Medicine, University of Kuopio, Kuopio, Finland; the [2]Department of Clinical Chemistry, University of Kuopio, Kuopio, Finland; and the [3]Department of Clinical Radiology, University of Kuopio, Kuopio, Finland.

Address correspondence and reprint requests to Markku Laakso, MD, Academy Professor, Department of Medicine, University of Kuopio, 70210 Kuopio, Finland. E-mail: markku.laakso@kuh.fi.

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Abbreviations: CRP, C-reactive protein; IGT, impaired glucose tolerance; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; NGT, normal glucose tolerance; TNF, tumor necrosis factor; WBGU, whole-body glucose uptake.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Characteristics of the study population*

	Control	Offspring of patients with type 2 diabetes		*P*
		NGT	IGT	
n	19	109	20	
Sex (M/F)	8/11	53/56	8/12	0.715
Age (years)	34.5 ± 4.5	35.0 ± 6.1	38.6 ± 6.6*	0.042
Waist-to-hip ratio	0.84 ± 0.1	0.87 ± 0.8	0.88 ± 0.1	0.161
BMI (kg/m^2)	24.6 ± 2.6	25.8 ± 4.3	28.0 ± 6.2*	0.075
Fat percent	29 ± 9	29 ± 8	32 ± 9	0.275
Systolic blood pressure (mmHg)	124 ± 10	126 ± 11	133 ± 18*	0.020
Diastolic blood pressure (mmHg)	82 ± 10	83 ± 9	90 ± 14*	0.004
Fasting glucose (mmol/l)	5.1 ± 0.6	5.2 ± 0.4	5.2 ± 0.5	0.746
120-min glucose (mmol/l)	5.6 ± 1.1	5.8 ± 1.0	8.7 ± 0.8†	<0.001
Fasting insulin (pmol/l)	47.9 ± 23.0	44.6 ± 19.1	57.9 ± 34.4	0.087
120-min insulin (pmol/l)	194.0 ± 107.2	219.2 ± 159.4	400.1 ± 261.7†	<0.001
Total cholesterol (mmol/l)	4.73 ± 0.96	4.89 ± 0.91	4.94 ± 0.64	0.731
LDL cholesterol (mmol/l)	2.80 ± 0.7	3.19 ± 0.81*	3.16 ± 0.60	0.116
HDL cholesterol (mmol/l)	1.37 ± 0.33	1.27 ± 0.28	1.26 ± 0.32	0.343
Total triglycerides (mmol/l)	1.24 ± 0.84	1.12 ± 0.62	1.25 ± 0.53	0.461

Data are means ± SD. NGT/IGT vs. control subjects: *$P < 0.05$; †$P < 0.001$.

already abnormal in nondiabetic offspring of type 2 diabetic subjects.

RESEARCH DESIGN AND METHODS

The subjects were healthy nondiabetic offspring of patients with type 2 diabetes. Type 2 diabetic probands were randomly selected among patients living in the region of the Kuopio University Hospital. Spouses of the probands had to have normal glucose tolerance (NGT), as determined by an oral glucose tolerance test. One to three offspring from each family were included in this study. The exclusion criteria for the offspring were as follows: *1*) diabetes or any other disease that could potentially disturb carbohydrate metabolism, *2*) diabetes in both parents, *3*) pregnancy, *4*) any ongoing infection, and *5*) age <25 or >50 years. A total of 129 offspring (61 men and 68 women) from 78 families (43 families with one child, 29 families with two children, and 6 families with three children) were studied. The control group included 19 healthy volunteers (8 men and 11 women) with NGT and without a family history of diabetes. The inclusion criteria were identical to the selection of offspring described above. The study protocol was approved by the ethics committee of the University of Kuopio and the Kuopio University Hospital.

Subjects were admitted to the metabolic ward of the Department of Medicine of the Kuopio University Hospital on three different occasions, 1–2 months apart. The order of the tests was the same for each subject. On day 1, a standardized interview was conducted to collect information on medical history, smoking, alcohol consumption, and physical activity. Weight and height were measured to the nearest 0.5 cm and 0.1 kg, respectively. Other clinical parameters were measured as previously described in detail (29). Fasting blood samples were drawn after a 12-h fast followed by an oral glucose tolerance test (75 g glucose). Glucose tolerance status was evaluated according to World Health Organization criteria (30).

On day 2, an intravenous glucose tolerance test was performed to evaluate first-phase insulin secretion capacity after an overnight fast. Immediately after an intravenous glucose tolerance test, the euglycemic-hyperinsulinemic clamp was started to determine the degree of insulin sensitivity, as previously described (29). After a priming dose of insulin, plasma insulin was maintained at 5.0 mmol/l by a continuous insulin infusion (insulin infusion rate of 40 mU/min per m^2 body surface area) and blood glucose kept constant for the next 120 min by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. The amount of glucose infused was used to calculate the rates of whole-body glucose uptake (WBGU). Body composition was determined by bioelectrical impedance (RJL Systems, Detroit, MI) in the supine position after a 12-h fast.

On day 3, abdominal fat distribution was evaluated by computed tomography (Siemens Volume Zoom; Siemens, Erlangen, Germany) at the level of fourth lumbal vertebra according to the method of Sjöström et al. (31). Subcutaneous and intraabdominal fat areas were calculated as previously described (32).

Plasma glucose was measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus; Yellow Springs Instrument, Yellow Springs, OH) and plasma insulin and C-peptide by radioimmunoassay (Phadeseph Insulin RIA 100; Pharmacia Diagnostics, Uppsala, Sweden, and 125J RIA kit; Incstar, Stillwater, MN, respectively). Cholesterol and triglyceride levels from whole serum and lipoprotein fractions were assayed by automated enzymatic methods (Roche Diagnostics, Mannheim, Germany).

For the determination of cytokines, blood was collected into EDTA tubes on ice and immediately centrifuged and the plasma stored at −70°C (maximum storage time 3 years). Plasma concentrations of TNF-α, IL-1β, IL-1Ra, IL-6, IL-10, and IL-18 were measured using assay kits from R&D Systems (Minneapolis, MN). IL-8 was measured using a kit from Biosource International (Camarillo, CA). CRP was measured using an Immulite analyzer and a DPC High Sensitivity CRP assay (Diagnostic Products, Los Angeles, CA).

Genotyping was performed by direct sequencing (ABI prism genetic analyzator) (IL-1Ra gene: G114C), restriction length polymorphism (IL-6 gene: C-174G; IL-10 gene: A-592C; TNF-

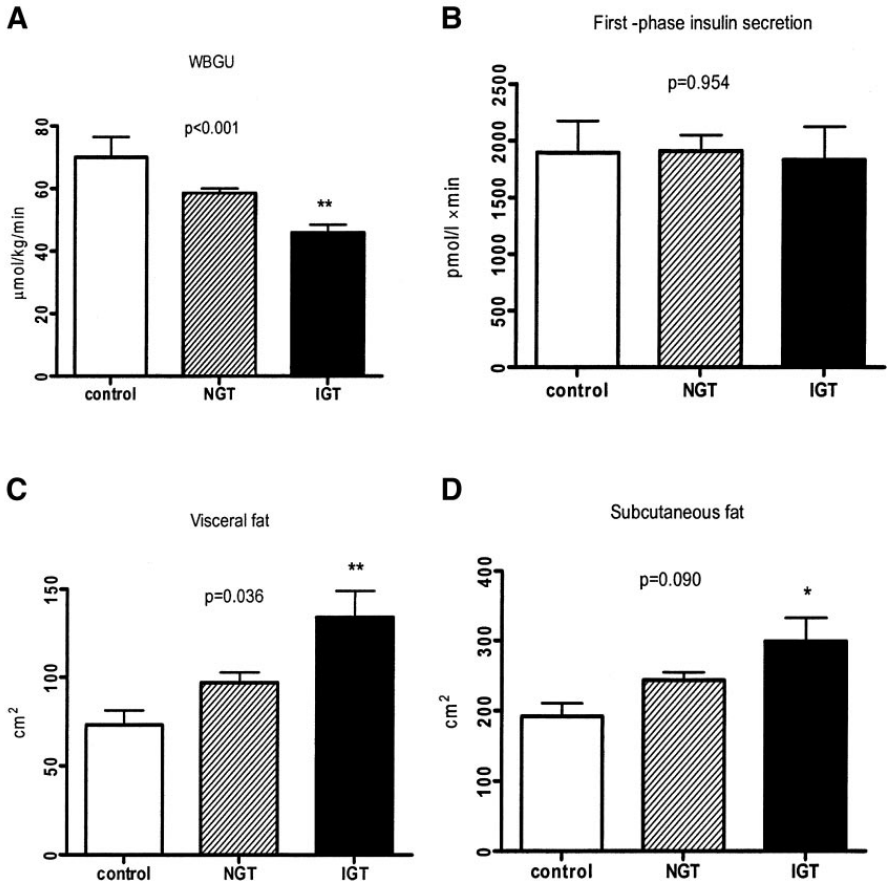

Figure 1— *Rates of WBGU (A), first-phase insulin secretion (B), visceral fat (C), and subcutaneous fat (D) in offspring of type 2 diabetic patients. □, control group; ▨, NGT group; ■, IGT group. P value after the adjustment for age, sex, BMI, and family relationship (mixed linear model). Data are means ± SE. *P < 0.05, **P < 0.01 for IGT vs. control group.*

receptor 2 gene: M196R), or by TaqMan assays (CRP gene: G942C, G1059C; IL-1β gene: T511C, C3954T; IL-10 gene: A1082G; TNF-α gene: G-308A). Details of the genotyping procedures can be obtained from the authors by request.

Statistical analysis

All calculations were performed with SPSS 11.0 for Windows. The results for continuous variables are shown as means ± SD, if not stated otherwise. The differences between the three groups were assessed by ANOVA for continuous variables and the χ^2 test for noncontinuous variables. Variables with skewed distribution (triglycerides and BMI), insulin, CRP, TNF-α, IL-6, IL-1Ra, IL-8, and IL-10 were logarithmically transformed for statistical analyses. The incremental insulin areas under the curve were calculated by the trapezoidal method. Linear mixed-model analysis was applied to test the differences between the groups to adjust for confounding factors. Pedigree membership was included in the model as a random factor and sex as a fixed factor. A P value <0.05 was considered statistically significant.

RESULTS — Table 1 reports anthropometric and metabolic characteristics of the study subjects. The groups were comparable with respect to sex, but subjects with IGT were older ($P < 0.05$), had higher BMI ($P < 0.05$), and had higher levels of systolic ($P < 0.05$) and diastolic ($P < 0.05$) blood pressure than the control subjects. There were no significant differences between the groups in waist-to-hip ratio and fat percent. LDL cholesterol was higher in the NGT group ($P < 0.05$) than in the control group, whereas total cholesterol, HDL cholesterol, and triglycerides did not differ among groups. Plasma glucose and insulin levels at 120 min were significantly elevated in the IGT group ($P < 0.001$ vs. control group).

Figure 1 presents the results of metabolic studies. A significant decrease was found in the rates of WBGU in the IGT group, and a similar, but not statistically significant, trend was observed in the NGT group compared with the control group. No compensatory increase in first-phase insulin secretion was observed in the IGT group. The areas of both visceral and subcutaneous fat were significantly higher in the IGT group compared with the control group. The differences per-

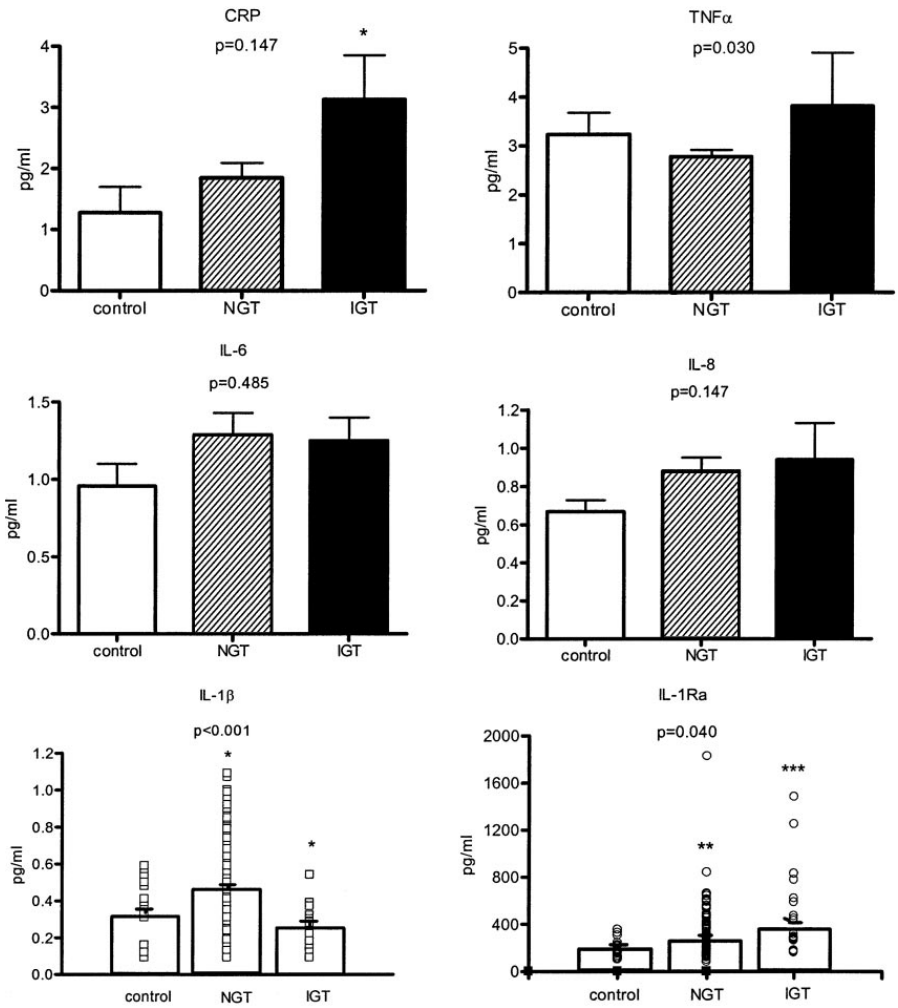

Figure 2—*Fasting cytokines in offspring of type 2 diabetic patients.* □*, control group;* ▨*, NGT group;* ■*, IGT group. The individual data for IL-1β and IL-1Ra is shown in scattergrams (*□ *in all respective groups).* P *value after the adjustment for sex, BMI, and familiality (mixed linear model). Means ± SE.* *P < *0.05,* **P < *0.01,* ***P < *0.001 for NGT or IGT vs. control group.*

sisted after adjustment for age, sex, BMI, and familiarity. The ratio of subcutaneous to visceral fat did not differ among groups (data not shown).

Levels of fasting cytokines are shown in Fig. 2. CRP level was significantly higher in the IGT group than in the control group. Levels of TNF-α did not differ significantly among the three groups, but after adjustment for age, sex, BMI, and familiality, a statistically significant difference was observed among the three groups. There were no significant differences in fasting levels of IL-6, IL-8, IL-10, or IL-18 (data not shown) among the three groups. Compared with the control group, levels of IL-1β were significantly higher in the NGT group, whereas there was a significant decrease in IL-1β levels in the IGT group. Levels of IL-1Ra increased linearly in the NGT and IGT groups compared with the control group.

The correlations of fasting cytokines with metabolic parameters are shown in Table 2. In the NGT group, IL-6 ($P < 0.05$), CRP ($P < 0.01$), IL-1β ($P < 0.05$), and IL-1Ra levels ($P < 0.05$) correlated inversely with WBGU. Inverse correlations were also significant among IL-6, IL-1Ra, and WBGU in the IGT group ($P < 0.05$ and $P < 0.01$, respectively). Significant correlations between IL-6 level and the amount of visceral and subcutaneous fat were found in both NGT and IGT groups (visceral fat: $P < 0.01$ and $P < 0.05$, respectively; subcutaneous fat: $P < 0.01$ in both groups). CRP ($P < 0.05$) and IL-1Ra ($P < 0.05$) correlated significantly with first-phase insulin secretion in the NGT group. The correlation was even stronger between IL-1Ra and first-phase insulin secretion in the IGT group ($P < 0.01$), whereas correlation with CRP was not statistically significant in the IGT group. In the NGT group, IL-6 ($P <$

Table 2—*Spearman correlations of fasting cytokines, visceral fat, subcutaneous fat, rates of WBGU, and first-phase insulin secretion in offspring with NGT and IGT*

	IL-6	CRP	IL-1β	IL-1Ra	TNF-α	IL-8	Visceral fat	Subcutaneous fat	WBGU	First-phase insulin secretion
NGT										
IL-6	1.000	0.236*	0.088	0.278†	0.196*	0.166	0.310†	0.310†	−0.241*	0.024
CRP		1.000	0.264†	0.233*	0.188	0.121	0.189	0.254†	−0.339†	0.223*
IL-1β			1.000	0.164	0.116	0.230*	0.290†	0.173	−0.239*	0.172
IL-1Ra				1.000	0.214*	0.347†	0.172	0.277†	−0.205*	0.212*
TNF-α					1.000	0.160	0.121	0.034	−0.082	0.076
IL-8						1.000	0.140	0.109	−0.154	−0.137
Visceral fat							1.000	0.326†	−0.452†	0.234*
Subcutaneous fat								1.000	−0.275†	0.274†
WBGU									1.000	−0.312†
First-phase insulin secretion										1.000
IGT										
IL-6	1.000	0.557*	−0.204	0.437	−0.214	−0.381	0.524*	0.609†	−0.463*	0.357
CRP		1.000	−0.122	0.525*	−0.421	0.015	0.261	0.381	−0.334	0.179
IL-1β			1.000	0.389	0.325	0.289	0.161	0.155	−0.137	0.234
IL-1Ra				1.000	−0.293	0.081	0.549*	0.547*	−0.645	0.672†
TNF-α					1.000	0.180	−0.112	−0.181	0.042	−0.432
IL-8						1.000	0.105	0.097	0.023	−0.197
Visceral fat							1.000	0.570*	−0.668†	0.437
Subcutaneous fat								1.000	−0.782†	0.608†
WBGU									1.000	−0.640†
First-phase insulin secretion										1.000

*$P < 0.05$; †$P < 0.01$.

0.05), IL-1β ($P < 0.01$), and IL-1Ra levels ($P < 0.05$) correlated significantly with CRP. In the IGT group, CRP correlated significantly with IL-6 ($P < 0.05$) and IL-1Ra ($P < 0.05$) but not with IL-1β levels.

Common polymorphisms that have been previously associated with insulin resistance, insulin resistance–related quantitative traits, or risk of diabetes in the CRP (G942C and G1059C), TNF-α (G-308A), TNF-receptor 2 (M-196R), IL-1β (T511C and C3954T), IL-1Ra (G114C), IL-6 (C-174G), or IL-10 (A-592C) genes were not associated with corresponding cytokine levels (data not shown).

CONCLUSIONS — The offspring of type 2 diabetic subjects are at increased risk of diabetes. Our study showed that CRP and proinflammatory cytokine levels are elevated in nondiabetic offspring compared with the control group, supporting the concept that low-grade inflammation is one of the earliest findings in the pathogenesis of type 2 diabetes. The novel finding of our study was that the level of IL-1Ra is the most sensitive marker of cytokine response in the prediabetic state.

Low-grade inflammation is linked to the onset of type 2 diabetes (33). To our knowledge, there are only three previously published studies that have investigated the role of inflammatory cytokines in nondiabetic offspring of patients with type 2 diabetes. However, these studies have measured only levels of TNF-α but not those of other proinflammatory cytokines. Kellerer et al. (27) showed that circulating TNF-α level did not contribute to obesity-induced insulin resistance. Maltezos et al. (28) observed significantly elevated concentrations of TNF-α in healthy nondiabetic offspring of type 2 diabetic subjects. Costa et al. (26) showed that the TNF-α pathway could predispose to the development of type 2 diabetes in the first-degree relatives of type 2 diabetic patients. In our study, we did not find increased levels of TNF-α or IL-6 in the offspring of type 2 diabetic individuals. However, we found that glucose-intolerant offspring of type 2 diabetic patients had elevated CRP levels, which is in line with previous studies (3–8,25).

In our study, the level of IL-1β was increased in the NGT group, whereas it was decreased in the IGT group. To determine the biological activity of IL-1β, we calculated the ratio of IL-1Ra to IL-1β. Eizirik et al. (34) have shown that a 10- to 100-fold excess of IL-1Ra over IL-1β suffices to block the effects of IL-1β on pancreatic islets. We found >100-fold excess of the ratio of IL-1Ra to IL-1β, indicating a decreased biological activity of IL-1β in the NGT group (999) and, more markedly, in the IGT group (2,538). The excess of IL-1Ra should block the biological activity of IL-1β by human islets. In line with recently published studies, we suggest that it is unlikely that IL-1β would mediate β-cell failure during progression to type 2 diabetes.

Decreased concentrations of IL-1Ra have been reported in type 2 diabetes (14), whereas IL-1Ra overproduction has been observed in men with the insulin resistance syndrome (35). The level of IL-Ra has been shown to be markedly and reversibly elevated in human obesity and predicted by lean body mass and insulin levels (16). In our study, IL-1Ra levels

were elevated in normoglycemic offspring and even more so in offspring with IGT compared with those in the control group. IL-1Ra had an inverse correlation with WBGU in the NGT and IGT groups. IGT offspring had a significantly higher amount of visceral and subcutaneous fat than control subjects, which, together with increased IL-1Ra levels, supports the finding that adipose tissue is an important source of IL-1Ra (15). It is possible that the elevation of IL-1Ra and the negative correlation between IL-1Ra and WBGU reflects insulin resistance in the NGT and IGT groups. Although IL-1Ra is considered a protective cytokine, increased levels of IL-1Ra might rather expose than protect the offspring at high risk of diabetes from insulin resistance.

Promoter polymorphisms of the TNF-α and IL-6 genes have been shown to predict the conversion from IGT to type 2 diabetes (36), and variants in the TNF-α gene regulate insulin sensitivity (26). Therefore, we analyzed the effects of common polymorphisms of CRP and cytokine genes (TNF-α, TNF-receptor 2, IL-β, IL-1Ra, IL-6, and IL-10) on fasting levels of CRP and cytokines, but no associations were found.

Our study has some limitations. First, the control group and the IGT group are small, limiting the statistical power of our study. Second, subjects in the control group were significantly younger and thinner than those in the IGT group, which could explain some of the differences between the groups, although adjustment for age and BMI was done in statistical analyses of the data.

In summary, our results add new insights to the understanding of inflammatory mechanisms in the pathogenesis of type 2 diabetes. Our study is the first to show that the offspring of type 2 diabetic patients have changes in levels of CRP, IL-1β, and IL-1Ra. It remains to be proven whether this cytokine imbalance is one of the fundamental defects in the pre-diabetic state and in type 2 diabetes.

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III

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Markers of endothelial dysfunction and low-grade inflammation are associated in the offspring of type 2 diabetic subjects

Eija Ruotsalainen [a], Ilkka Vauhkonen [a], Urpu Salmenniemi [b], Jussi Pihlajamäki [a], Kari Punnonen [c], Sakari Kainulainen [d], Sirpa Jalkanen [e], Marko Salmi [f], Markku Laakso [a,*]

[a] *University of Kuopio, Department of Medicine, 70210 Kuopio, Finland*
[b] *University of Turku, Deparment of Medicine, Turku, Finland*
[c] *University of Kuopio, Department of Clinical Chemistry, Kuopio, Finland*
[d] *University of Kuopio, Department of Clinical Radiology, Kuopio, Finland*
[e] *University of Turku, MediCity Research Laboratory, Turku, Finland*
[f] *National Public Health Institute, Turku, Finland*

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Abstract

The offspring of type 2 diabetic patients are at elevated risk for type 2 diabetes and cardiovascular disease. The aim of our study was to characterize the role of various biomarkers of endothelial activation in a cohort of offspring of type 2 diabetic subjects and to assess the association of adhesion molecules with inflammatory markers and metabolic parameters.

Cytokine and adhesion molecule levels were measured in 19 healthy subjects and in 129 offspring of patients with type 2 diabetes (109 with normal glucose tolerance and 20 with impaired glucose tolerance). Insulin sensitivity was determined with the hyperinsulinemic-euglycemic clamp, insulin secretion with the intravenous glucose tolerance test, and abdominal fat distribution with computed tomography.

The levels of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-Selectin and vascular adhesion protein-1 were not increased in offspring of type 2 diabetic subjects, but they correlated with inflammatory markers (C-reactive protein, tumor necrosis-alpha, interleukin-6, interleukin-1beta, interleukin-1 receptor antagonist, interleukin-8, interleukin-10 and interleukin-18). In conclusion, the levels of adhesion molecules were not elevated in the prediabetic state. Inflammatory markers and adhesion molecules were correlated suggesting that low-grade inflammation may precede the elevation of levels of adhesion molecules.
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Keywords: Offspring of type 2 diabetic patients; Endothelial dysfunction; Adhesion molecules; Inflammation; Type 2 diabetes; Insulin resistance

1. Introduction

The offspring of type 2 diabetic patients are at elevated risk for type 2 diabetes and cardiovascular disease (CVD) [1]. Insulin resistance and clustering of CVD risk factors with it are predictors of the development of type 2 diabetes and CVD [2]. Similarly, low-grade inflammation and endothelial dysfunction precede the development of type 2 diabetes [3,4] and CVD [5,6]. A unifying factor in the pathogenesis of these diseases is inflammation in the vascular endothelium, which might contribute to the disease process [3].

As a sign of endothelial damage, soluble cellular adhesion molecules are released from the endothelial cells. Increased levels of cellular adhesion molecules have been detected in several studies in patients with insulin resistance [7,8], type 2 diabetes [9], and CVD [10]. Among the adhesion molecules, E-Selectin is only found in the endothelium, whereas intercellular adhesion molecule-1 (ICAM-1) and vascular cell

* Corresponding author. Tel.: +358 17 172151; fax: +358 17 173993.
E-mail address: markku.laakso@kuh.fi (M. Laakso).

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adhesion molecule-1 (VCAM-1) represent a wider tissue distribution [11]. High soluble E-Selectin level is a strong independent predictor of type 2 diabetes [3,4], but the role of other soluble adhesion molecules remains controversial.

Vascular adhesion protein-1 (VAP-1) is located at the endothelial membrane [12]. It may contribute to the pathogenesis of atherosclerosis by guiding inflammatory cells into atherosclerotic lesions [13]. VAP-1 is also found in a soluble form in serum. Elevated levels of soluble VAP-1 have been measured in patients with type 1 diabetes, and the clearance of VAP-1 is regulated by insulin [12,14]. Moreover, in patients with type 1 diabetes, increased levels of soluble VAP-1 have been observed in patients with poor metabolic control. By normalizing blood glucose with exogenous insulin, a rapid decrease in soluble VAP-1 concentrations has been observed [12].

Previous studies that have investigated the levels of adhesion molecules have demonstrated that the offspring of type 2 diabetic patients have features of endothelial dysfunction, estimated either by non-invasive methods or by measuring blood levels of adhesion molecules, or both [15–19]. Adhesion molecules are regulated by inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) [20]. Thus, the evaluation of endothelial dysfunction requires the measurement of multiple biomarkers of endothelial activation [3]. The association of inflammatory cytokines with adhesion molecules has not been previously studied in first-degree relatives of type 2 diabetic subjects. Therefore, the aim of our study was to characterize various biomarkers of endothelial activation in a cohort of offspring of type 2 diabetic subjects and to assess the association of adhesion molecules with inflammatory markers and metabolic parameters.

2. Methods

2.1. Subjects

The subjects were 129 healthy non-diabetic offspring of patients with type 2 diabetes (men/women 61/68, age 35 ± 6 years, body mass index (BMI) 26.1 ± 4.6 kg/m^2). The control group consisted of 19 healthy normoglycemic subjects with no family history of type 2 diabetes (men/women 8/11, age 34 ± 4 years, BMI 24.6 ± 2.6 kg/m^2). Probands were randomly selected among type 2 diabetic subjects living in the region of the Kuopio University Hospital. The spouses of patients with type 2 diabetes had to have normal glucose tolerance in an oral glucose tolerance test (OGTT). The exclusion criteria for the selection of the offspring were: (1) diabetes mellitus or any other disease that could potentially disturb carbohydrate metabolism; (2) diabetes mellitus in both parents; (3) pregnancy; (4) age less than 25 or more than 50 years. The Ethics Committee of the University of Kuopio approved the study protocol. All study subjects gave informed consent.

2.2. Study design

On the first day, the subjects were interviewed about their medical history, smoking, alcohol consumption, and exercise habits. After a 5-min rest, blood pressure was measured in a sitting position with a mercury sphygmomanometer. The average of three measurements was used to calculate systolic and diastolic blood pressure. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. BMI was calculated as weight in kilograms divided by height in meters squared. Waist (at the midpoint between the lateral iliac crest and the lowest rib) and hip circumference (at the level of the trochanter major) were measured to the nearest 0.5 cm. Fasting blood samples were drawn after 12 h of fasting, followed by an OGTT (75 g of glucose). On the second day, after a 12-h fast, an intravenous glucose tolerance test (IVGTT) and the hyperinsulinemic euglycemic clamp, including indirect calorimetry, were performed. On day 3, a CT scan was performed to evaluate the amount and distribution of abdominal fat.

2.3. Metabolic studies

An IVGTT was performed to determine the first-phase insulin secretion capacity after an overnight fast. After baseline blood collection, a bolus of glucose (300 mg/kg in a 50% solution) was given within 30 s into the antecubital vein. Samples for the measurement of blood glucose and plasma insulin (arterialized venous blood) were drawn at −5, 0, 2, 4, 6, 8 and 10 min.

Immediately after an IVGTT, the euglycemic hyperinsulinemic clamp (insulin infusion rate of 40 mU/min/m^2 body surface area) was started to determine the degree of insulin sensitivity as previously described [21]. Blood glucose was clamped at 5.0 mmol/l for the next 120 min by infusion of 20% glucose at various rates according to blood glucose measurements performed at 5-min intervals. The mean amount of glucose infused during the last hour was used to calculate the rates of whole-body glucose uptake (WBGU). Indirect calorimetry was performed with a computerized flow-through canopy gas analyzer system (DELTATRAC, TM Datex) as previously described [21,22]. The mean value of the data during the last 20 min of the clamp was used to calculate glucose and lipid oxidation [23]. The rates of non-oxidative glucose disposal during the clamp were estimated by subtracting the rates of glucose oxidation from the glucose infusion rate.

2.4. Body composition and fat distribution

Body composition was determined by bioelectrical impedance (RJL Systems, Detroit, MI) in the supine position after a 12-h fast. Abdominal fat distribution was evaluated by computed tomography (Volume Zoom, Siemens, Erlanger, Germany) at the level of fourth lumbar vertebra. Subcuta-

neous and visceral fat areas were calculated as previously described [22].

2.5. Laboratory determinations

Blood and plasma glucose levels were measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat PLUS, Yellow Springs Instruments Co, Inc.) and plasma insulin and C-peptide were determined by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Biotech, Uppsala, Sweden). Plasma concentrations of cytokines (TNF-α, IL-1β, interleukin-1 receptor antagonist (IL-1Ra), IL-6, interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-18 (IL-18)) were measured by solid phase ELISA (Quantikine, R&D Systems; and IL-8 Ultrasensitive ELISA, BioSource International). C-reactive protein (CRP) was determined by an Immulite 2000 High Sensitivity CRP assay (Diagnostic Products Corp). Levels of soluble adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) were measured with high-sensitivity assay kits from R&D Systems. Soluble VAP-1 was measured using an in-house sandwich ELISA, as described [24].

2.6. Statistical analysis

All data analyses were performed with the SPSS 13.0 for Windows programs. The results for continuous variables are given as mean ± S.D., if not stated otherwise. Variables with skewed distribution were logarithmically transformed for statistical analyses. The differences between the three groups were assessed by one-way ANOVA for continuous variables and by χ^2 test for non-continuous variables. Linear mixed model was applied to test the differences between the groups to adjust for confounding factors. Pedigree membership was included into the model as a random factor and BMI, smoking and medication for hypertension as a fixed factor. Correlations were calculated by Spearman correlation analysis.

3. Results

Among 129 offspring included in the study, 109 subjects had normal glucose tolerance (NGT) and 20 impaired glucose tolerance (IGT) as assessed by an OGTT. The groups were comparable with respect to gender, but subjects with IGT were older (control: 34.0 ± 4.5; NGT: 35.0 ± 6.1; IGT: 38.6 ± 6.6 years, $p < 0.05$), and had higher BMI (control: 24.6 ± 2.6; NGT: 25.8 ± 4.3; IGT: 28.0 ± 6.2 kg/m^2, $p < 0.05$) than control subjects. Plasma glucose (control: 5.6 ± 1.1; NGT: 5.8 ± 1.0; IGT: 8.7 ± 0.8 mmol/l, $p < 0.001$) and insulin levels at 120 min (control: 194.0 ± 107.2; NGT: 219.2 ± 159.4; IGT: 400.1 ± 261.7 pmol/l, $p < 0.001$) were significantly elevated in the IGT group compared to those in the control group.

Table 1
Spearman correlations between the levels of fasting adhesion molecules

	VCAM	ICAM	E-Selectin	VAP-1
Control				
sVCAM-1	1	0.268	−0.023	0.141
sICAM-1		1	−0.112	−0.273
sE-Selectin			1	−0.211
sVAP-1				1
NGT				
sVCAM-1	1	0.130	0.056	0.053
sICAM-1		1	0.168	0.132
sE-Selectin			1	0.029
sVAP-1				1
IGT				
sVCAM-1	1	0.030	0.341	−0.120
sICAM-1		1	−0.046	0.064
sE-Selectin			1	0.214
sVAP-1				1

Fig. 1 presents the levels of fasting adhesion molecules by glucose tolerance status. The fasting levels of VCAM-1, ICAM-1, E-Selectin and VAP-1 did not differ significantly among the three groups. The subjects with IGT tended to have higher E-Selectin levels than control subjects (63.4 ± 6.8 ng/ml versus 46.6 ± 5.1 ng/ml, $p = 0.052$).

Table 1 gives correlations among fasting adhesion molecules. No statistically significant correlations among fasting adhesion molecule levels were found in any of the three groups. Significant correlations of VCAM-1 with TNF-α (0.482, $p < 0.05$) and the rates of whole body glucose uptake (WBGU) were found in control subjects (0.732, $p < 0.01$). Inverse correlation was observed between VCAM-1 level and fasting glucose (−0.549, $p < 0.05$), 120 min glucose (−0.528, $p < 0.05$) and visceral fat (−0.639, $p < 0.01$). ICAM-1 correlated significantly with IL-1Ra (0.526, $p < 0.05$), BMI (0.633, $p < 0.01$) and subcutaneous fat (0.695, $p < 0.01$). The only significant correlation of E-Selectin was observed between the first-phase insulin secretion (−0.586, $p < 0.01$). VAP-1 correlated inversely with levels of 120 min plasma glucose (−0.461, $p < 0.05$).

Table 2 shows correlations of fasting cytokines, clinical and metabolic parameters with adhesion molecules in normoglycemic offspring of type 2 diabetic patients. VCAM-1 correlated significantly with TNF-α (0.214, $p < 0.05$) and IL-1β (0.224, $p < 0.05$), whereas ICAM correlated with CRP (0.271, $p < 0.01$) and IL-6 (0.292, $p < 0.01$). E-Selectin correlated significantly with IL-8 (0.282, $p < 0.01$) and IL-18 (0.268, $p < 0.01$), and VAP-1 with IL-18 (0.222, $p < 0.05$). Among the metabolic parameters, VCAM-1 correlated with 120 min plasma glucose (0.218, $p < 0.05$), fasting plasma insulin (0.229, $p < 0.05$) and 120 min plasma insulin (0.227, $p < 0.05$). ICAM-1 correlated significantly with BMI (0.278, $p < 0.01$), fat percent (0.237, $p < 0.05$), visceral fat (0.243, $p < 0.05$) and subcutaneous fat (0.212, $p < 0.05$), and with fasting plasma insulin (0.220, $p < 0.05$). A significant correlation between E-Selectin and fasting plasma glucose (0.307, $p < 0.01$), and visceral fat (0.281, $p < 0.01$) was observed.

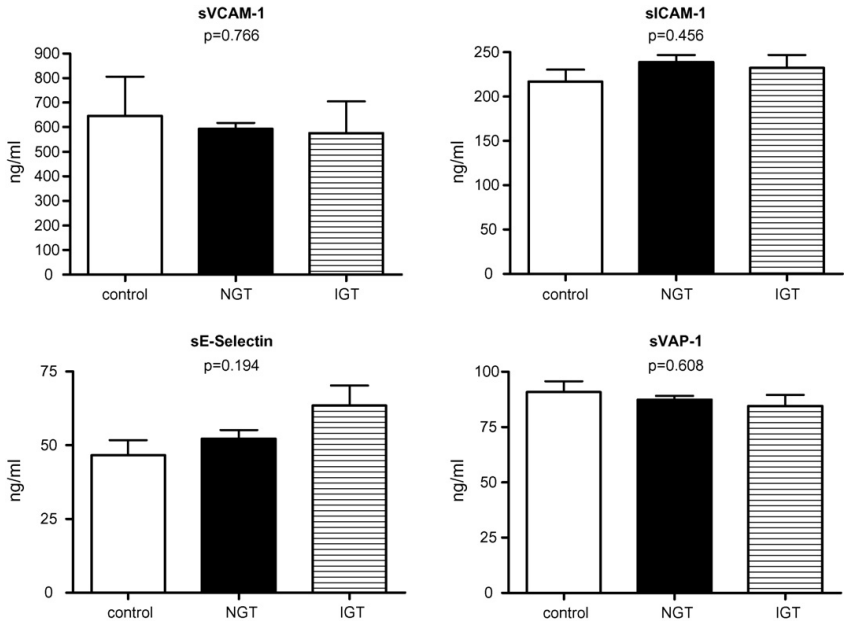

Fig. 1. The levels of fasting adhesion molecules by glucose tolerance status. *p*-Value after adjustment for sex, BMI, smoking, antihypertensive medication and family relationship (mixed linear model). Data are mean ± S.E.M.

Table 2
Spearman correlations among fasting cytokines, clinical and metabolic parameters with adhesion molecules in normoglycemic offspring of type 2 diabetic patients

	s-VCAM-1	s-ICAM-1	sE-Selectin	s-VAP-1
CRP	0.121	0.271**	−0.037	−0.163
TNF-α	0.214*	0.016	−0.018	0.043
IL-6	0.054	0.292**	−0.035	−0.073
IL-1β	0.224*	0.023	0.128	−0.062
IL-1Ra	0.049	0.048	0.095	0.028
IL-8	−0.022	−0.026	0.282**	−0.071
IL-10	0.180	0.093	0.173	0.088
IL-18	−0.046	0.130	0.268**	0.222*
RRs	−0.007	−0.031	0.005	0.078
RRd	0.044	−0.038	0.024	−0.063
BMI	0.022	0.278**	0.047	0.039
Fasting plasma glucose	0.074	0.095	0.307**	0.045
120 min plasma glucose	0.218*	0.101	0.096	−0.007
Fasting plasma insulin	0.229*	0.220*	0.129	0.081
120 min plasma insulin	0.227*	0.168	−0.023	0.062
LDL cholesterol	−0.193*	0.076	0.221*	0.021
HDL cholesterol	−0.103	−0.186	−0.130	−0.139
Total triglycerides	−0.095	0.192*	0.158	−0.004
Visceral fat	−0.013	0.243*	0.281**	0.106
Subcutaneous fat	0.114	0.212*	−0.013	0.012
First-phase insulin secretion	0.053	0.190	−0.036	0.077
Whole body glucose uptake 40 mU/L clamp/LBM	−0.134	0.176	−0.191*	0.128

* $p < 0.05$.
** $p < 0.01$.

E-Selectin correlated inversely with the rates of WBGU (−0.191, $p < 0.05$). VAP-1 did not have any significant correlations with metabolic or inflammatory factors in the NGT group.

Table 3 shows that among offspring with IGT, ICAM-1 correlated with IL-6 (0.605, $p < 0.01$), and E-Selectin with IL-1β (0.565, $p < 0.01$). ICAM-1 correlated also significantly with BMI (0.565, $p < 0.01$), subcutaneous fat (0.570, $p < 0.05$), fasting insulin (0.652, $p < 0.01$), and 120 min insulin (0.620, $p < 0.01$), and inversely with the rates of whole body glucose uptake (−0.569, $p < 0.01$). VCAM-1 and VAP-1 did not correlate significantly with cytokines or metabolic parameters in the IGT group.

4. Discussion

In our study the levels of adhesion molecules were not increased in offspring of type 2 diabetic subjects with varying glucose tolerance compared to those of control subjects, but they correlated with inflammatory markers. Because low-grade inflammation is an early finding in the offspring of type 2 diabetic subjects [22] it might be the primary abnormality preceding the increase of endothelial biomarkers in individuals at high risk of type 2 diabetes. The levels of VAP-1 were not increased in offspring of type 2 diabetic subjects, and they were not systematically associated with metabolic or inflammatory factors.

Endothelial dysfunction has been shown to precede the development of type 2 diabetes [15–18]. In agreement with this notion endothelial dysfunction has been consistently reported in offspring of type 2 diabetic subjects. In contrast, the results with respect to adhesion molecule levels have been conflicting. In two relative small studies on offspring of type 2 diabetic patients (number of subjects 19 and 14, respectively), the levels of ICAM-1 were elevated or had a trend towards higher levels of ICAM-1 [16,17]. VCAM-1 was increased in the offspring of type 2 diabetic patients ($n = 39$) in a study by Caballero et al. [15], whereas ICAM-1 was increased in subjects with IGT or type 2 diabetes. The levels of E-Selectin were increased in type 2 diabetic patients, but not in the offspring of type 2 diabetic subjects ($n = 60$) [19]. We measured the levels of endothelial biomarkers in a large cohort of non-diabetic offspring of type 2 diabetic subjects ($n = 129$) and found no increase in adhesion molecule levels. The reasons for these opposite findings are unclear, but they could be related to different protocols and small sample sizes of previous studies. Because we did not measure endothelial function using non-invasive methods, it is impossible to conclude whether or not the levels of adhesion molecules are reflecting endothelial activation in our study.

We found a significant correlation between ICAM-1 and BMI in all three study groups. This finding agrees with previous reports in healthy men [25], in obese individuals [26] and in type 2 diabetic patients [27]. We also demonstrated a significant correlation of ICAM-1 with subcutaneous fat, which together with an association of ICAM-1 with BMI supports the notion that overall adiposity could contribute to high levels of ICAM-1 [27]. This agrees with the findings on transgenic obese mice. These mice produced 10-fold higher soluble ICAM-1 than did wild type mice, and were more susceptible to weight gain and fat accumulation [28]. Adipose tissue may be a possible source of ICAM-1 [29]. Interestingly, in our study, ICAM-1 was associated with visceral fat in the NGT group, but not in the IGT group. The probands of the IGT group had higher BMI and larger amount of visceral and subcutaneous fat than probands in the NGT group. The mechanisms how adhesion molecules and adiposity are linked remain to be determined. Adipose tissue is a source of pro-inflammatory cytokines [30] and adhesion molecules are released in response to cytokines. Therefore, endothelial dysfunction may be a consequence of adiposity-induced chronic inflammation.

In young first-degree relatives of type 2 diabetic individuals, an association of non-invasively measured endothelial dysfunction and clamp-derived insulin resistance has been demonstrated [18]. Moreover, a significant association between the degree of insulin resistance and increased levels of E-Selectin, ICAM-1 and VCAM-1 has been observed in healthy, non-diabetic subjects suggesting that an increase in circulating adhesion molecules is secondary to insulin resistance [8]. In type 2 diabetic subjects, E-Selectin has been associated with insulin resistance, but results with respect to ICAM-1 and VCAM-1 have been conflicting [29]. In the present study, E-Selectin correlated inversely with the rates of WBGU in normoglycemic offspring, and ICAM-1 in offspring of type 2 diabetic patients having IGT. ICAM-1 was also associated with fasting and 120 min plasma insulin levels in the IGT group. None of the adhesion molecules was associated with the first-phase insulin secretion in offspring with NGT or IGT. Our results not only confirm previous findings that adhesion molecules and insulin resistance are linked, but also extend these data to individuals at high risk of diabetes and CVD.

A strong association between markers of endothelial dysfunction and inflammatory activity has been demonstrated [22]. This is not surprising given the fact that endothelial dysfunction is induced by inflammatory cytokines. Moreover, endothelial dysfunction itself may increase inflammatory activity, thus generating a vicious circle. Also in our study inflammatory markers were associated with elevated levels of adhesion molecules in the offspring of type 2 diabetic subjects. Low-grade inflammation is an early finding in the offspring of type 2 diabetic subjects, and in the present study we showed that inflammatory markers and adhesion molecules are related. Therefore, we suggest that low-grade inflammation may be the primary abnormality preceding the elevation of levels of adhesion molecules in the prediabetic state.

This study is the first to report the levels of VAP-1 in the offspring of type 2 diabetic subjects. VAP-1 is an ectoenzyme with adhesive and enzymatic properties. Ele-

Table 3
Spearman correlations among fasting cytokines, clinical and metabolic parameters with adhesion molecules in offspring of type 2 diabetic patients having impaired glucose tolerance

	s-VCAM-1	s-ICAM-1	sE-Selectin	s-VAP-1
CRP	−0.083	0.351	0.024	−0.138
TNF-α	0.101	−0.127	0.438	0.245
IL-6	−0.199	0.605**	−0.329	0.042
IL-1β	0.214	0.153	0.565**	0.187
IL-1Ra	0.087	0.343	0.086	−0.077
IL-8	0.033	−0.152	0.288	−0.295
IL-10	−0.031	−0.297	0.316	−0.182
IL-18	0.229	−0.087	0.405	0.071
RRs	0.073	−0.135	0.471*	−0.239
RRd	−0.160	−0.063	0.305	−0.172
Fat percent	−0.062	0.416	0.044	0.110
BMI	−0.068	0.565**	0.141	0.018
Fasting plasma glucose	0.194	−0.036	0.348	0.010
120 min plasma glucose	0.022	0.202	−0.216	−0.249
Fasting plasma insulin	0.080	0.652**	0.194	−0.028
120 min plasma insulin	−0.096	0.620**	0.092	−0.106
LDL cholesterol	−0.138	0.019	−0.152	0.117
HDL cholesterol	0.263	−0.508*	0.154	0.010
Total triglycerides	−0.052	0.133	−0.022	−0.365
Visceral fat	−0.125	0.419	0.010	−0.018
Subcutaneous fat	−0.151	0.570*	0.041	−0.144
First-phase insulin secretion	−0.325	0.246	−0.133	−0.180
Whole body glucose uptake 40 mU/L clamp/LBM	0.296	−0.569**	−0.177	0.121

* $p < 0.05$.
** $p < 0.01$.

vated levels of VAP-1 have been observed in alcoholic liver cirrhosis, infectious hepatitis, primary biliary cirrhosis, liver adenocarcinoma, and in type 1 diabetes and VAP-1 seems to be a more specific marker of certain types of inflammation than are other endothelial adhesion molecules [13]. Moreover, transgenic mice overexpressing human VAP-1 on endothelium have increased BMI and subcutaneous abdominal fat pad weights that are independent of food consumption. The increased SSAO (semicarbazide-sensitive amine oxidase) activity also leads to diabetes-like complications, including advanced glycation end product formation, elevated blood pressure, altered atherosclerosis progression, and nephropathy [14]. In the present study, the levels of VAP-1 were not elevated in the offspring of type 2 diabetic subjects. Furthermore, the only significant correlation was observed between IL-18 and VAP-1 in normoglycemic offspring of type 2 diabetic subjects. These findings together with the published information regarding diabetic individuals suggest that elevated serum concentrations of VAP-1 can only be detected when inflammation process is more advanced than in offspring of type 2 diabetic subjects. Thus, on the basis of our results, VAP-1 levels do not reflect endothelial dysfunction in offspring of type 2 diabetic subjects.

In conclusion, our findings indicate that the levels of adhesion molecules are not increased in the offspring of type 2 diabetic subjects. Thus, they are not the best markers of endothelial dysfunction in the prediabetic state. Inflammatory markers and adhesion molecules were correlated suggesting that low-grade inflammation may precede the elevation levels of adhesion molecules.

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IV

Changes in Cytokine Levels During Acute Hyperinsulinemia in Offspring of Type 2 Diabetic Subjects

Eija Ruotsalainen, MD[a], Ilkka Vauhkonen, MD, PhD[a], Urpu Salmenniemi, MD, PhD[b], Jussi Pihlajamäki, MD, PhD[a], Kari Punnonen, MD, PhD[c], Markku Laakso, MD, PhD[a]

[a] University of Kuopio, Department of Medicine, Kuopio, Finland
[b] University of Turku, Department of Medicine, Turku, Finland
[c] University of Kuopio, Department of Clinical Chemistry, Kuopio, Finland and Eastern Finland Laboratory Centre (ISLAB)

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Correspondence:

Eija Ruotsalainen, MD
Consultant Physician
Department of Medicine
University of Kuopio
70210 Kuopio, Finland
phone: +358-17-173311
fax: +358-173931
e-mail: Eija.Ruotsalainen@kuh.fi

Abstract

Objective To investigate the changes in the levels of cytokines and adhesion molecules in response to acute hyperinsulinemia in the offspring of type 2 diabetic subjects.

Methods Forty healthy offspring of type 2 diabetic subjects and 19 healthy controls were included in the study. Twenty offspring had normal glucose tolerance (NGT) and twenty offspring impaired glucose tolerance (IGT). Insulin sensitivity was determined by the hyperinsulinemic euglycemic clamp and insulin secretion with the intravenous glucose tolerance test. The levels of cytokines and adhesion molecules were measured before and at the end of the clamp.

Results Acute hyperinsulinemia induced by the euglycemic hyperinsulinemic clamp reduced the levels of TNF-α, IL-8, IL-10 and IL-18 in healthy controls but not in the offspring of type 2 diabetic subjects having NGT or IGT. In response to insulin, levels of hs-CRP decreased and levels of IL-6 increased significantly in all study groups. The levels of adhesion molecules (ICAM-1, VCAM-1, E-Selectin) remained unchanged in response to hyperinsulinemia.

Conclusions The suppression of cytokine levels (particularly proinflammatory cytokines) during acute hyperinsulinemia differs between offspring of type 2 diabetic patients and healthy controls. This emphasizes the crucial role of low-grade inflammation in insulin resistance in subjects with high risk of developing diabetes.

Key words: Offspring of type 2 diabetic patients, type 2 diabetes, cytokines, hyperinsulinemia

Introduction

Insulin resistance or hyperinsulinemia predicts the development of type 2 diabetes [1] and cardiovascular disease [2], independently of other risk factors. We have previously shown that offspring of type 2 diabetic patients are insulin resistant and characterized by increased levels of markers of low-grade inflammation [3]. In contrast, the levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-Selectin were not increased in the offspring of type 2 diabetic subjects [4].

Previous studies suggest that markers of low-grade inflammation are associated with insulin resistance [3,5]. However, these studies are based on samples drawn in the fasting state. To our knowledge, there is only one previous study reporting the effects of acute hyperinsulinemia on the levels of interleukin 8 (IL-8) [6], whereas the information on other cytokines remains unclear, although previous studies have addressed the changes in expression of genes regulating cytokines [7]. Furthermore, it is not clear whether hyperinsulinemia modulates the levels of adhesion molecules during acute hyperinsulinemia in the offspring of subjects with type 2 diabetes. Therefore, we performed detailed metabolic studies in healthy nondiabetic offspring of type 2 diabetic subjects and measured the levels of proinflammatory cytokines and adhesion molecules in the fasting state and during acute hyperinsulinemia induced by the hyperinsulinemic euglycemic clamp.

Methods

Forty healthy nondiabetic offspring of patients with type 2 diabetes (men/women 19/20, age 36.6 ± 6.6 years, body mass index [BMI] 28.1 ± 6.1 kg/m²) were included in the study. The control group consisted of 19 healthy normoglycemic subjects with no family history of type 2 diabetes (men / women 8/11, age 34 ± 4.5 years, BMI 24.6 ± 2.6 kg/m²). Probands (men or women) were randomly selected from type 2 diabetic subjects living in the region of the Kuopio University Hospital. The spouses of the patients with type 2 diabetes had NGT in an OGTT . The exclusion criteria for the selection of the offspring were: 1) diabetes mellitus or any other disease or drug treatment that could potentially disturb carbohydrate metabolism; 2) diabetes mellitus in both parents; 3) pregnancy; 4) age less than 25 or more than 50 years. The Ethics Committee of the University of Kuopio approved the study protocol. All study subjects gave informed consent.

Blood pressure was measured in the sitting position with a mercury sphygmomanometer after a 5 min rest. The average of 3 measurements was used to calculate systolic and diastolic blood pressure. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. BMI was calculated as weight in kilograms divided by height in meters squared. Waist (at the midpoint between the lateral iliac crest and the lowest rib) and hip (at the level of the trochanter major) circumferences were measured to the nearest 0.5 cm. Initial blood samples were drawn after 12 hours of fasting, and an oral glucose tolerance test (OGTT) using 75 g of glucose was performed**.** On the second day, after a 12-hour fast, an intravenous glucose tolerance test (IVGTT) and the hyperinsulinemic euglycemic clamp were performed.

An IVGTT was performed to determine the first-phase insulin release after an overnight fast. After baseline blood collection, a bolus of glucose (300 mg/kg in a 50 % solution) was infused over 30 seconds into the antecubital vein. Samples for the measurement of blood glucose and plasma insulin (arterialized venous blood) were drawn at –5, 0, 2, 4, 6, 8 and 10 min. Immediately after the IVGTT, the euglycemic hyperinsulinemic clamp (insulin infusion rate of 240 pmol/ min / m² body surface area) was started to determine the degree of insulin sensitivity, as previously described [8]. Blood glucose was clamped at 5.0 mmol/l for the next 120 minutes using an infusion of 20% glucose, with rate adjusted according to blood glucose measurements performed at 5-minute intervals. The mean amount of glucose infused during the final hour was used to calculate the rate of whole-body glucose uptake (WBGU). Blood samples for cytokine measurements were drawn before the clamp and at the end of the clamp.

Blood and plasma glucose levels were measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat PLUS, Yellow Springs Instruments Co, Inc), and the levels of plasma insulin by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Biotech, Uppsala, Sweden). Plasma concentrations of cytokines (tumor necrosis factor-α [TNF-α], IL-1β, IL-1 receptor antagonist [IL-1Ra], IL-6, IL-8, IL-10 and IL-18) were measured by solid phase ELISA (Quantikine, R&D Systems) and IL-8 by Ultrasensitive ELISA (BioSource International), as previously described [3]. The levels of C-reactive protein (hs-CRP) were determined by an Immulite 2000 High Sensitivity CRP assay (Diagnostic Products Corp). The levels of soluble adhesion molecules (s-ICAM-1, s-VCAM-1 and sE-Selectin) were measured by high-sensitivity assay kits from R&D Systems.

All data analyses were performed with the SPSS 14.0 for Windows programs. The results for continuous variables are given as means ± SD, if not stated otherwise. Variables with skewed distributions (hs-CRP, TNF-α, IL-1Ra, IL-6, IL-8 and IL-10) were logarithmically transformed for statistical analyses. The differences between the three groups were assessed by one-way ANOVA for continuous variables and by the χ^2 test for noncontinuous variables. A linear mixed model was applied to test the differences between the groups to adjust for confounding factors. Pedigree membership was included into the model as a random factor**,** and BMI and smoking as cofactors. Correlations were calculated by Spearman correlation analysis.

Results

Clinical and biochemical characteristics of the offspring of type 2 diabetic patients and control subjects are given in Table 1. Twenty subjects had normal glucose tolerance (NGT) and 20 impaired glucose tolerance (IGT), as assessed by an OGTT. The groups were comparable with respect to gender, but differed significantly with respect to age (Control 34.5 ± 4.5, NGT 34.6 ± 6.1, IGT 38.6 ± 6.6 years, $P< 0.05$), and tended to differ with respect to BMI (24.6 ± 2.6, 28.2 ± 6.1, 28.0 ± 6.2 kg/m², $P=0.064$). In addition**,** the offspring in the NGT and IGT groups were markedly insulin resistant**,** with significantly higher plasma insulin levels at 120 min in the OGTT. The NGT and IGT groups also had higher systolic blood pressure levels than did the control group (Control 124 ± 10, NGT

133 ± 13, IGT 133 ± 18 mmHg, P< 0.05). No difference in the first-phase insulin release between the study groups was found.

Spearman correlations among the cytokine levels in the fasting state and during hyperinsulinemia are shown in Table 2. The NGT and IGT groups were pooled together in statistical analysis. Fasting cytokine levels correlated positively with the levels of cytokines during hyperinsulinemia. Furthermore, hs-CRP levels showed a positive correlation with the levels of IL-6 and IL-1Ra, both in the fasting state and during hyperinsulinemia**.** Similarly, a positive correlation was found between the levels of IL-1Ra and IL-18, both in the fasting state (r= 0.330, P< 0.05) and during hyperinsulinemia (r = 0.760, P<0.01). TNF-α levels were associated with IL-1Ra (r= 0.321, P<0.05) and IL-8 (r=0.351, P<0.05) levels only during hyperinsulinemia.

Correlations between the adhesion molecule levels of the NGT and IGT groups are shown in Table 3. Fasting sICAM-1 (r=0.778, P< 0.01), sVCAM-1 (r= 0.558, P< 0.01) and sE-Selectin (r= 0.703, P<0.01) levels showed positive correlations with the respective adhesion molecule levels during hyperinsulinemia. Fasting s ICAM-1 levels were also positively correlated with sVCAM-1 levels during hyperinsulinemia.

The changes in cytokine levels between the fasting state and hyperinsulinemia are shown in Figure 1. Levels of hs-CRP decreased significantly during hyperinsulinemia compared to the levels in the fasting state in all study groups (P<0.001). In contrast, levels of IL-6 increased significantly during hyperinsulinemia (P<0.001) in all groups**,** and the increase was similar in the NGT and IGT groups to that in the control group (P=0.294). Fasting and hyperinsulinemic levels of IL-1ß or IL-1Ra did not differ in any of the groups. TNF-α and IL-

8 levels decreased significantly (P<0.05) in the control group during hyperinsulinemia, but remained unchanged in the offspring with NGT or IGT. Hyperinsulinemia also decreased significantly the levels of IL-10 and IL-18 in the control group (P<0.05 and P<0.001, respectively), but not in the NGT and IGT groups. To investigate the effect of obesity on our results we performed further statistical analyses between non-obese and obese (cut-off point of BMI of 27.0 kg/ m²) subjects in all study groups. As shown in Supplemental Table 1 changes in cytokine levels did not systematically differ between non-obese and obese subjects in any glucose tolerance category giving further evidence that obesity can not explain our findings. Insulin did not have significant effects on the levels of any of the adhesion molecules in any group (Figure 2).

Discussion

We showed for the first time that acute hyperinsulinemia induced by the euglycemic hyperinsulinemic clamp lowers significantly the levels of TNF-α, IL-8, IL-10 and IL-18 in healthy control subjects. However, in both normoglycemic and glucose intolerant offspring of type 2 diabetic subjects hyperinsulinemia was unable to suppress cytokine levels. Therefore, we suggest that the offspring of type 2 diabetic patients are insulin resistant not only with regard to glucose metabolism, but also with regard to the inhibition of cytokine responses during hyperinsulinemia.

Previous studies have shown that hs-CRP is elevated in insulin resistant states [9]. In agreement with these studies, we showed that fasting levels of hs-CRP were elevated in insulin resistant offspring of type 2 diabetic patients and correlated strongly with hs-CRP

levels during hyperinsulinemia. CRP was believed to be synthesized only in the liver in response to IL-6 [10]. However, recent studies have shown that also adipose tissue (both adipocytes and stromal cells) can produce CRP [11]. We observed a similar statistically significant decrease in hs-CRP levels in response to hyperinsulinemia in all groups. Therefore, we suggest that in the offspring of type 2 diabetic subjects insulin resistance is not characterized by an impaired hs-CRP response to insulin.

Adipose tissue is a major source of TNF-α, IL-8, IL-10 and IL-18 [12-14]. IL-8 and IL-18 are proinflammatory cytokines that are associated with insulin resistance and type 2 diabetes [12,15], whereas IL-10 is an anti-inflammatory cytokine produced by macrophages and lymphocytes. IL-10 level was decreased during hyperinsulinemia in control subjects, but not in the offspring of type 2 diabetic patients. High TNF-α level stimulates IL-10 production[14] Therefore, reduction in TNF-α level leads to a reduction in IL-10 level observed in control subjects, whereas in offspring of type 2 diabetic patients no statistically significant reduction in IL-10 level was observed due to impaired suppression of TNF-α response during hyperinsulinemia. The effect of TNF-α on IL-10 levels is likely to explain the high correlation between these cytokines both in the fasting state and during hyperinsulinemia. We found that in normal subjects hyperinsulinemia reduced the levels of IL-8 and IL-18, whereas in the offspring of type 2 diabetic subjects this reduction was not found. Therefore, our results suggest that insulin resistance in adipose tissue manifests itself as impaired suppression of the cytokine responses to hyperinsulinemia. Previously Straczkowski et al [6] reported that acute hyperinsulinemia increased the levels of IL-8 similarly in subjects with NGT and IGT. We do not have an obvious explanation for these conflicting findings.

IL-6 is a pleiotropic cytokine that is produced and released by macrophages, adipocytes and skeletal muscle cells [16]. Only 30 % of total circulating IL-6 originates from adipose tissue in healthy subjects [17]. Thus, it differs from other cytokines investigated in our study although also contradictory finding have been published [5]. IL-6 stimulates the production of acute-phase proteins in the liver. Circulating IL-6 levels are elevated in type 2 diabetes [18] and in the presence of insulin resistance [19], although the role of IL-6 in the development of insulin resistance has remained controversial [20]. We observed a statistically significant increase in IL-6 levels during hyperinsulinemia in all groups which may indicate that high insulin level induces the production of IL-6 independently of the degree of insulin resistance.

In our study the levels of adhesion molecules were not significantly altered by acute hyperinsulinemia in any of the groups. sVCAM-1 level during hyperinsulinemia decreased in controls, but the change was not statistically significant. Previous reports regarding the effect of hyperinsulinemia on adhesion molecule levels are contradictory. In two studies insulin increased the levels of sE-Selectin in IGT subjects and in type 2 diabetic subjects, whereas the levels of sICAM-1 and sVCAM-1 remained unchanged [21,22]. High insulin has been shown to increase the expression of ICAM-1 potentially leading to vascular injury [23]. On the other hand, insulin activates endothelial nitric oxide synthesis [24], which inhibits the expression of adhesion molecules [25]. Our findings are in line with previous studies that the levels of sICAM-1, sVCAM-1 and sE-Selectin remained unaffected during hyperinsulinemia, and extend the data to healthy individuals who are genetically predisposed to develop type 2 diabetes.

In conclusion, we demonstrate for the first time that the offspring of type 2 diabetic subjects are not only insulin resistant with regard to glucose metabolism but also with regard to the suppression of cytokine responses. This emphasizes the crucial role of low-grade inflammation in insulin resistance in subjects with high risk of developing diabetes. However, the role of impaired insulin secretion can not be entirely excluded given the fact that the offspring of type 2 diabetic subjects was not able to compensate their first-phase insulin release in our study. The disturbed cytokine response was especially linked with fat-derived cytokines highlighting the crucial role of adipose tissue in this process.

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Table 1

Characteristics of the study groups

		Offspring of patients with type 2 diabetes		
	Control n=19	NGT n=20	IGT n=20	P
Gender (M/F)	8/11	11/9	8/12	0.603
Age (years)	34.5 ± 4.5	34.6 ± 6.1	38.6 ± 6.6*	0.048
Body mass index (kg/m2)	24.6 ± 2.6	28.2 ± 6.1	28.0 ± 6.2	0.064
Waist to Hip ratio	0.84 ± 0.1	0.90 ± 0.10	0.88 ± 0.08	0.062
Systolic blood pressure (mmHg)	124 ± 10	133 ± 13	133 ± 18	0.060
Diastolic blood pressure (mmHg)	82 ± 10	87 ± 9	90 ± 14	0.070
Fasting plasma glucose (mmol/L)	5.1 ± 0.6	5.3 ± 0.4	5.2± 0.5	0.486
120min plasma glucose (mmol/L)	5.6 ± 1.1	6.4 ± 0.7**	8.7 ± 0.8***	<0.001
Fasting plasma insulin (pmol/L)	46.9 ± 23.0	52.4± 24.5	57.9 ± 34.4	0.474
120 min plasma insulin (pmol/l)	106.98 ± 24.6	238.92 ± 53.4*	261.72 ± 58.5**	0.014
WBGU (µmol/kg/min)	70.1 ± 27.9	50.1 ± 15.6*	45.9 ± 11.1**	0.001
First-phase insulin release (pmol/lxmin)	1897.0 ± 1207.4	2636.6 ± 2339.0	1830.5 ± 1273.2	0.338

Data are mean ± SD. NGT/IGT vs controls *P < 0.05, **P < 0.01, ***P < 0.001 (P value was calculated if P value for ANOVA was < 0.05). NGT = normal glucose tolerance, IGT = impaired glucose tolerance, WBGU = rates of whole body glucose uptake

Table 2

Spearman correlations between the levels of cytokines in the fasting state and during the hyperinsulinemic euglycemic clamp in offspring of type 2 diabetic subjects. Offspring with normal and impaired glucose tolerance were pooled in statistical analyses.

Fasting state

	hs-CRP	TNF-α	IL-6	IL-8	IL-1β	IL-1Ra	IL-10	IL-18
hs-CRP	1	-0.055	0.473**	0.259	-0.039	0.500**	-0.107	0.029
TNF-α		1	-0.029	0.293	0.172	0.006	0.475**	0.109
IL-6			1	-0.024	-0.035	0.288	-0.269	-0.076
IL-8				1	0.034	0.183	0.222	0.280
IL-1β					1	0.106	0.018	-0.093
IL-1Ra						1	0.149	0.330*
IL-10							1	0.285
IL-18								1

*p< 0.05, ** p <0.01

During the clamp

	hs-CRP	TNF-α	IL-6	IL-8	IL-1β	IL-1Ra	IL-10	IL-18
hs-CRP	1	-0.063	0.232	0.138	0.003	0.416**	-0.117	-0.012
TNF-α		1	0.038	0.351*	0.321*	0.073	0.644***	0.191
IL-6			1	0.349*	0.083	0.280	0.014	0.053
IL-8				1	0.338*	0.075	0.261	0.210
IL-1β					1	0.290	0.281	0.225
IL-1Ra						1	0.268	0.421**
IL-10							1	0.328
IL-18								1

*p <0.05, ** p <0.01, *** p< 0.001

Table 3

Spearman correlations between the levels of adhesion molecules in the fasting state and during the hyperinsulinemic euglycemic clamp in offspring of type 2 diabetic subjects. Offspring with normal and impaired glucose tolerance were pooled in statistical analyses.

	sVCAM-1 fasting	sVCAM-1 clamp	sICAM-1 fasting	sICAM-1 clamp	sE-Selectin fasting	sE-Selectin clamp
sVCAM-1 fasting	1	0.558**	0.201	0.076	0.017	-0.154
sVCAM-1 clamp		1	0.343**	0.189	-0.091	-0.132
sICAM-1 fasting			1	0.778**	0.031	-0.020
sICAM-1 clamp				1	0.141	0.098
sE-Selectin fasting					1	0.703**
sE-Selectin clamp						1

** $p<0.01$

Supplemental Table 1. Cytokine levels (ng/ml) in the fasting state and during hyperinsulinemia. BMI cut-off point of 27 kg/m2 for Non-obese and Obese subjects. Data are mean ± SD.

		Control	NGT	IGT	p
Fasting hs-CRP:	Non-Obese	1.10 ±1.60	2.83 ± 3.54	2.75 ± 3.43	0.257
	Obese	2.21 ± 2.82	2.00 ± 1.77	3.51 ± 3.15	0.365
Clamp hs-CRP:	Non-obese	1.01 ± 1.62	2.72 ± 3.46	2.44 ± 3.12	0.225
	Obese	2.14 ± 2.87	1.85 ± 1.66	3.28 ± 2.97	0.324
Fasting TNF-α:	Non-obese	3.41 ± 2.04	4.41 ± 2.78	5.12 ± 6.80	0.702
	Obese	2.38 ± 0.38	2.32 ± 0.26 a)	2.51 ± 0.51	0.610
Clamp TNF-α:	Non-obese	3.22 ± 1.90	4.57 ± 3.17	5.14 ± 7.07	0.559
	Obese	2.21 ± 0.56	2.40 ± 0.28	2.40 ± 0.64	0.755
Fasting IL-1-ß:	Non-obese	0.34 ± 0.19	0.65 ± 0.22	0.22 ± 0.15	0.000
	Obese	0.45 ± 0.87	0.67 ± 0.23	0.23 ± 0.09	0.000
Clamp IL-1-ß:	Non-obese	0.26 ± 0.16	0.67 ± 0.34	0.19 ± 0.12	0.000
	Obese	0.45 ± 0.17	0.67 ± 0.28	0.28 ± 0.18	0.004
Fasting IL-1Ra:	Non-obese	187.53 ± 63.86	358.56 ± 112.00	370.47 ± 342.53	0.003
	Obese	256.43 ± 92.43	345.01 ± 242.31	574.57 ± 380.02*	0.087
Clamp IL-1Ra:	Non-obese	182.47 ± 56.44	436.12 ± 251.47	350.38 ± 380.97	0.004
	Obese	259.10 ± 132.62	349.63 ± 208.05	630.32 ± 415.77* b)	0.047
Fasting IL-6:	Non-obese	0.86 ± 0.86	1.31 ± 0.64	0.82 ± 0.39	0.058
	Obese	1.53 ± 1.45	1.21 ± 0.82	1.68 ± 0.66**c)	0.235
Clamp IL-6 clamp:	Non-obese	2.57 ± 1.65	3.63 ± 2.85	4.11± 3.94	0.338
	Obese	1.82 ± 0.99	3.24 ± 1.99	4.20 ± 2.17	0.112
Fasting IL-8:	Non-obese	0.65 ± 0.27	1.18 ± 1.29	1.06 ± 1.09	0.951
	Obese	0.79 ± 0.40	0.76 ± 0.64	0.82 ± 0.54	0.692
Clamp IL-8:	Non-obese	0.53 ± 0.27	1,46 ± 1.40	0.93 ± 0.48	0.249
	Obese	0.58 ± 0.33	0.60 ± 0.38	1.18 ± 1.46	0.282
Fasting IL-10:	Non-obese	1.04 ± 0.61	1.47 ± 1.57	1.51 ± 1.66	0.825
	Obese	1.06 ± 0.55	0.98 ± 0.82	1.05 ± 0.86	0.929
Clamp IL-10:	Non-obese	0.97 ± 0.60	1.28 ± 1.23	1.39 ± 1.83	0.963
	Obese	0.79 ± 0.41	1.02 ± 0.88	0.97 ± 0.76	0.907
Fasting IL-18:	Non-obese	259.76 ± 89.14	224.03 ± 94.29	243.20 ± 0.67	0.602
	Obese	278.43 ± 122.85	213.05 ± 50.54	298.93 ± 109.46	0.099
Clamp IL -18:	Non-obese	234.63 ± 97.90	204.86 ± 75.32	201.66 ± 75.12	0.568
	Obese	246.20 ± 112.24	194.55 ± 51.95	292.73 ± 106.75*d)	0.051

*P<0.05, compared to Control subjects within the same obesity status (non-obese or obese)

a) P= 0.040, b) P= 0.028, c) P= 0.001, d) P=0.042 comparing non-obese vs. obese subjects within the same glucose tolerance group

Figure 1

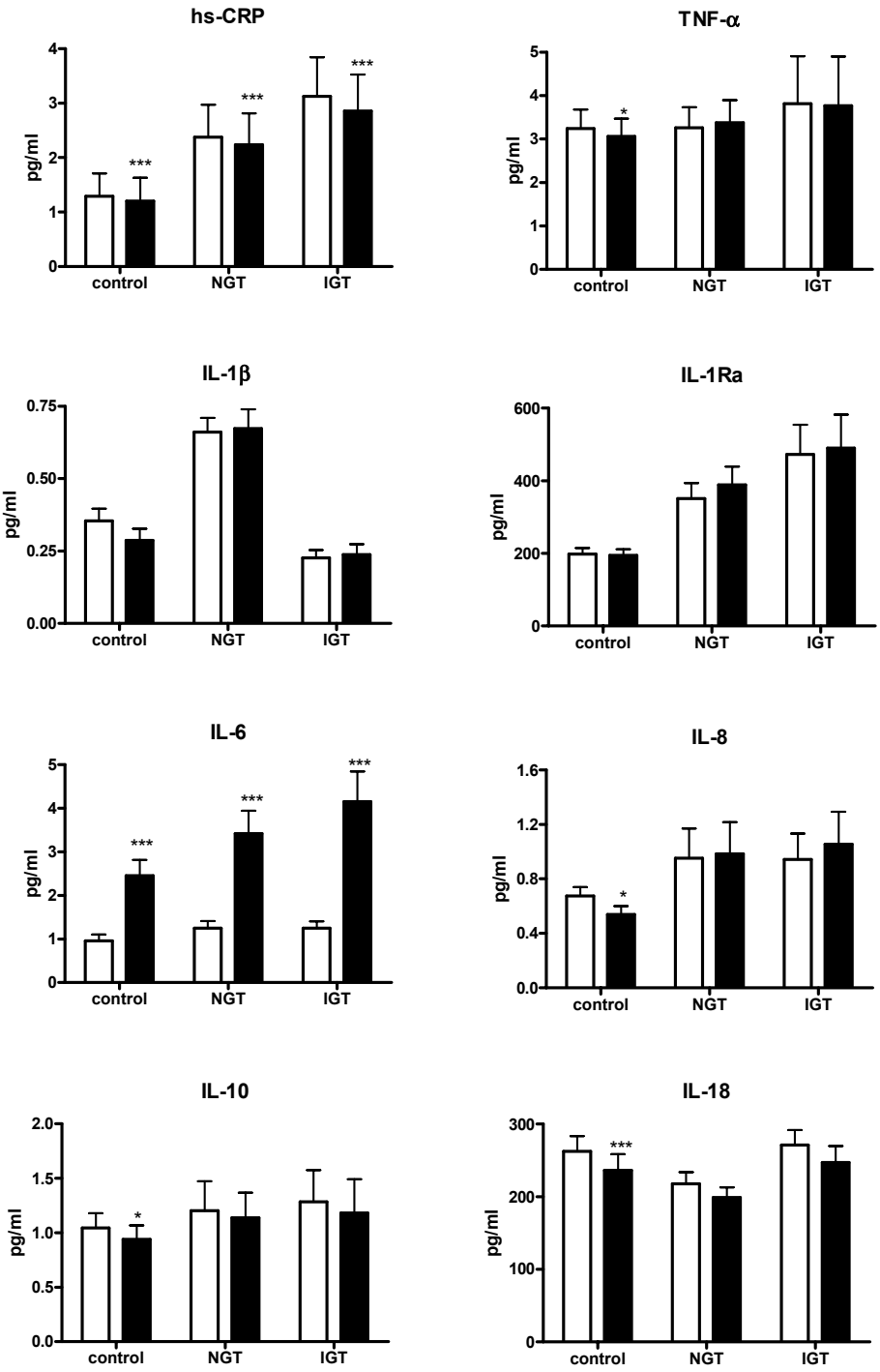

Figure 2

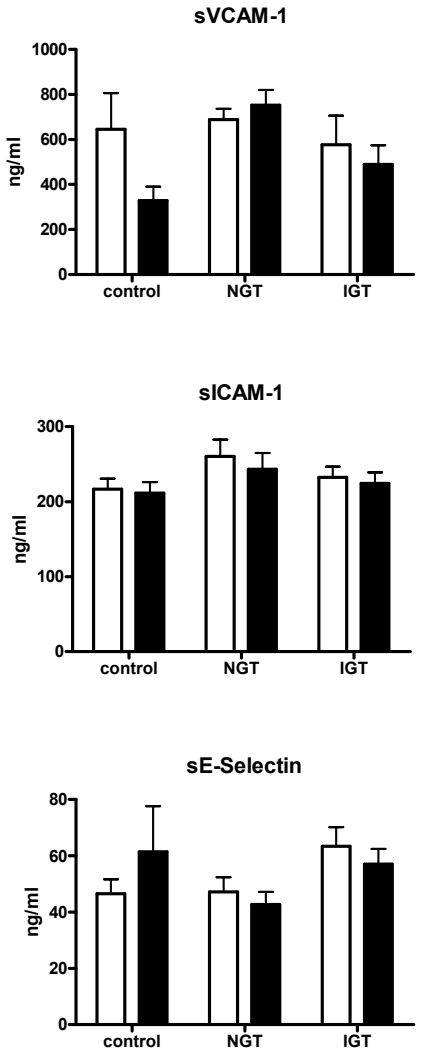
sVCAM-1
1000
800
600
400
200
0
ng/ml
control
NGT
IGT
sICAM-1
300
200
100
0
ng/ml
control
NGT
IGT
sE-Selectin
80
60
40
20
0
ng/ml
control
NGT
IGT

Figure legends

Figure 1. Changes in cytokine levels during the hyperinsulinemic euglycemic clamp. White bars = fasting levels of cytokines in control, NGT and IGT groups. Black bars = levels of cytokines during the hyperinsulinemic euglycemic clamp in the control, NGT and IGT groups. P-value after the adjustment for body mass index, smoking and family relationship (mixed model). Mean ± SEM. *P < 0.05, *** P < 0.001.

Figure 2. Changes in adhesion molecules during hyperinsulinemic euglycemic clamp. White bars = fasting levels of adhesion molecules in the control, NGT and IGT groups. Black bars = levels of adhesion molecules during the hyperinsulinemic euglycemic clamp in the control, NGT and IGT groups. Mean ± SEM.

Supplemental Table 1. Cytokine levels (pg/ml) as actual concentrations before stratification (shaded rows), in the fasting state and during hyperinsulinemia. BMI cut-off point of 27 kg/m^2 for Non-obese and Obese subjects. Data are mean ± SD.

		Control n (before stratification) = 19 n (non-obese) = 15 n (obese) = 4	**NGT** n (before stratification) = 20 n (non-obese)= 9 n (obese) = 11	**IGT** n (before stratification)= 20 n (non-obese)= 10 n (obese) = 10	P
Fasting hs-CRP before stratification		1.29 ± 1.79	2.37 ± 2.67 (n=20)	3.12 ± 3.27* (n=20)	0.112
Fasting hs-CRP:	Non-Obese	1.10 ±1.60	2.83 ± 3.54 (n=9)	2.75 ± 3.43 (n=10)	0.257
	Obese	2.21 ± 2.82	2.00 ± 1.77 (n=11)	3.51 ± 3.15 (n=10)	0.365
Clamp hs-CRP before stratification		1.20 ± 1.82	2.24 ± 2.57	2.86 ± 3.0*	0.139
Clamp hs-CRP:	Non-obese	1.01 ± 1.62	2.72 ± 3.46	2.44 ± 3.12	0.225
	Obese	2.14 ± 2.87	1.85 ± 1.66	3.28 ± 2.97	0.324
Fasting TNF-α before stratification		3.24 ± 1.91	3.26 ± 2.11	3.82 ± 4.88	0.822
Fasting TNF-α:	Non-obese	3.41 ± 2.04	4.41 ± 2.78	5.12 ± 6.80	0.702
	Obese	2.38 ± 0.38	2.32 ± 0.26 a)	2.51 ± 0.51	0.610
Clamp TNF-α before stratification		3.06 ± 1.78	3.38 ± 2.35	3.76 ± 5.08	0.812
Clamp TNF-α:	Non-obese	3.22 ± 1.90	4.57 ± 3.17	5.14 ± 7.07	0.559
	Obese	2.21 ± 0.56	2.40 ± 0.28	2.40 ± 0.64	0.755
Fasting IL-1ß before stratification		0.35 ± 0.18	0.66 ± 0.22***	0.23 ± 0.12*	0.000
Fasting IL-1-ß:	Non-obese	0.34 ± 0.19	0.65 ± 0.22	0.22 ± 0.15	0.000
	Obese	0.45 ± 0.87	0.67 ± 0.23	0.23 ± 0.09	0.000
Clamp IL-1ß before stratification		0.29 ± 0.18	0.67 ± 0.30***	0.24 ± 0.16	0.000
Clamp IL-1-ß:	Non-obese	0.26 ± 0.16	0.67 ± 0.34	0.19 ± 0.12	0.000
	Obese	0.45 ± 0.17	0.67 ± 0.28	0.28 ± 0.18	0.004
Fasting IL-1 Ra before stratification		198.41 ± 70.81	351.06 ± 190.34**	472.52 ± 367.35**	0.004
Fasting IL-1Ra:	Non-obese	187.53 ± 63.86	358.56 ± 112.00	370.47 ± 342.53	0.003
	Obese	256.43 ± 92.43	345.01 ± 242.31	574.57 ± 380.02*	0.087
Clamp IL-1Ra before stratification		194.57 ± 73.71	388.55 ± 226.62**	490.35 ± 413.83***	0.006
Clamp IL-1Ra:	Non-obese	182.47 ± 56.44	436.12 ± 251.47	350.38 ± 380.97	0.004
	Obese	259.10 ± 132.62	349.63 ± 208.05	630.32 ± 415.77* b)	0.047
Fasting IL-6 before stratification		0.96 ± 0.63	1.25 ± 0.73	1.25 ± 0.69	0.320
Fasting IL-6:	Non-obese	0.86 ± 0.86	1.31 ± 0.64	0.82 ± 0.39	0.058
	Obese	1.53 ± 1.45	1.21 ± 0.82	1.68 ± 0.66**c)	0.235
Clamp IL-6 before stratification		2.45 ± 1.57	3.42 ± 2.35	4.16 ± 3.10	0.101
Clamp IL-6 clamp:	Non-obese	2.57 ± 1.65	3.63 ± 2.85	4.11± 3.94	0.338
	Obese	1.82 ± 0.99	3.24 ± 1.99	4.20 ± 2.17	0.112
Fasting IL-8 before stratification		0.67 ± 0.28	0.95 ± 0.98	0.94 ± 0.85**	0.447
Fasting IL-8:	Non-obese	0.65 ± 0.27	1.18 ± 1.29	1.06 ± 1.09	0.951
	Obese	0.79 ± 0.40	0.76 ± 0.64	0.82 ± 0.54	0.692
Clamp IL-8 before stratification		0.54 ± 0.27	0.98 ± 1.04	1.06 ± 1.07	0.152
Clamp IL-8:	Non-obese	0.53 ± 0.27	1,46 ± 1.40	0.93 ± 0.48	0.249
	Obese	0.58 ± 0.33	0.60 ± 0.38	1.18 ± 1.46	0.282
Fasting IL-10 before stratification		1.04 ± 0.59	1.20 ± 1.21	1.28 ± 1.31	0.787
Fasting IL-10:	Non-obese	1.04 ± 0.61	1.47 ± 1.57	1.51 ± 1.66	0.825
	Obese	1.06 ± 0.55	0.98 ± 0.82	1.05 ± 0.86	0.929
Clamp IL-10 before stratification		0.94 ± 0.57	1.14 ± 1.03	1.18 ± 1.38	0.750
Clamp IL-10:	Non-obese	0.97 ± 0.60	1.28 ± 1.23	1.39 ± 1.83	0.963
	Obese	0.79 ± 0.41	1.02 ± 0.88	0.97 ± 0.76	0.907
Fasting IL-18 before stratification		262.71 ± 91.36	217.99 ± 71.55	271.06 ± 92.96	0.120
Fasting IL-18:	Non-obese	259.76 ± 89.14	224.03 ± 94.29	243.20 ± 0.67	0.602
	Obese	278.43 ± 122.85	213.05 ± 50.54	298.93 ± 109.46	0.099
Clamp IL-18 before stratification		236.45 ± 96.96	199.19 ± 61.94*	247.20 ± 101.27	0.207
Clamp IL -18:	Non-obese	234.63 ± 97.90	204.86 ± 75.32	201.66 ± 75.12	0.568
	Obese	246.20 ± 112.24	194.55 ± 51.95	292.73 ± 106.75*d)	0.051

*P<0.05, **P<0.01, ***P<0.001, compared to Control subjects within the same obesity status (non-obese or obese) or Control vs NGT/IGT before stratification

a) P= 0.040, b) P= 0.028, c) P= 0.001, d) P=0.042 comparing non-obese vs. obese subjects within the same glucose tolerance group