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URPU SALMENNIEMI

Metabolic and Genetic Abnormalities Clustering with Intra-abdominal Obesity

Doctoral dissertation

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ABSTRACT

Metabolic syndrome (MetS) is an important risk factor cluster that increases the risk of cardiovascular disease and type 2 diabetes (T2DM). An increase in the prevalence of obesity worldwide is associated with increased incidence of atherogenic dyslipidemia, elevated blood pressure and glucose intolerance, making the identification of subjects with the MetS important. Several definitions of the MetS have been presented. According to current understanding abdominal obesity and insulin resistance are the most probable causes of the syndrome. In the latest diagnostic criteria of the MetS presented by the International Diabetes Federation, abdominal obesity is considered prerequisite for the diagnosis of the syndrome. In addition, several new candidates such as low-grade inflammation, hypoadiponectinemia, endothelial dysfunction and protrombotic factors have been suggested to be associated with the MetS. Thus, there are still uncertainty concerning the pathogenesis of the syndrome and the spectrum of metabolic abnormalities relating to the MetS. Furthermore, genetic factors are likely to contribute to abdominal obesity and the MetS.

The aim of this study was to perform detailed metabolic characterization and assessment of body composition and body fat distribution in offspring of subjects with T2DM in order to investigate the metabolic defects related to abdominal obesity and the MetS. Furthermore the aim was to assess contribution of certain genetic variations previously linked to the risk of T2DM, to abdominal obesity and related traits.

Altogether 158 nondiabetic offspring of T2DM patients were studied. Insulin sensitivity was assessed with 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 bioelectrical impedance and CT, respectively. Bicycle ergometer test was performed to assess maximal oxygen uptake. Furthermore, levels of adiponectin, C-reactive protein, inflammatory cytokines and adhesion molecules were measured.

We applied factor analysis to identify the MetS factor characterized by the following variables: 2-hour glucose, fasting insulin, body mass index, waist circumference, high density lipoprotein and triglycerides. Subjects with the MetS were insulin resistant, had more 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 than subjects without the MetS. When study subjects were grouped according to adiponectin tertiles or by the total amount and distribution of adipose tissue similar defects in metabolic variables were observed in subjects with hypoadiponectinemia or abdominal obesity as in subjects with the MetS. Single nucleotide polymorphisms of calpain-10 gene were associated with abdominal obesity. No associations of adiponectin gene variants with metabolic variables were observed.

In conclusion, the MetS is associated with multiple changes in metabolic variables that are closely related to abdominal obesity independently of total body fat. Because low levels of adiponectin reflect an increase in abdominal obesity and features of insulin resistance, adiponectin appears to be a reliable marker of the MetS. The MetS, hypoadiponectinemia and abdominal obesity were associated with low levels of energy expenditure suggesting susceptibility to further weight gain in these subjects.

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Medical Subjects Headings: abdominal fat/metabolism; adiponectin; body mass index; cytokines; intra-abdominal fat/metabolism; body composition; body fat distribution; diabetes mellitus, type 2/genetics; dyslipidemias/epidemiology; Finland; genetic predisposition to disease; glucose intolerance; hyperinsulinism/genetics; hypertension/genetics; insulin resistance; metabolic syndrome x; obesity/genetics; obesity/metabolism; polymorphism, single nucleotide; risk factors

Mietittyäni asiaa viisikymmentä vuotta voin nyt sanoa että maailma on sana.

Samuli Paronen

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This book is dedicated to my family: mother and father, Siru, Arttu and Sisu, Salla and Einari.

Kuopio, February 2006

Urpu Salmenniemi

LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme	11HSD-1	11β-hydroxysteroid dehydrogenase type 1
ADP	adenosine diphosphate	IL	interleukin
AMP1	adiponectin gene	IL-1 RA	interleukin-1 receptor antagonist
AMPK	adenosine monophosphate-activated protein kinase	IRS	insulin receptor substrate
ANCOVA	analysis of covariance	IVGTT	intravenous glucose tolerance test
ANOVA	analysis of variance	LBM	lean body mass
ApoB	apoprotein B	LDL	low-density lipoprotein
BAT	brown adipose tissue	LPL	lipoprotein lipase
BMI	body mass index	MAP	mitogen-activated pathway
BMR	basal metabolic rate	MetS	metabolic syndrome
CAPN10	calpain-10 gene	MRI	magnetic resonance imaging
CETP	Cholesteryl ester transfer protein	mRNA	messenger ribonucleic acid
CHD	coronary heart disease	NCEP	National Cholesterol Education Program
CNS	central nervous system	NGT	normal glucose tolerance
CRP	C-reactive protein	NO	nitric oxide
CT	computed tomography	OGTT	oral glucose tolerance test
CVD	cardiovascular disease	PAI-1	plasminogen activator inhibitor-1
DNA	deoxyribonucleic acid	PCR	polymerase chain reaction
EE	energy expenditure	PI3K	phosphatidylinositol-3-kinase
EGIR	European Group for the Study of Insulin Resistance	QTDT	quantitative transmission disequilibrium test
ELISA	enzyme-linked immunosorbent assay	RQ	respiratory quotient
FDR	first-degree relative	SNP	single nucleotide polymorphism
FFA	free fatty acid	TFM	total fat mass
HDL	high-density lipoprotein	TNF-α	tumor necrosis factor-α
HL	hepatic lipase	T2DM	type 2 diabetes mellitus
HOMA	homeostasis model assessment	UCP-1	uncoupling protein-1
HSL	hormone-sensitive lipase	VCAM-1	vascular cell adhesion molecule-1
IAF	intra-abdominal fat	VLDL	very-low-density lipoprotein
IAFM	intra-abdominal fat mass	VO_2max	maximal oxygen uptake
ICAM-1	intercellular adhesion molecule-1	WBGU	whole body glucose uptake
IDF	International Diabetes Federation	WHO	World Health Organization
IGT	impaired glucose tolerance	WHR	waist to hip ratio

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to 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 Salmenniemi U, Zacharova J, Ruotsalainen E, Vauhkonen I, Pihlajamäki J, Kainulainen S, Punnonen K, Laakso M. Association of adiponectin level and variants in the adiponectin gene with glucose metabolism, energy expenditure, and cytokines in offspring of type 2 diabetic patients. J Clin Endocrinol Metab. 2005; 90: 4216-23.

III Salmenniemi U, Ruotsalainen E, Vänttinen M, Vauhkonen I, Pihlajamäki J, Kainulainen S, Punnonen K, Laakso M. High amount of visceral fat mass is associated with multiple metabolic changes in offspring of type 2 diabetic patients. Int J Obes Relat Metab Disord, 2005; 29: 1464-70.

IV Pihlajamäki J, Salmenniemi U, Ruotsalainen E, Vauhkonen I, Kainulainen S, Ng M, Cox N, Bell G, Laakso M. Common polymorphisms of calpain-10 are associated with visceral obesity in offspring of patients with type 2 diabetes. Submitted.

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1. INTRODUCTION

The metabolic syndrome (MetS) is a constallation of cardiovascular risk factors (abdominal obesity, insulin resistance, glucose intolerance, dyslipidemia and elevated blood pressure) associated with 2-fold increased risk of cardiovascular death (2;3) and even higher risk of type 2 diabetes mellitus (T2DM) (4;5). The age-adjusted prevalence rate of the MetS has generally been around 25% (6-8), although varying significantly depending on the diagnostic criteria used and the population studied (8). The current epidemic of obesity is associated with increased incidence of the MetS (9), and the syndrome may be identifiable even in young adults and children (10). Thus, the understanding of the pathophysiology of the MetS and identification of subjects with the MetS is highly important.

The pathophysiology of the MetS is complex and not completely understood. Originally, when Reaven introduced the concept 'Syndrome X', insulin resistance was suggested to be the cause of the syndrome (11). Importantly, obesity was not included in syndrome X (11). As the detrimental metabolic effects of abdominal fat accumulation have been acknowledged, the relative impact of subcutaneous and intra-abdominal fat (IAF) tissue on metabolic consequences of obesity has been a matter of dispute (12-16). According to current understanding the distict morphological and functional features of IAF may serve as a link to pathogenesis of the MetS (17). IAF is associated with an increased release of free fatty acids (FFAs) and altered production and release of multiple polypeptides such as adiponectin, tumor necrosis factor-α (TNF-α), interleukins (ILs), angiotensinogen, plasminogen activator inhibitor-1 (PAI-1), leptin, resistin and visfatin. These metabolic mediators contribute to the components of the MetS and to endothelial dysfunction, and proinflammatory and protrombotic state, conditions closely associated with the MetS.

Genetic factors also contribute to the MetS. Insulin resistance and abdominal fat accumulation, central features of the thrifty phenotype, are inherited (18;19). The concept of the thrifty phenotype has been introduced to explain how metabolic abnormalities predisposing to obesity and T2DM in times of abundance of food supply have provided selection advantage to our ancestors in times of famine (20). The thrifty genotype, the genetic variations predisposing to thrifty phenotype, is, however, largely unknown.

In this study we performed detailed metabolic characterization of offspring of T2DM subjects to investigate the metabolic abnormalities related to the MetS, intra-abdominal fat accumulation and hypoadiponectinemia. Furthermore, we analyzed the associations of variants in the adiponectin and Calpain-10 genes, previously linked to increased risk of

T2DM, with insulin resistance and abdominal obesity and other metabolic features of the MetS. The review of literature will discuss the evolution of the concept of the MetS and different diagnostic criteria, epidemiology, pathophysiological aspects of different components of the MetS and closely related features, and genetics of the MetS.

2. REVIEW OF LITERATURE

2.1 Metabolic syndrome

2.1.1. Evolution of the concept

Clustering of cardiovascular risk factors has been known for decades. Kylin (21) was the first to report a syndrome comprising of hypertension, hyperglycemia and hyperurikemia in 1923. Since then several different constellations of cardiovascular risk factors have been suggested. In 1988 Gerald Reaven postulated that insulin resistance is the underlying cause of 'syndrome X' comprising of hyperinsulinemia, hyperglycemia, dyslipidemia and high blood pressure (11). Later on the term 'insulin resistance syndrome' has been commonly used for this risk factor clustering. However, the etiology of the syndrome has remained unclear. In 1998, the World Health Organization (WHO) consultation proposed the first internationally accepted criteria for the syndrome (22). The term 'metabolic syndrome' was preferred over 'insulin resistance syndrome'. Since then several new components of the MetS have been proposed which are summarized in Figure 1. Each of these components is separately discussed in Chapter 2.3.

2.1.2. Definitions of the metabolic syndrome (Table 1)

Despite general recognition of the syndrome, the lack of knowledge of the etiology and uniform diagnostic criteria hampered epidemiological studies of the MetS for years. The WHO diagnostic criteria published in 1998 were the first attempt to achieve some agreement on the definition particularly for research purposes (22). The prerequisite for the diagnosis of this syndrome was insulin resistance coupled with any two other features of the syndrome (central obesity, elevated blood pressure, dyslipidemia and microalbuminuria). However, the report clearly stated that the definition did not imply causal relationships and that the definition could be modified as new information becomes available.

The WHO criteria were not accepted without criticisms. Particularly the inclusion of microalbuminuria as a component of the syndrome was debated, since microalbuminuria in non-diabetic individuals is uncommon (23) and its association with other central components of the syndrome is not consistent (24-26). Furthermore, the requirement of the assessment of insulin resistance with the hyperinsulinemic euglycemic clamp technique (27) in glucose

tolerant subjects made the criteria impractical for epidemiological studies. For these reasons, soon after the WHO criteria were published, the European Group for the Study of Insulin Resistance (EGIR) presented a simpler definition particularly for epidemiological studies (28). Insulin resistance was still the central element of the diagnosis, but it was defined as the presence of fasting hyperinsulinemia (28;29). The use of fasting insulin level restricts the criteria for nondiabetic subjects. Other differences compared to the WHO criteria were the cut-off points for hypertension and dyslipidemia (28). Furthermore, instead of waist to hip ratio (WHR), waist circumference was proposed as a measure of abdominal obesity because of simplicity and better correlation with intra-abdominal adipose tissue mass (30). Microalbuminuria was not included in the EGIR criteria.

The Nation Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) published their diagnostic criteria for the MetS in 2001 (31). This definition is based on fasting values of triglycerides, high density lipoprotein (HDL) cholesterol, plasma glucose and measurements of waist circumference and blood pressure with equal weight on each component. Insulin resistance was not included in the definition of the syndrome.

Recently, the International Diabetes Federation (IDF) revised the definition of the MetS (32). In the IDF criteria abdominal obesity with insulin resistance are recognized causal factors for the syndrome. Therefore, a large waist circumference was defined as a prerequisite risk factor for the diagnosis of the MetS. For the first time ethnic specific cut-points for waist circumference were given. For European men the cut-off point is ≥94 cm and for European women ≥80 cm, based on the sensitivity and specificity of these cut-off points to identify subjects with body mass index (BMI) ≥25 kg/m^2 or WHR ≥0.9 for men and ≥0.85 for women (33). The given cut-off points for waist circumference for Asian populations are lower since their risk of DM2 and cardiovascular disease (CVD) is apparent at lower levels of adiposity than in Europeans (34). The criteria for dyslipidemia and elevated blood pressure are identical to those of the NCEP definition, but the limit for hyperglycemia is lowered to ≥5.6 mmol/l according to the new definition of impaired fasting glucose by the American Diabetes Association (35). A similar revision on the cut-off point of fasting glucose of the ATP III criteria for the MetS was recently done (36).

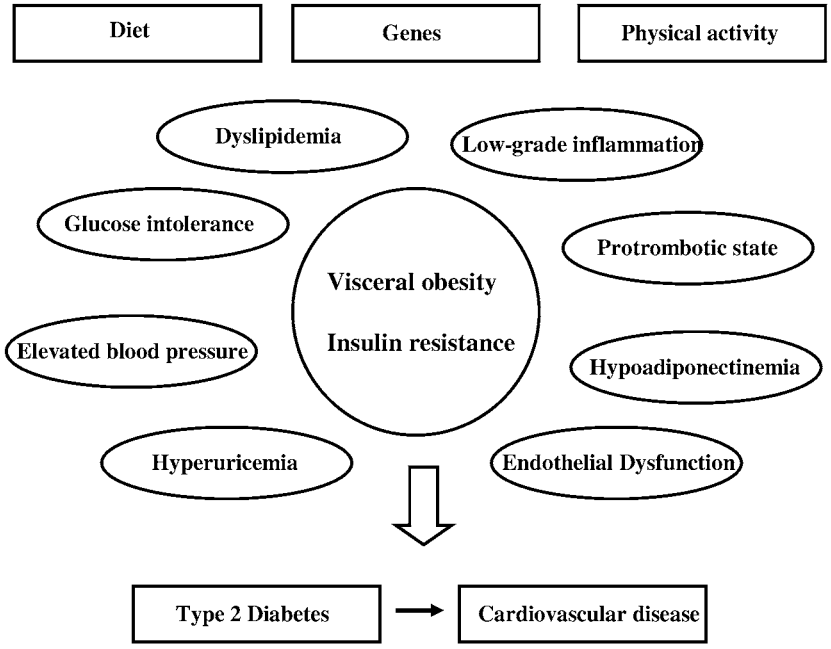

Figure 1. Components of the metabolic syndrome. Diet, genetic factors and physical inactivity contribute to the pathophysiology of the metabolic syndrome. Visceral obesity and insulin resistance are the core components having at least partial etiological role in the syndrome. Dyslipidemia, glucose intolerance and elevated blood pressure are features included in the diagnostic criteria. Hyperuricemia, low-grade inflammation, protrombotic state, hypoadiponectinemia and endothelial dysfunction are other characteristics of the syndrome. Cardiovascular disease and type 2 diabetes are the adverse outcomes of the syndrome.

Table 1. Different criteria of the metabolic syndrome

World Health Organization (WHO) criteria (22)

Glucose intolerance or diabetes mellitus (WHO criteria) **and/or insulin resistance** (glucose uptake below lowest quartile for background population) **and 2 or more of the following:**

- Elevated blood pressure (≥ 160/90 mmHg)
- Elevated plasma triglycerides (≥1.7 mmol/l) and/or low HDL cholesterol (<0.9 mmol/l in men and <1.0 mmol/l in women)
- Central obesity (WHR >0.9 in men and >0.85 in women) and/or BMI> 30 kg/m^2
- Microalbuminuria (urinary albumin excretion rate ≥ 20 µg/min or albumin/creatinine ratio ≥ 20 mg/g)

European Group for the Study of Insulin Resistance (28)

Fasting hyperinsulinemia (highest quartile for background population) **and 2 or more of the following criteria:**

- Hyperglycemia (fasting plasma glucose ≥ 6.1 but < 7.0 mmol/l)
- Central obesity (waist circumference ≥ 94 cm in men and ≥ 88 in women)
- Elevated blood pressure (≥ 140/90 mmHg or drug treatment for hypertension)
- Dyslipidemia (triglycerides >2.0 mmol/l or HDL cholesterol <1.0 mmol/l or drug treatment for dyslipidemia)

National Cholesterol Education Program Adult Treatment Panel III (31)

3 or more of the following:

- Central obesity (waist circumference ≥ 102 cm in men and ≥ 88 cm in women)
- High triglycerides (≥ 1.7 mmol/l)
- Low HDL cholesterol (<1.03 mmol/l in men and < 1.29 mmol/l in women)
- Elevated blood pressure (≥ 130/85 mmHg)
- Hyperglycemia (fasting plasma glucose ≥ 6.1 mmol/l)*

International Diabetes Federation (32)

Central obesity (waist circumference ≥ 94 cm in European men and ≥ 80 cm in European women, with ethnicity specific values for other groups) **and 2 or more of the following criteria:**

- High triglycerides (≥ 1.7 mmol/l or specific treatment for this lipid abnormality)
- Low HDL cholesterol (<1.03 mmol/l in men and < 1.29 mmol/l in women or specific treatment for this lipid abnormality)
- Elevated blood pressure (systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg or drug treatment of previously diagnosed hypertension)
- Hyperglycemia (fasting plasma glucose ≥ 5.6 mmol/l or previously diagnosed type 2 diabetes)

HDL= high-density lipoprotein, WHR= waist to hip ratio, BMI= body mass index
* In updated criteria (36) fasting plasma glucose ≥ 5.6 mmol/l.

2.1.3. Epidemiology of the metabolic syndrome

2.1.3.1. Prevalence of the metabolic syndrome

As stated above, the prevalence and consequences of the MetS was difficult to assess prior the generally accepted definition of the syndrome was published. However, it became obvious that simultaneously with an increase of obesity the prevalence of the MetS was also increasing. Analysis of data on a representative sample of 8814 men and women from the United States (the Third National Health and Nutrition Examination Survey from 1988-1994), estimated that the age-adjusted prevalence of the MetS according to the NCEP criteria was 23.7% (6). The prevalence increased from 6.7% among the participants aged from 20 to 29 years to 43.5% for participants aged from 60 to 69 years. In addition, there were considerable differences in the prevalence of MetS among ethnic groups (6). The prevalence was highest among Mexican Americans (31.9%) and lowest among whites (21.6%), African Americans (21.6%) and people reporting "another" race or ethnicity (20.3%). Comparison of the WHO (insulin resistance estimated with homeostasis model assessment, HOMA) and the NCEP definitions showed that the age-adjusted prevalence of the MetS was quite similar 25.1 and 23.9%, respectively, but 15-20% of individuals were classified as having the syndrome by one definition but not the other, with equal discordance (7). In a Finnish study of 1209 men aged from 42-60 years the prevalence of MetS according to the WHO (insulin resistance estimated as hyperinsulinemia) and NCEP definitions were relatively low 14.2% and 8.8%, respectively (3). However, subjects with diabetes and CVD were excluded from the cohort. A recent study of 9669 subjects representing Greek population reported the prevalence of the MetS of 24.5% according to the NCEP criteria, which is very similar to the prevalence in US adults (8). By the new IDF definition, the age-adjusted prevalence in the same population was substantially higher (43.4%) and included a majority of subjects older than 50 years. The most prevalent trait of the syndrome defined by IDF was abdominal obesity. The significant difference in the prevalence using the different definitions of the syndrome was attributable to the lower cut-off points of waist circumference and fasting glucose as defined by IDF since most subjects with only one or two diagnostic criteria by the NCEP had the MetS according to the IDF definition (8).

2.1.3.2. Predictive significance of the metabolic syndrome

The main reason to identify subjects with the metabolic syndrome is the fact that these individuals are at a high risk of developing CVD and T2DM (22). Several prospective population-based studies have investigated the association of metabolic risk factors with CVD (37-40). In a large cohort of about 20 000 men and women, mortality was followed for an average of 7 years in relation to the components of the MetS. The risk of all cause and CVD mortality increased with increasing number of abnormalities associated with the MetS (37).

In the Botnia study (family study of T2DM) 4483 subjects aged 35-70 years, were evaluated for cardiovascular risk associated with the MetS by the WHO definition (insulin resistance estimated by HOMA) (41). The prevalence of coronary heart disease (CHD) and stroke was increased by 3-fold in subjects with the syndrome and their mortality markedly increased during the 7-year prospective follow-up compared to subjects without the syndrome (12.2% versus 2.2%, respectively).

In another, aforementioned Finnish study, men with the MetS defined by the NCEP and WHO criteria had 4.2 and 2.9 times higher risk to die from CHD, respectively, than men without the MetS after the adjustment for conventional cardiovascular risk factors (3). Relative risks of CVD death and all-cause mortality were increased by 2.9- and 2.6-fold, respectively, using the WHO criteria, but the NCEP definition predicted these outcomes less consistently.

The predictive value of the WHO and NCEP criteria were also compared in the San Antonio Heart Study participants (2815 subjects aged 25-64 years, average follow-up time 12.7 years), where the prevalence rate of the MetS in the general population were identical, 25.2% by both definitions (2). In the general population the NCEP criteria were predictive of all-cause mortality (hazard ratio 1.5) and CVD mortality (hazard ratio 2.5), whereas the WHO criteria was only predictive of CVD mortality (hazard ratio 1.6). In primary prevention population (neither T2DM, nor history of CVD at baseline) the prevalence of the MetS was similar 17.5 and 17.9% by the NCEP and WHO criteria, respectively. In this low risk population the NCEP criteria predicted CVD mortality (hazard ratio 2.0), but not all-cause mortality, whereas the WHO criteria predicted neither.

Besides CVD, the MetS also predicts T2DM (4;5).The close relationship between the MetS and glucose tolerance was demonstrated in the Botnia study, where the prevalence of the MetS was 10% in subjects with normal glucose tolerance compared to 50% in subjects with impaired glucose tolerance (IGT) and 80% in subjects with T2DM (41).

2.1.4. Application of factor analysis in studies of the metabolic syndrome

High intercorrelation between the components of the MetS complicates the use of traditional statistical methods and therefore, factor analysis, a multivariate correlation method, has been used to investigate the relationship of the variables included the syndrome (42;43). Factor analysis identifies statistically independent latent "factors", underlying the associations of included variables. If an analysis reveals only one single factor this supports the hypothesis of the common etiology for the MetS e.g. insulin resistance. Alternatively, several factors represent probably distinct physiological phenotypes and suggest a more complex etiology. However, if a variable (a trait of the syndrome) is associated with more than one factor, this overlap indicates unifying commonalities between physiological domains, and this pattern of overlap might provide insight into the underlying structure of the syndrome (42).

Factor analysis have rather consistently yielded from 2 to 4 factors (38;44-49) for the MetS with a separate obesity-hyperinsulinemia factor (38;39;44;47;48;50-52), that in most cases has also included dyslipidemia (38;39;44;47;50;51). Blood pressure has most consistently loaded on a separate factor (38;44;45;48-52). Much of the variability of results may, however, be accounted for the diversity of variables included in analyses, and most analyses have included only surrogate measures of insulin sensitivity and visceral obesity.

2.1.5. Criticism and future challenges

Although the diagnosis of MetS has been established and accepted by international organizations, the reasons for the diagnosis have been recently questioned (53). Diagnosis is warranted if a syndrome reflects a unifying pathological process or predicts future adverse event(s) better than the sum of its components (53). According to criticism current data on the MetS does not fulfill either of these criteria (53), and thus labeling of subjects with the term "Mets" shoud be avoided.

As reviewed above, subjects with the MetS have a higher risk for CVD than subjects without the syndrome. However, convincing data are lacking to indicate that the MetS itself conveys a greater risk for CVD than the individual components of the syndrome (53-55). Furthermore, the separate components of the syndrome may convey different risk profile (54;56;57), suggesting that the different combinations that lead to the diagnosis of the syndrome do not carry equal risk. The dichotomous nature of the diagnosis of MetS has also received criticisms, since as the variables included in the definition are continuous variables,

so is also the associated risk for different end points a continuum. Furthermore, the risk of CVD depends on the number of components of the syndrome (54;58;59). It is not known whether substituting an old risk factor or addition of any of the new risk factors associated with the MetS, (e.g. inflammation markers, adiponectin or protrombotic factors) would improve the predictive power of the syndrome. Thus, more studies are needed to understand the pathophysiology of the syndrome, and to identify the most predictive combinations of the MetS related risk factors.

2.2. Etiology of the metabolic syndrome

The original description of the MetS was based on the assumption that the syndrome is a clinical manifestation of insulin resistance. However, given the complex nature of insulin resistance, it is likely that insulin resistance can only partially explain the components of the MetS. As more data have been published and many new features have been proposed to belong to the syndrome (low-grade inflammation, endothelial dysfunction and protrombotic state), abdominal obesity has become the central focus of attention for its close association with insulin resistance and other components of the MetS. Both environmental and genetic factors are also involved in the pathophysiology of the MetS.

2.3. Components of the metabolic syndrome

2.3.1. Obesity and abdominal fat distribution

The dramatic worldwide increase in the prevalence of obesity, defined as BMI $\geq$ 30 kg/m^2 by the WHO, is the most important cause for increasing prevalence of the MetS and T2DM (60). Obesity results from the imbalance between energy intake and energy expenditure. This balance is modified by numerous genetic, environmental and psychosocial factors (60). Total energy expenditure (EE) composes of basal metabolic rate (BMR, representing 60-70% of EE), dietary-induced thermogenesis (10% of EE) and physical activity (20-30% of EE) (61). Low BMR, low physical activity, low ratio of lipid oxidation, low leptin level, low activity of the sympathetic nervous system and high insulin sensitivity are all predictors of weight gain (62). In contrast, in cross-sectional studies obesity is related to high metabolic rate, high fat oxidation, low insulin sensitivity and high plasma leptin concentration (62).

While BMI provides an indicator of overall obesity for epidemiological studies, a more accurate assessment of total body fat mass can be obtained with bioelectrical impedance (63), underwater weighing (64) or dual X-ray absorptiometry (64). Waist circumference as a surrogate marker for abdominal obesity is probably the most important anthropometric measure, because the impact of body fat distribution is crucial for metabolic complications of obesity. Waist circumference correlates with abdominal obesity better than WHR (30). Accurate assessment of abdominal fat distribution can be obtained with computed tomography (CT) (65) or magnetic resonance imaging MRI (66).

Some 50 years ago Juhan Vaque demonstrated that android or upper body fat deposition is associated with increased risk of chronic diseases, including T2DM, atherosclerosis and gout (67). Since then a large number of both cross-sectional and prospective studies have assessed the impact of body fat distribution and confirmed the link of abdominal obesity with insulin resistance (13;68;69), the MetS (70;71), T2DM (72;73) and CVD (74;75). Furthermore, an increase in visceral fat mass, despite similar total fat mass, has been demonstrated in subjects with family history of T2DM compared to subjects without family history of T2DM (81). This suggests that increased visceral fat mass may be an early sign of predisposition to the development of insulin resistance and T2DM (76). Despite the well-established association of abdominal obesity with various metabolic abnormalities there has been some controversy about the contribution of abdominal subcutaneous and IAF depots to metabolic disturbances (12-16).

In addition to its function as storage of surplus energy, adipose tissue has multiple regulatory functions in metabolism. Changes in biologic properties of adipocytes are now recognized to contribute to adverse metabolic effects of abdominal fat tissue. In the normal state the balance between adipose tissue lipolysis and triglyceride synthesis is carefully governed by energy status, different hormones and the autonomic nervous system. This balance seems to be disrupted in abdominal obesity due to an increase in both fasting and postprandial FFA levels (77-79). Accordingly, visceral adipose tissue has been shown to be more sensitive to β-adrenergic lipolysis than subcutaneous adipose tissue because of a larger amount of β-adrenergic receptors on the cell surfaces (80). Furthermore, the antilipolytic effect of insulin is impaired in visceral adipose tissue (81;82), resulting in an excess supply of FFAs and causing multiple adverse events closely associated with insulin resistance. The flux of FFAs contributes to increased hepatic very low-density-lipoprotein (VLDL) production and gluconeogenesis, decreased hepatic insulin clearance, impairment of muscle glucose utilization and pancreatic β-cell insulin secretion, and endothelial dysfunction.

Adipose tissue contributes to metabolism also by secreting a variety of polypeptides that have multiple paracrine and endocrine functions (Figure 2). These polypeptides, collectively termed adipokines, include adiponectin, leptin, TNF-α, ILs, angiotensinogen, PAI-1, visfatin and resistin. The adipose depot specific differences in the amount of adipokines secreted have been suggested to contribute to the association of visceral obesity and insulin resistance (17). Accordingly, the secretion of most adipokines is increased in visceral adipose tissue compared to subcutaneous adipose tissue, whereas the secretion of adiponectin is decreased in visceral fat. Furthermore, an increase in adipocyte size has been associated with increased secretion of adipokines (83;84), and visceral adipocyte size has been shown to correlate with insulin resistance (85).

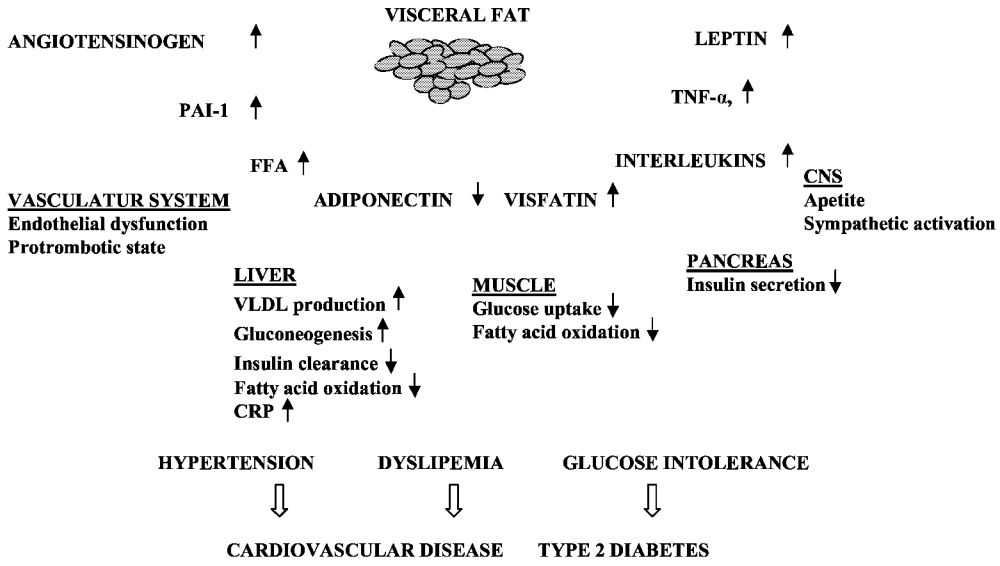

Figure 2. Visceral adipose tissue is associated with increased release of free fatty acids (FFA) and certain adipokines, and decreased release of adiponectin contributing to multiple metabolic changes and increased risk of the metabolic syndrome, type 2 diabetes and cardiovascular disease. PAI-1= plasminogen activator inhibitor-1, TNF-α= tumor necrosis factor-α, CNS= central nervous system, VLDL= very low density lipoprotein, CRP= c-reactive protein.

Both adiponectin and inflammatory cytokines (primarily TNF-α and IL-6) have a pivotal role in glucose homeostasis, insulin resistance and vascular disorders associated with the MetS, which are discussed separately in Sections 2.4.2. and 2.4.1, respectively.

Leptin is an important exception among the adipokines, because it is more abundantly expressed and secreted from subcutaneous adipose tissue than from visceral adipose tissue (86;87). Adipose tissue is the only known source of leptin, and its secretion is proportional to adipocyte size (87). Accordingly, leptin secretion is increased in hypertrophic adipocytes of obese subjects. This seems to contradict the proposed function of leptin as a signal of positive energy balance from the periphery to the central nervous system (CNS), which ultimately leads to an anorexigenic effect (88;89). However, there are data indicating that hyperleptinemia in obesity is coupled with central leptin resistance (89;90). Leptin may also contribute to the development of vascular disease. In animal studies hyperleptinemia has been associated with enhanced thickening of the vascular wall after induced arterial injury (91). In humans leptin levels have been associated with impaired vascular function, independently of other metabolic and inflammatory disturbances associated with obesity (92). Furthermore, hyperleptinemia may contribute to impaired insulin secretion from pancreatic β-cells (93).

High levels of PAI-1 contribute to the procoagulant state in the MetS. Although PAI-1 is synthesized in many cell types, adipose tissue is thought to be the major source of PAI-1 in obese subjects, and circulating PAI-1 levels correlate with visceral obesity (94). Increased levels of TNF-α contribute to PAI-1 production (94).

Angiotensinogen is a precursor of angiotensin II that is a powerful vasoconstrictor and has many proatherogenic properties in endothelial cells such as the induction of adhesion molecule expression and free oxygen radical production (95). Angiotensinogen gene expression correlates with generalized and abdominal obesity (96;97).

Resistin is a protein that is secreted exclusively by adipocytes in the mouse, but is primarily expressed in macrophages and monocytes in humans (98). Rodent and *in vitro* studies have suggested that resistin contributes to insulin resistance (98) and endothelial dysfunction (99), but studies in humans have failed to link resistin to insulin resistance.

Visfatin is a recently identified adipokine that is particularly expressed in visceral fat and has insulin-like metabolic effects (100). Its physiological role has, however, not been established.

Finally, human visceral adipose tissue has been reported to contribute substantially to the regeneration of cortisol from its inactive 11-keto-metabolite cortisone via the activity of 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), thus amplifying glucocorticoid receptor activation independently of the circulating cortisol level (101). The consequences of an effect of excess cortisol is illustrated in Cushing's syndrome characterized by the redistribution of fat tissue from peripheral to central parts of the body and subsequent development of other

features of the MetS. Similarly, transgenic mice overexpressing 11HSD1 in adipocytes develop central obesity with hyperinsulinemia, hyperglycemia, hyperlipidemia and hypertension (102).

To summarize, most evidence suggests that an increase in visceral abdominal fat mass is the primary perturbation in the pathogenesis of the MetS, providing mediators for cross-talk with other tissues and finally leading to insulin resistance and vascular disorders.

2.3.2. Insulin resistance

2.3.2.1. Definition and measurement

Insulin resistance can be defined as impaired insulin action in target tissues, particularly skeletal muscle, liver and adipose tissue. Insulin action is influenced by a number of factors including age, weight, physical activity and medication. The impact of genetic factors is demonstrated by reduced insulin sensitivity in certain ethnic groups (103) and in first-degree relatives (FDRs) of T2DM patients (18). Insulin resistance is the strongest predictor of the risk to develop T2DM (104). In large prospective population studies hyperinsulinemia (a surrogate marker of insulin resistance) has also predicted CVD and mortality (39;105-107).

The golden standard for the measurement of whole-body glucose uptake is the euglycemic hyperinsulinemic clamp (108). This procedure is complex and time consuming and therefore impractical for epidemiological studies. Many alternative methods have been suggested (109) (110). The minimal model (111) is a computerized modeling of individual glucose and insulin data during intravenous glucose tolerance test (IVGTT) yielding estimates of insulin sensitivity (insulin sensitivity index, S_I and glucose effectiveness, S_G). S_I correlates strongly with the rates of whole body glucose uptake (WBGU) during the hyperinsulinemic euglucemic clamp (112). Like the clamp study, the frequent sampling IVGTT is time-consuming and expensive test, though simplified modification has been presented (113). Homeostasis model assessment of insulin resistance is simple and inexpensive method and therefore widely used in epidemiological studies. HOMA-R gives an estimate of basal insulin resistance from mathematical modeling of fasting plasma glucose and insulin concentrations (114). The correlation to clamp-measured insulin sensitivity has been variable, but generally around -0.80 (114;115). Furthermore, fasting plasma insulin level has been used as a marker of insulin resistance in epidemiological studies. The correlation between fasting insulin levels and the hyperinsulinemic euglycemic clamp derived measure of insulin sensitivity has been

around -0.70 in subjects with normal glucose tolerance (29). Hyperinsulinemia and insulin resistance may, however, partly represent different phenotypes (116). In the EGIR study the overlap between different definitions of insulin resistance were around 60%, i.e. a subject who was identified to be insulin resistant according to the hyperinsulinemic clamp was insulin resistant also according to his/her insulin levels. When subjects with insulin resistance without hyperinsulinemia and subjects with hyperinsulinemia without insulin resistance were compared important differences emerged. Insulin resistant subjects showed more central fat and an increase in the rate of lipolysis and endogenous glucose production. In contrast, subjects with hyperinsulinemia had suppressed lipolysis, endogenous glucose production, higher blood pressure and lower HDL levels. Hypertriglyceridemia was the only abnormality common to both phenotypes was (116).

The challenge in studies of insulin resistance is the diversity of metabolic pathways regulated by insulin and the variation in the severity of insulin resistance. Further complexity comes from the specificity of the defects to some pathways whereas other insulin signaling pathways remain intact (117). Furthermore, some manifestations of insulin resistance may be caused by compensatory hyperinsulinemia (118).

2.3.2.2. Insulin signaling

The biological action of insulin is exerted via the insulin receptor, which is widely expressed in human tissues. The binding of insulin to its cell surface receptor initiates a cascade of phosforylation and dephosforylation events, second messenger generation and protein-protein interaction that lead to diverse metabolic events. Insulin binds to the extracellular α-subunits of the receptor leading to activation of the intracellular β-subunits and subsequent autophosphorylation. This in turn leads to phosphorylation of several insulin receptor-docking proteins including insulin receptor substrates (IRS1-4) and Shc (119). Phosphorylation of these proteins leads to docking of several SH2 domains containing molecules (119). Activation of the phosphatidylinositol-3-kinase (PI3K) pathway by IRS-1 and IRS-2 leads to most of the physiological actions of insulin, including activation of glucose transport and stimulation of glycogen, protein and lipid synthesis (120). However, there are compelling data that activation of the glucose transport requires also activation of PI3K-independent pathway that appears to involve tyrosine phosphorylation of the Cbl protooncogene (121) and subsequent association with adapter protein CAP (122). The Cbl-CAP complex translocates to the lipid raft of the plasma membrane and finally G protein TC10 provides a signal to the

glucose transporter protein 4 (123). Activation of Ras and mitogen-activated protein (MAP) kinase pathway contributes solely to the nuclear and mitogenic effects of insulin and has no role in conveying the metabolic actions of insulin (124). In the setting of insulin resistance, insulin has reduced effects on PI3K-mediated pathways while maintaining MAP kinase activity (117). Thus, the activation of the MAP kinase pathway conveys many of the adverse events seen in insulin resistance (120;125). FFAs (126), low-grade inflammation (127), hypoadiponectinemia (128) and hyperglycemia (129) all contribute to impaired insulin signalling in the MetS.

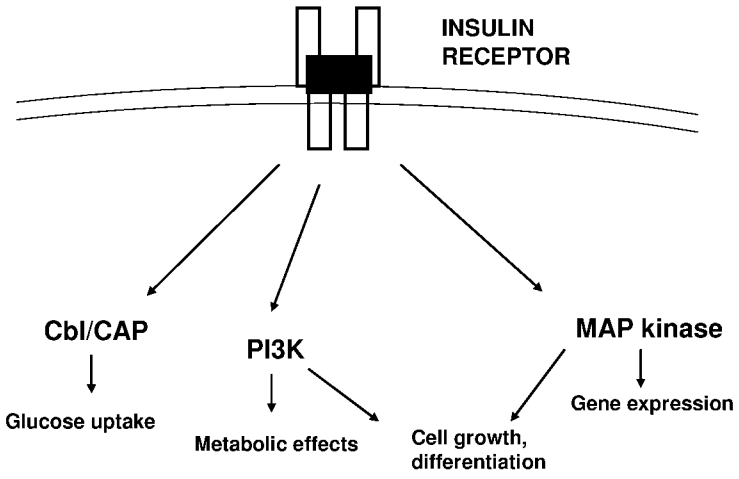

Figure 3. Simplified presentation of insulin signalling pathways. Phosphatidylinositol-3-kinase (PI3K) pathway mediates the acute metabolic effects of insulin with Cbl/CAP pathway activity contributing to insulin-stimulated glucose uptake. Mitogen-activated protein (MAP) kinase is responsible of the chronic growth effects of insulin. Modified from ref (1).

2.3.2.3. Insulin action at the tissue level

The metabolic effect of insulin is to promote substrate storage in adipose tissue, muscle and liver by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown.

Skeletal muscle

Approximately 75% of the cellular glucose disposal occurs in skeletal muscle under hyperinsulinemic conditions (130). Insulin stimulates both cellular glucose uptake and glucose storage as glycogen. Glycogen synthesis accounts for more than 90% of non-oxidative glucose metabolism (46). Studies using indirect calorimetry during the hyperinsulinemic-euglucemic clamp have demonstrated that the impairment of non-oxidative glucose metabolism mostly accounts for the impairment of glucose uptake in insulin resistant subjects (131;132). However, impaired glycogen synthesis seems to be secondary to a defect in glucose transport (133).

A major contributor to the development of insulin resistance is an excess of circulating FFAs. According to the original Randle's hypothesis elevated circulating FFAs compete with glucose for substrate oxidation and lead secondarily to the inhibition of muscle glucose uptake. However, recent studies have shown that FFAs decrease primary glucose transport (134). An increase in FFA availability may also alter the equilibrium between FFA oxidation and reesterification, resulting in an increase in intramyocellular triglyceride content that has been shown to be inversely related to insulin-stimulated glucose uptake (135;136). Furthermore, alterations in adipokine levels may directly impair insulin signaling (128;137).

Liver

Insulin blocks glycogenolysis and gluconeogenesis, and stimulates glycogen synthesis in liver. Insufficient suppression of endogenous glucose production by insulin leads to glucose intolerance (130). FFAs, adipokines and cortisol secreted from the adipose tissue contribute to hepatic insulin resistance. Increase in FFA availability leads to an increase in VLDL and apoprotein B (Apo B) production. FFA availability also increases gluconeogenesis (138) and impairs hepatic insulin extraction leading to hyperinsulinemia (139;140). In animal models there is an inverse relationship between hepatic triglyceride content and hepatic insulin resistance. An increase in insulin sensitivity has been observed after a decrease in triglyceride content or administration of adiponectin or leptin, suggesting that hepatic triglyceride accumulation is a causal factor involved in hepatic insulin resistance (141).

Adipose tissue

Insulin enhances lipogenesis by stimulating lipoprotein lipase (LPL) (142) and inhibiting hormone sensitive lipase (HSL) activity (143). A decrease in LPL activity and increase in HSL activity in insulin resistance leads to increased release of FFAs from adipose tissue. As a source of FFAs and adipokines adipose tissue contributes significantly to insulin resistance in other tissues. Adipokines also have auto- and paracrine functions and thereby interfere with insulin signalling locally in the adipose tissue. Accordingly, insulin`s antilipolytic effect was impaired in the FDRs of T2DM patients compared to controls. In vitro experiments with isolated adipocytes showed no difference in the effects of insulin, however, suggesting that some factors in vivo contribute to insulin resistance (144).

Non-classical target tissues

Insulin receptors are widely distributed throughout the CNS. Neuron-spesific disruption of the insulin receptor in mice (NIRCO mice) results in diet-sensitive obesity and insulin resistance with increases in plasma leptin and triglyceride levels (145). In pancreatic β-cells insulin promotes its own biosynthesis by enhancing gene transcription (122). Insulin resistance in β-cells may contribute to impaired insulin secretion (146). Insulin also possesses anti-inflammatory (147), anti-oxidant (147) and other vascular protective functions (148). Resistance to these nonmetabolic actions of insulin contributes to the protrombotic state, endothelial dysfunction and atherosclerosis. (149).

2.3.3. Glucose intolerance

Glucose intolerance develops when increased insulin secretion from pancreatic β cells fails to compensate for insulin resistance in target tissues. Insufficient insulin action in the liver leads to increased gluconeogenesis and to a lesser extent glycogenolysis of stored glycogen. An increase in FFA levels, due to adipose tissue insulin resistance, contribute to glucose production by stimulating gluconeogenesis (138). Increased hepatic glucose output coupled with decreased peripheral glucose uptake leads to hyperglycemia.

Insulin secretion from the pancreatic β cells is mainly regulated by glucose entry via its transporter. Multiple adverse metabolic changes in insulin resistance may contribute to the impairment of insulin secretion. First, insulin itself has regulatory functions in pancreatic β cells, and insulin resistance at the β cell level may contribute to alterations in insulin secretion

(146). Hyperglycemia (so-called glucose toxicity) or prolonged exposure of β cells to excessive concentration of FFAs may also result in impaired insulin secretion (150;151). Furthermore, cytokines and leptin may contribute to the suppression of insulin secretion (93;152;153).

The temporal pattern of insulin secretion seems to be altered in glucose intolerant subjects. In an oral glucose tolerance test (OGTT) there is a delay in the peak insulin response (154) and the first-phase insulin response to intravenous glucose bolus is diminished (155). Furthermore, there is a loss of a coordinated insulin secretory response during an oscillatory glucose infusion, indicating an impairment of the pancreatic β cells to sense and respond appropriately to changes is plasma glucose level (156). These qualitative changes in insulin secretion have also been shown in first-degree relatives of T2DM even during euglycemia (157-159).

2.3.4. Dyslipidemia

Atherogenic dyslipidemia comprises hypertriglyceridemia, low HDL cholesterol, and small dense low-density lipoprotein (LDL) particles. The predominant mechanism for dyslipidemia is accelerated hepatic synthesis of VLDL in response to increased availability of FFAs from adipose tissue (160). Insulin resistance may also lead to impaired LPL activity and thus reduced triglyceride clearance may contribute to hypertriglyceridemia (161). An increase in VLDL triglycerides and thereby excessive postprandial lipemia leads secondarily to qualitative and quantitative changes in HDL and LDL particles. Cholesteryl ester transfer protein (CETP) mediates the transfer of excess triglycerides from VLDL to HDL in exchange of cholesterol esters making HDL particles small and dense, and leading to increased clearance of HDL from the circulation (162). Composition of LDL particles is modified similarly. The preponderance to small dense LDL particles is closely associated with triglyceride level. When fasting triglyceride level exceeds 2.0 mmol/l almost all subjects have a predominance of small, dense LDL. All these changes in lipid metabolism are independently associated with cardiovascular risk. Propensity of small dense LDL is attributable to its facilitated transit through the endothelium and a tight adherence to intimal proteoglycans, in addition to its susceptibility to oxidation. Therefore, atherogenic dyslipidemia activates several mechanisms that can lead to endothelial dysfunction (163). Furthermore, reduced levels of HDL impair reverse cholesterol transport, and compositional changes of HDL particles reduce antioxidative ability (164).

2.3.5. Blood pressure

Blood pressure in also associated with insulin resistance independently of obesity, although not as strongly as dyslipidemia. About 50% of hypertensive subjects are insulin resistant (165-168). Normotensive FDRs of patients with hypertension also have insulin resistance and dyslipidemia (169), whereas patients with secondary forms of hypertension do not (170). There are several mechanisms that directly link insulin resistance and hyperinsulinemia to increased blood pressure. Under physiological conditions, insulin has vasodilatory and anti-inflammatory functions in the vasculature that are lost in insulin resistance (171). Insulin mediates vasodilatation by stimulating the release of endothelium-derived nitric oxide (NO). Loss of this vasodilatory effect can lead to hypertension (172). Hyperinsulinemia can also result in increased sodium reabsorption in renal tubular cells and thereby volume-dependent hypertension (173;174). Finally, hypertension may be due to sympathetic hyperactivity associated with hyperinsulinemia and insulin resistance (175).

The presence of obesity further increases the likelihood of hypertension in insulin-resistant subjects (176). The pathophysiological mechanisms linking hypertension and obesity beyond hyperinsulinemia relate to central obesity. Intra-abdominal adipose tissue as a source of a variety of hormones and polypeptides contribute to hypertension and vascular disease. Adipose tissue releases angiotensinogen and also possesses angiotensin-converting enzyme (ACE) activity (177). Increased activity of the renin-angiotensin system may lead to hypertension via an increase in oxidative stress, vasoconstriction, renal sodium reabsorption and sympathetic stimulation (178). An increase in sympathetic stimulation has also been associated with leptin (179). Excess delivery of FFAs can impair NO production and thereby mediate vasoconstriction (180). Furthermore, locally enhanced activity of glucocorticoids in adipose and muscle tissue of obese subjects contribute to metabolic abnormalities associated with the MetS and may lead to hypertension via the activation of renin-angiotensin system (181-183).

2.4. Other features of the metabolic syndrome

2.4.1. Low-grade inflammation

Adipose tissue, especially intra-abdominal adipose tissue is an abundant source of adipokines that may serve as a link between obesity and insulin resistance and atherosclerosis. Amongst the adipokines there is a large variety of inflammatory cytokines, and obesity can be classified

as an inflammatory condition. Most data have been published on TNF-α and IL-6, but secretion of IL-1β, IL-8, IL-10 and IL-1 receptor antagonist (IL-1 RA) has also been reported from the adipose tissue (184).

C-reactive protein (CRP) is an acute-phase reactant that is synthesized in the liver under IL-6 stimulation. CRP has been found to be the best correlate of visceral fat in subjects with MetS (185), and it is one of the best independent predictors of CVD (59;186;187). CRP may not be merely a marker of inflammation, but itself could directly contributes to atherosclerosis by modulating endothelium function (188). CRP induces endothelial expression of adhesion molecules (188), angiotensin type 1 receptor (188), and PAI-1 (189), and attenuates basal and induced NO production (190) at concentrations known to predict CVD events.

Serum level of TNF-α is elevated in obesity, insulin resistance and T2DM, and a significant decrease is observed in TNF-α level after weight loss (191-193). TNF-α may contribute to insulin resistance since it has been shown to impair the initial steps of insulin signaling in animal models (137;194). Knock-out models have demonstrated increased kinase activity of the insulin receptor and preserved insulin sensitivity (195). Furthermore, TNF-α increases lipolysis by inhibiting LPL and stimulating hepatic lipase (HL) (196;197). TNF- α apparently contributes also to endothelial dysfunction (198;199). IL-6 has many properties identical to TNF-α (192;194;196), but most importantly it induces CRP production in hepatocytes.

2.4.2. Hypoadiponectinemia

Adiponectin is the most abundant adipokine in the plasma (average concentration of 5-10 μg/ml, higher in women than in men (200)). White adipose tissue is the only source of adiponectin, and in contrast to other adipokines, adiponectin level is inversely associated with insulin resistance, obesity, the MetS (201), T2DM (202) and atherosclerosis. In prospective studies low adiponectin level has been predictive of the development of insulin resistance, T2DM and CVD (203-205).

Adiponectin is composed of a collagen-like fibrous domain and C1q-like globular domain, and in circulation molecules form a wide range of multimers (206). Recently, two adiponectin receptors have been identified. AdipoR1, a receptor for globular adiponectin, is abundantly expressed in skeletal muscle, whereas AdipoR2, a receptor for full-length adiponectin, is mainly expressed in the liver (207). The mechanisms of adiponectin action have not yet been fully elucidated. However, rodent studies have established that adiponectin improves insulin

signaling in both skeletal muscle and liver. In skeletal muscle, adiponectin has been shown to increase the expression of molecules involved in FFA transport and energy dissipation leading to a decrease in tissue triglyceride content and improvement of insulin sensitivity (208). In hepatic tissue adiponectin has been shown to inhibit endogenous glucose output (209;210) and decrease lipid synthesis (211). Furthermore, a wide range of anti-atherosclerotic effects of adiponectin has been suggested (207). In mouse studies the administration of adiponectin or its globular domain has resulted in a significant weight loss without affecting food intake. This suggests that adiponectin may also play a role in the regulation of energy expenditure. A recent study suggested that adiponectin increases energy expenditure by acting in the brain (212). Adiponectin injected in intracerebrospinal fluid increased substantially expression of uncoupling protein-1 (UCP-1) in brown adipose tissue (BAT) and hypothalamic expression of corticotropin-releasing hormone (212) that has been shown to control sympathetic outflow from CNS leading to induction UCP-1 in BAT (213;214). Human studies have not established the role of adiponectin in intermediary metabolism or energy expenditure. A summary of different actions of adiponectin, based on animal models, is presented in figure 4.

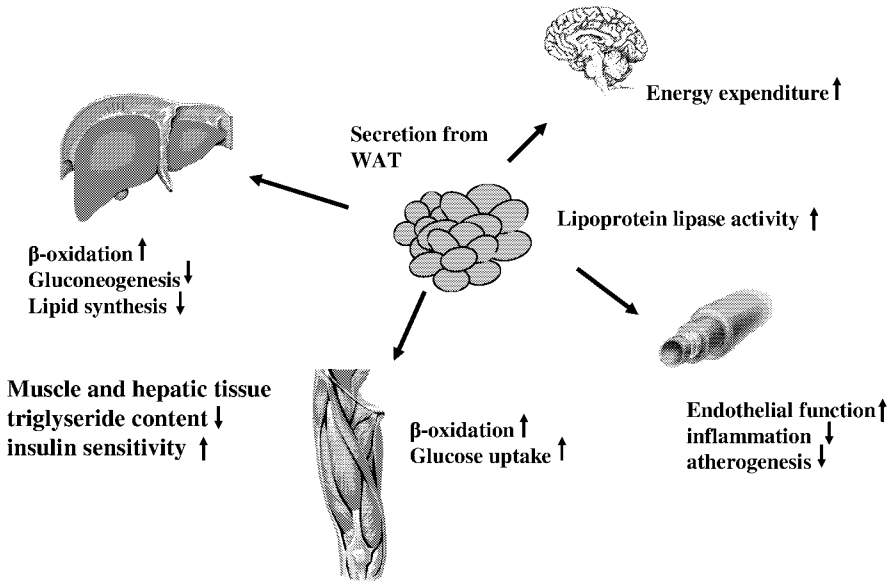

Figure 4. Actions of adiponectin based on animal studies. Adiponectin improves insulin sensitivity in hepatic and skeletal muscle tissue by decreasing tissue triglyceride content. Adiponectin enhance lipid clearance also by stimulating lipoprotein lipase activity. In vasculature adiponectin has many antiatherogenic functions. Adiponectin has also been suggested to increase energy expenditure via central nervous system activity. (Modified from ref 20-23; 194-195)

2.4.3. Prothrombotic state

The prothrombotic state present in the MetS is due to numerous perturbations in the coagulation pathway, fibrinolytic pathway and platelet function (215). Increased plasma levels of fibrinogen reflect the activation of the coagulation pathway. Fibrinogen provides the final substrate for thrombus formation and polymerizes to fibrin fibers after the activation by thrombin. Increased levels of fibrinogen are associated with both low-grade chronic inflammation and insulin resistance in the MetS (216). It also has independent predictive value for future CVD (217). Furthermore, insulin resistance has been associated with increased level of coagulation factors VII-IX (218). Increased level of soluble tissue factor that has a pivotal role in the activation of the external coagulation pathway has been associated with obesity (219;220).

Hypofibrinolysis, as a consequence of increased level of plasminogen activator inhibitor-1 PAI-1, is well documented in the MetS (221) and contributes to increased risk of CVD (217). The physiological role of PAI-1 is to control the degradation of fibrin and inhibit endogenous thrombolysis. Chronic inflammation, insulin resistance and visceral obesity contribute to increased levels of PAI-1 in the MetS (221;222).

Platelet function is also disturbed in insulin resistance. Normally, insulin decreases platelet aggregability through the reduction of platelet responses to adenosine diphosphate (ADP) and thrombin. Platelets from obese insulin-resistant subjects have reduced sensitivity to this anti-aggregatory effect of insulin (223). Dyslipidemia and angiotensin II may also contribute to increased platelet activation (224;225). Furthermore, the levels of von Willebrand factor may also be increased in the MetS (218).

2.4.5. Endothelial dysfunction

The endothelium is involved in the regulation of vascular tone, platelet adhesion, coagulation, fibrinolysis and leukocyte adherence, but the term endothelial dysfunction specifically refers to impaired endothelium-dependent relaxation caused by a loss of NO bioactivity in the vessel wall (226). NO is the mediator of many other functions besides vasodilatation exerted by intact endothelium. Disruption of functional integrity of the vascular endothelium plays an integral role in all stages of arteriosclerosis, from lesion initiation to plaque rupture. Endothelial dysfunction also predicts future adverse CVD events (227). Endothelial dysfunction characterizes all insulin resistant conditions (226) and links insulin resistance to

CVD. Potential contributors to decreased vascular NO bioactivity (decreased synthesis and/or accelerated degradation) in the MetS include impaired insulin action in vasculature (148), low HDL cholesterol (164), oxidized LDL particles (163), reactive oxygen species, FFAs, cytokines (228) and hyperglycemia (229). Microalbuminuria is also associated with MetS, and is often regarded as a manifestation of endothelial dysfunction. Microalbuminuria is a strong independent predictor of CVD events (230).

2.4.6. Hyperuricemia

Hyperuricemia clusters with other components of the MetS in population-based studies (231) and correlates significantly with insulin resistance assessed by the hyperinsulinemic clamp (232). At least partially hyperuricemia is due to reduced insulin stimulated renal uric acid clearance (233).

2.5. Factors associated with lifestyle

Sedentary lifestyle is associated with several features of the MetS (234-236). Both a low level of leisure-time physical activity and poor cardiorespiratory fitness assessed by maximal oxygen uptake (VO_2max) predict the development of the MetS (237). Moderate alcohol consumption has been associated with a low risk of the MetS in some (236;238), but not in all studies (239). Studies concerning the relationship of cigarette smoking and MetS risk are controversial (238;240;241). In one randomized single-blind trial a Mediterranean-like diet reduced the risk of the MetS during the 2-year follow-up compared to a control diet (242).

2.6. Genetics of the metabolic syndrome

Genetic factors influence the components of the MetS and predispose to the MetS together with environmental and behavioral factors. Genetic factors contributing to the syndrome are still largely unknown. However, it is likely that a large number of genes interact with each other and environment, and thus contribute to the risk of the MetS.

Insulin resistance clusters in families. In FDRs of patients with T2DM 45% are insulin resistant compared with 20% of individuals without a family history of diabetes (18). The estimates of the heritability of obesity vary greatly, but generally about one-half of the variation in body weight is explained by genetic factors (243;244). Genetic factors influence also the distribution of adipose tissue. Genes are considered to explain about 60% of the

variance in abdominal fat in menopausal women (19). Furthermore, first-degree relatives of T2DM have an increased WHR compared to their spouses without a family history of T2DM (18). Heritability also influences other components of the MetS such as blood pressure (245), HDL cholesterol, triglycerides (246), and microalbuminuria (247).

According to the 'thrifty gene' hypothesis, genes that gave a survival benefit to our ancestors by maximizing the ability to store energy predispose to obesity and T2DM when exposed to sedentary lifestyle and high caloric intake typical to the Western world (20). Thus, the 'thrifty genes' could also predispose to the MetS.

Two major approaches have been used in the search of the thrifty genes, the candidate gene approach and genome-wide scanning (248). The candidate gene approach aims to identify genes based on information about their function. Genes affecting body weight and fat distribution, lipolysis, fuel oxidation or skeletal muscle glucose metabolism could predispose to the MetS (249). Such candidate genes include β_2- and β_3 -adrenergic receptors (250;251), hormone-sensitive lipase (252), uncoupling proteins (253), peroxisome proliferators-activated receptor gamma-2 (254), TNF-α (255), insulin receptor substrates (256) and glycogen synthase (257). In addition, some rare forms of monogenic obesity as a result of mutations in leptin, leptin receptor, melanocortin 4-receptor, pro-opiomelanocortin and endopeptidase prohormone convertase-1 have been described, but these genes may not contribute to the thrifty genotype (258).

Variation in the adiponectin gene (*AMP1*) could also contribute to increased risk of the MetS. In fact, recent genome-wide scans have mapped a susceptibility locus of the MetS and T2DM on chromosome 3q27, where *AMP1* is located (259;260). Subsequently, several single nucleotide polymorphisms (SNPs, 45T→G, 276G→T, 517T→C) and haplotypes of the *AMP1* have been associated with hypoadiponectinemia, insulin resistance and T2DM (261-265). Reduced circulating adiponectin levels have also been observed in FDRs of T2DM (266).

The Calpain-10 gene (*CAPN10*), located on chromosome 2q37, was the first T2DM susceptibility gene identified by a genome-wide scan and positional cloning (267;268). Calpain-10 protein is an atypical member of Ca^{2+}-activated cysteine proteases found ubiquitously in human tissues (269). However, the physiological function and potential molecular mechanisms by which calpain-10 contributes to the risk of T2DM are still largely unclear.

Several genetic variations in *CAPN10* have been suggested to contribute to increased risk of T2DM. Originally, a haplotype of *SNP-43*, *Indel-19* and *SNP-63* (112/121) was associated

with a 3-fold increased risk of T2DM in Mexican Americans and Europeans (Finnish and German populations) (268). The population-attributable risk in Mexican-Americans was estimated to be 14%, but only 4% in Europeans because of significantly lower frequency of the risk haplotype (268). Subsequently, the association of genetic variation of *CAPN10* with T2DM has been reproduced in other populations (270-272). In Pima Indians the G/G genotype of *SNP-43* was associated with decreased rates of glucose oxidation and preferential oxidation of lipids coupled with decreased rate of gluconeogenesis evidenced by a decreased sleeping metabolic rate (273). Furthermore, reduced CAPN10 mRNA expression in skeletal muscle was found in subjects with the G/G genotype of *SNP-43* (273). The G/G genotype of *SNP-43* was speculated to contribute to the thrifty genotype since the associated metabolic changes enable the preservation of muscle protein and glycogen stores, both advantageous traits during food deprivation (273). The G allele of *SNP-43* has also been associated with increased first-phase insulin secretion (274) and reduced expression of CAPN10 mRNA in subcutaneous and visceral fat coupled with elevated triglyceride levels in obese subjects (275). In animal models the exposure of mouse pancreatic islets to calpain inhibitors (not specific to calpain-10) has increased the insulin secretory response to glucose. In contrast, the inhibition of calpain in skeletal muscle cells or adipocytes has led to a reduction in insulin stimulated glucose uptake (276).

3. AIMS OF THE STUDY

This study was undertaken to perform detailed metabolic characterization and assessment of adipose tissue distribution in offspring of T2DM. The general aim was to investigate the metabolic consequences of abdominal obesity, the core element in pathophysiology of the MetS, and the association of genetic varians with abdominal obesity and related traits. The more specific aims were:

1. To investigate the clustering of cardiovascular risk factors associated with the MetS by using factor analysis and to investigate the alterations in substrate oxidation, insulin sensitivity, energy expenditure, adipose tissue distribution and levels of adipokines and adhesion molecules associated with the MetS (Study I).
2. To assess the alterations in substrate oxidation, insulin sensitivity, energy expenditure, adipose tissue distribution and biochemical measurements with respect to adiponectin levels (Study II)
3. To evaluate the alterations in substrate oxidation, insulin sensitivity, energy expenditure and biochemical measurements with respect to adipose tissue distribution (Study III)
4. To investigate the alterations in substrate oxidation, insulin sensitivity, energy expenditure, adipose tissue distribution and biochemical measurements with respect to variants in the adiponectin and calpain-10 genes (Studies II and IV)

4. SUBJECTS AND METHODS

4.1. Subjects

The subjects of this study were nondiabetic offspring of patients with T2DM. The probands were randomly selected among T2DM patients living in the Kuopio University Hospital region. The spouses of patients with T2DM had to have a normal glucose tolerance (NGT). From 1 to 3 offspring of each family were included in the study and altogether 158 offspring of T2DM and 20 control subjects with no family history of T2DM were studied. 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 under 25 or over 50 years.

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 the first visit subjects were interviewed regarding their medical history, tobacco and alcohol consumption and exercise habits. Blood pressure was measured in a sitting position after a 5-min rest with a mercury sphygmomanometer. 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). 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 lowest rib) and hip circumference (at the level of the trochanter major) were measured to the nearest 0.5 cm. Blood samples were drawn after 12-h fast followed by an OGTT. On the second occasion bioelectric impedance measurement and indirect calorimetry were performed after 12 hours fast followed by an IVGTT and the hyperinsulinemic euglycemic clamp, respectively. Indirect calorimetry was reperformed during the last 30-min of the euglycemic clamp. On the third occasion a CT scan for the evaluation of the abdominal fat area and exercise test to determine maximum oxygen uptake were performed.

4.3. Oral glucose tolerance test

In a 2-hour OGTT (75g 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 (22) were included in the study.

4.4. Intravenous glucose tolerance test

An IVGTT was performed to determine the first phase insulin secretion capacity (277). After an overnight fast an intravenous catheter was placed into the left antecupital 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 performance of indirect calorimetry a bolus of glucose (300 mg/kg in a 50% solution) was given within 30 seconds into the antecupital 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.

4.5. Euglycemic clamp

The degree of insulin sensitivity was evaluated with the euglycemic hyperinsulinemic clamp technique (108). 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/m^2 body surface area. The resulting average plasma insulin concentration was 67.9 ± 15.1 mU/l and 58.1 ± 7.76 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 WBGU. Samples for plasma lactate, insulin and serum FFA measurements were drawn at 0 and 120 min.

4.6. 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 nonprotein nitrogen excretion rate (278). The fraction of carbohydrate nonoxidation during the euglycemic clamp was estimated by subtracting the carbohydrate oxidation rate from the glucose infusion rate.

4.7. 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, Germany) at the level of fourth lumbal vertebra. Subcutaneous and IAF areas were calculated as previously described (65).

4.8. Cardiopulmonary exercise test

The cardiopulmonary test was performed with bicycle ergometer (Siemens Elema 380) until exhaustion. Respiratory gas exhange was analyzed continuously during the test with a computer-based system (Sensor Medics 2900, Metabolic Measurement Cart/System). The average values of oxygen uptake measured during the last 20 seconds of the exercise were used to calculate VO_2max.

4.9. 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 (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden). Serum lipid and lipoprotein concentrations were determined from fresh serum samples drawn after a 12-h overnight fast. Cholesterol and triglyceride levels from the whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Roche Diagnostics, Mannheim, Germany). Serum FFAs were determined by an enzymatic method from Wako Chemicals GmbH (Neuss, Germany). Plasma concentration of TNF-α, IL-1β, IL-1 RA, IL-6, IL-8 and 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 by solid phase

ELISA (Quantikine, R&D Systems, Inc, MN, US and IL-8 UltraSensitive ELISA, BioSource International, Inc., Camarillo, CA, US). CRP was determined by Immulite 2000 High Sensitivity CRP assay (DPC, Los Angeles, CA, US) and adiponectin by the Human Adiponectin ELISA Kit (B-Bridge International, Inc, San Jose, CA, US). Nonprotein urinary nitrogen was measured by automated Kjeldahl method (279).

4.10. DNA analyses

The SNaPshot ddNTP Primer Extension Kit technique was used to genotype SNP 45 T/G and SNP 276 G/T of the adiponectin gene. Forward, 5´-GGCTCAGGATGCTGTTGCTGG-´3, and reverse, 5´-GCT TTG CTT TCT CCC TGT GTC T-´3, primers were used to amplify a 328 bp size DNA fragment. Primers used to determine the genotypes were 5´-CTGCTATTAGCTCTGCCCGG-3´ for the SNP +45 T/G polymorphism and 5´-ACCTCCTACACTGATATAAACTAT-3´ for the SNP +276 G/T polymorphism. Primer extension products were analyzed with Abi PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA).

Genotyping of SNP-43, SNP-44 and SNP-63 of CAPN10 were determined with the TaqMan Allelic Discrimination Assays (Applied Biosystems, Foster City, CA). The TaqMan genotyping reaction was amplified on a GeneAmp PCR system 2700 and fluorescence was detected using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The following primers and probes were used:

SNP	Forward primer	Reverse primer
CAPN10-43	GCGCTCACGCTTGCT	CCTCACCAAGTCAAGGCTTAGC
CAPN10-44	GCAGGGCGCTCACG	CCTCACCAAGTCAAGGCTTAGC
CAPN10-63	TCGGTCAGAGCCCTAGCA	TCCAGCCTCTTAGGAAGCTTCT
	FAM-probe	**VIC-probe**
CAPN10-43	FAM-AAGTAAGGCATTTGAAG-NFQ	VIC-AAGTAAGGCGTTTGAAG-NFQ
CAPN10-44	FAM-CCTTACTTCACAGCAAG-NFQ	VIC-CCTTACTTCGCAGCAAG-NFQ
CAPN10-63	FAM-CCCCTCACTCCACC-NFQ	VIC-CCCCTCGCTCCACC-NFQ

The insertion/deletion polymorphism Indel-19 of CAPN10 was amplified by published PCR primers (280), and PCR products were separated on a 3% NuSieve agarose gel. We used the following coding for the alleles of CAPN10; SNP-43: G = allele 1, A = allele 2; SNP-44: T =

allele 1, C = allele 2; Indel-19: 2 copies of a 32bp repeat = allele 1, 3 copies of a 32bp repeat = allele 2; and SNP-63: C = allele 1, T = allele 2. Only four SNP-43, Indel-19 and SNP-63 haplotypes were found (111, 121, 112 and 221). In this study the C allele of the SNP-44 was observed only with haplotype 111.

4.11. Statistical analysis

All data analyses were performed with the SPSS 10.0 or 11.0 for Windows program (SPSS Inc, Chicago, Illinois, USA). The results for continuous variables are given as means ± SD. Variables with skewed distribution were logarithmically transformed for statistical analyses. The differences between the groups were assessed with one-way analysis of variance (ANOVA) for continuous variables and with 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) principal component method was used for extraction of the initial components. Factors with eigenvalues ≥1 were retained and varimax rotation was applied for the elucidation of factors. Variable loadings ≥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. Adiponectin haplotype frequencies were estimated using the EH software (available from **ftp://linkage.rockefeller.edu/software/eh/**). Linkage disequilibrium between the two adiponectin SNPs was calculated with the two-locus linkage disequilibrium calculator (from http://web1.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software.shtml). For the haplotype analysis of CAPN10, haplotype frequencies were estimated and likely haplotypes reconstructed for each individual using the MERLIN program (281). The effect of each haplotype on quantitative parameters was analyzed with the family based test of linkage disequilibrium using the quantitative transmission disequilibrium test (QTDT) program with age, gender and BMI as covariates, when appropriate (282).

4.12. Approval of the Ethics Committee

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

5. RESULTS

5.1. Characteristics of study subjects

The basic clinical and laboratory characteristics of study subjects are shown in Table 2. Among 158 subjects 24 (15.2%) had IGT. For further statistical analysis subjects were grouped according to the MetS factor score (Study I), levels of adiponectin (Study II) and total body fat and its distribution (Study III).

Table 2. Clinical and laboratory characteristics of the study subjects

	Offspring of T2DM			Controls
	Study I	**Study III**	**Studies II and IV**	**Study II**
	N=119	N=129	N=158	N=20
Men/Women	55/64	59/70	70/88	8/12
Age (years)	35.5 ± 6.0	35.7 ± 6.3	34.9 ± 6.3	34.9 ± 4.3
Body mass index (kg/m^2)	26.1 ±4.7	26.2 ±4.6	26.2 ±4.9	24.4 ±2.5
Waist (cm)	88 ± 12	89 ± 12	88 ± 12	82 ± 9
Systolic blood pressure (mmHg)	126 ± 11	127 ± 13	127 ± 12	123 ± 10
Diastolic blood pressure (mmHg)	84 ± 9	84 ± 10	84 ± 9	81± 10
Fasting plasma glucose (mmol/L)	5.1 ± 0.4	5.1 ± 0.4	5.1 ± 0.4	5.0 ± 0.54
120min plasma glucose (mmol/L)	6.2 ± 1.4	6.3 ± 1.4	6.3 ± 1.4	5.4 ± 1.06
Fasting plasma insulin (pmol/L)	46.2 ± 22.5	47.3 ± 22.7	51.0 ± 26.9	41.6 ± 20.2
120 min plasma insulin (pmol/L)	245.6 ± 195.2	252.8 ± 196.0	267.1 ± 201.8	171.6 ± 101.6
Total cholesterol (mmol/L)	4.90 ± 0.87	4.90 ± 0.87	4.90 ± 0.87	4.64 ± 0.94
HDL cholesterol (mmol/L)	1.27 ± 0.28	1.27 ± 0.28	1.27 ± 0.29	1.38 ± 0.32
Total triglycerides (mmol/L)	1.13 ± 0.60	1.13 ± 0.60	1.17 ± 0.60	1.17 ± 0.84

HDL= high density lipoprotein

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

The variables representing the components of the MetS were included in factor analysis (120 min glucose, fasting insulin, waist, BMI, HDL cholesterol, total triglycerides, mean blood pressure). There were significant correlations among the variables, with the highest correlations between the parameters measuring obesity (waist and BMI, r=0.523, P<0.001), whereas mean blood pressure correlated only weakly with other components of the MetS (<0.40). Fasting plasma insulin correlated significantly with the rates of WBGU during the clamp (r=–0.572, P<0.01) and waist circumference with intra-abdominal fat area assessed by CT (r=0.700, P<0.01).

Table 3 presents the results of factor analyses. Model 1, including, among the other variables, simple clinical measures of insulin resistance (fasting insulin) and abdominal fat (waist) resulted in one factor, the MetS factor, explaining 46.2% of total variance. Waist (0.830) and fasting insulin (0.760) had the highest loadings on this factor. Substituting waist by IAF area 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 as in Model 1 (43.3%). When BMI was excluded from the analyses the results remained essentially unchanged. However, when IAF was replaced by subcutaneous fat or when both systolic and diastolic blood pressures were included, a 2-factor solution was obtained.

Table 3. Results of factor analyses using different measurements of insulin sensitivity (fasting insulin or rates of whole body glucose uptake) and visceral obesity (waist circumference or intra-abdominal fat in CT)

	Model 1	**Model 2**
	Factor 1	Factor 1
120 min blood glucose	0.532	0.574
Fasting insulin (log)	0.760	
Body mass index	0.781	0.666
Waist	0.830	
HDL cholesterol	-0.637	-0.654
Total triglycerides (log)	0.646	0.719
Mean blood pressure	0.502	0.460
Whole body glucose uptake		-0.678
Intra-abdominal fat in CT (log)		0.802
% of variance explained	**46.2**	**43.3**

For further analysis, the study subjects were grouped into the tertiles according to the factor scores obtained in Model 1. Subjects in the highest factor score tertile were defined as having the MetS. During hyperinsulinemia the highest factor score tertile was associated with decreased rates of glucose oxidation (P<0.001, adjusted for gender, Figure 5 A) and nonoxidative glucose disposal (P<0.001, adjusted for gender, Figure 5 A), high rates of lipid oxidation (P=0.001, adjusted for gender, Figure 6 C), low energy expenditure (P=0.031, adjusted for gender, Figure 6 A) and impaired suppression of free fatty acids (P=0.003, adjusted for gender, Figure 6 B). Furthermore, subjects with the MetS had a high amount of IAF (P<0.001, adjusted for gender, Figure 5 C), high levels of CRP (P<0.001, adjusted for gender and IAF, Figure 7 A), pro-inflammatory cytokines (IL-1β: P=0.015, IL-1 receptor antagonist (IL-1 RA): P=0.002, IL-6: P=0.042, IL-8: P=0.014, all P values adjusted for gender and IAF, Figure 7 A-F) and adhesion molecules (Figure 8 A-D), hypoadiponectinemia (P=0.001, adjusted for gender and IAF, Figure 5 B), and low maximum oxygen uptake (P<0.001, adjusted for gender, Figure 6 D).

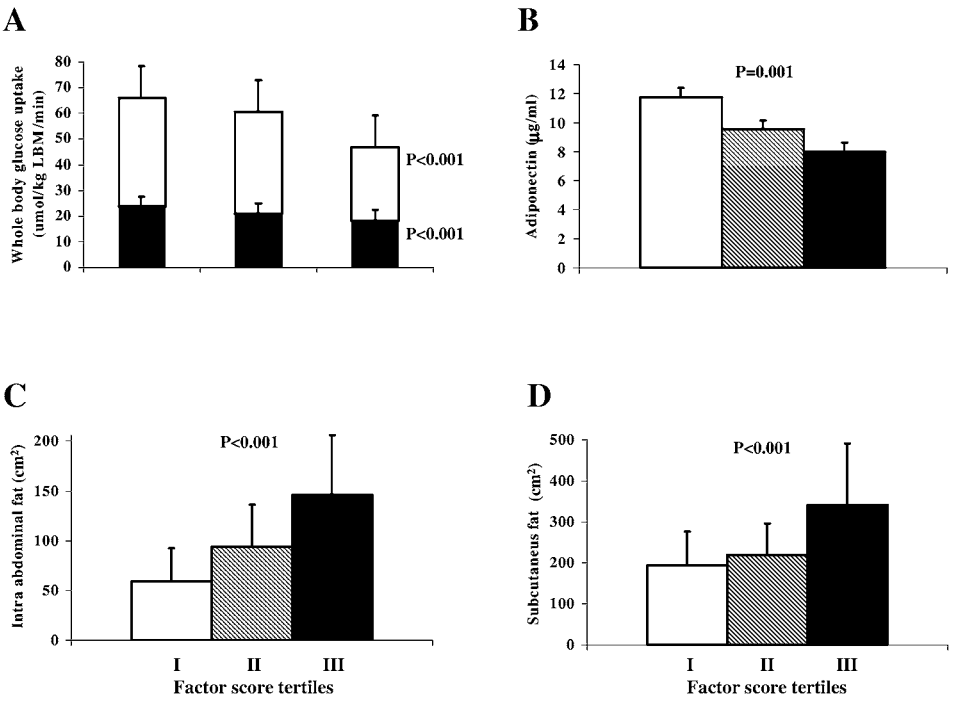

Figure 5. Rates of whole body glucose uptake (■ glucose oxidation, □ non-oxidative glucose disposal, P values given respectively) (A), adiponectin concentration (B), intra-abdominal fat mass (C), and subcutaneous fat mass (D) according to the factor score tertiles (I = lowest, II = middle, III = highest tertile) derived from factor analysis. P values are unadjusted.

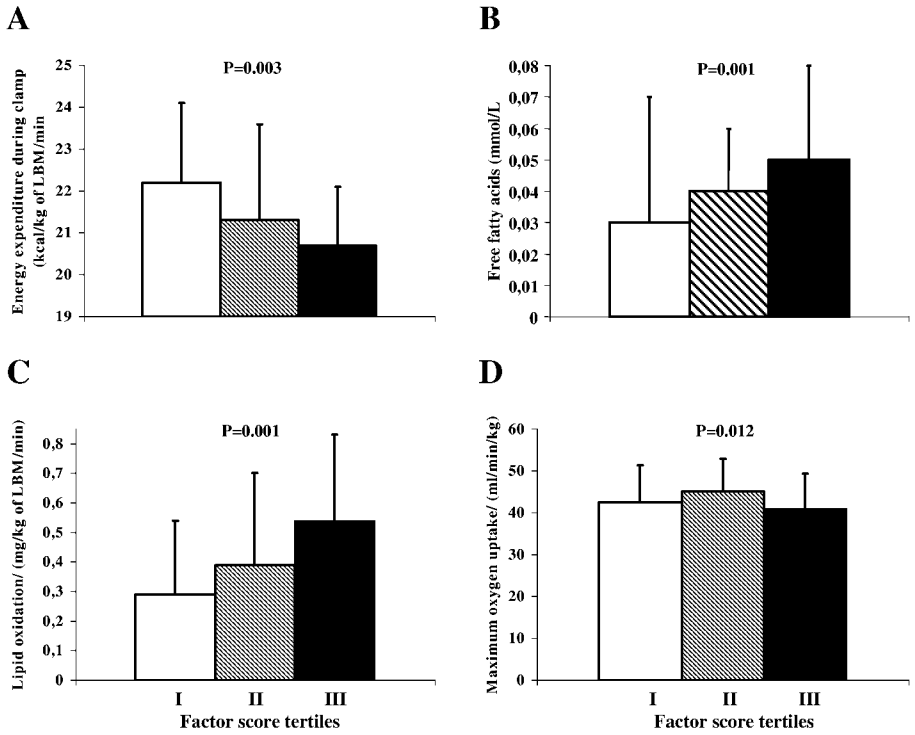

Figure 6. Energy expenditure during the hyperinsulinemic clamp (A), free fatty acid levels during the hyperinsulinemic clamp (B), lipid oxidation during the hyperinsulinemic clamp (C), and maximal oxygen uptake during exercise (D) according to the factor score tertiles (I = lowest, II = middle, III = highest) derived from factor analysis. P values are unadjusted.

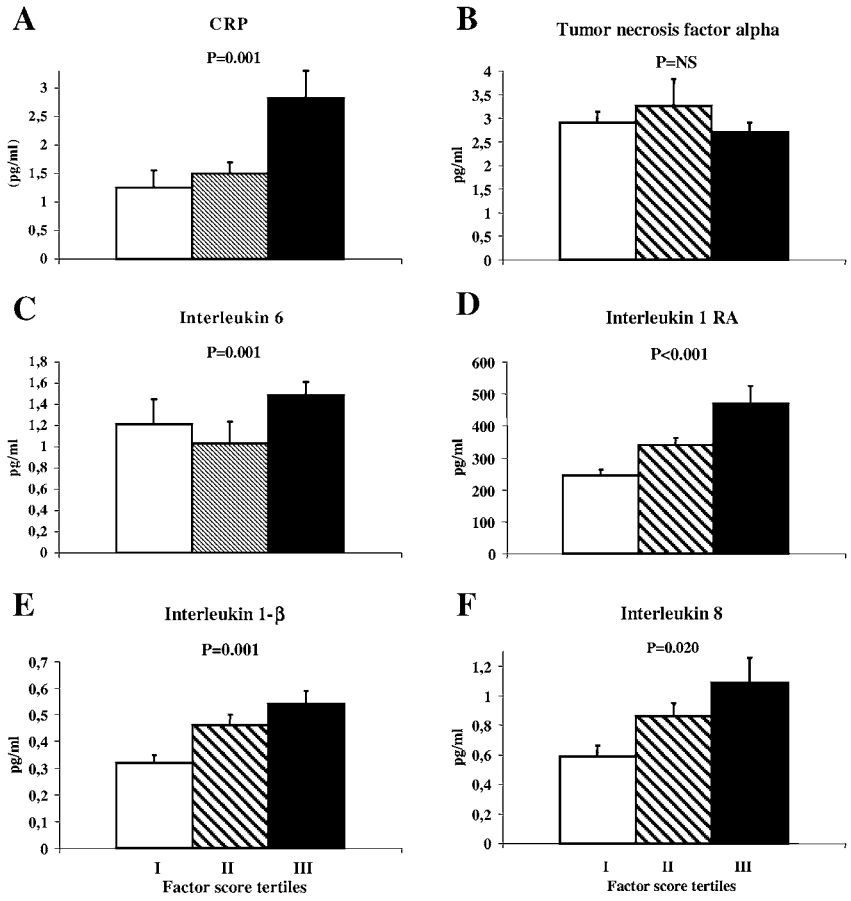

Figure 7. Fasting CRP (A) and cytokine levels (B-F) according to the factor score tertiles (I = lowest, II = middle, III = highest) derived from factor analysis. P values are unadjusted.

The gender -adjusted parameters between those with and without MetS according to the NCEP ATP III criteria (31) were also compared. Twenty-two subjects fulfilled the ATP III criteria. Subjects with the MetS had higher amount of IAF ($P < 0.001$), lower rates of WBGU ($P = 0.001$) and oxidative ($P=0.002$) and non-oxidative ($P=0.001$) 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 level ($P=0.002$) and maximum oxygen uptake ($P=0.001$) than subjects without the MetS.

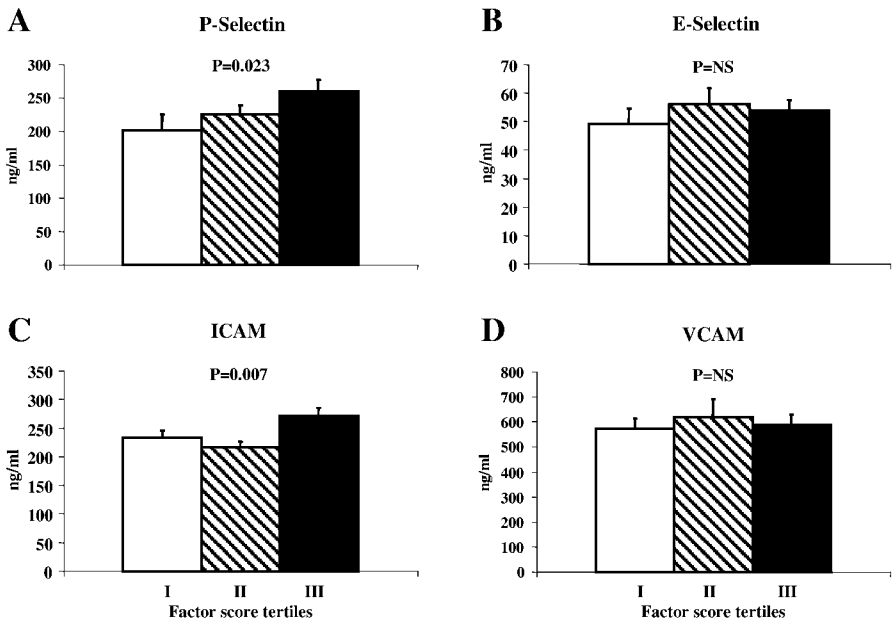

Figure 8. Fasting adhesion molecule levels (A-D) according to the factor score tertiles (I=lowest, II=middle, III=highest) derived from factor analysis. P values are unadjusted.

5.3. Metabolic profile stratified by adiponectin levels (Study II)

The basic characteristics of the offspring of T2DM patients according to the levels of adiponectin are presented in Table 4. Adiponectin levels were higher in women than in men (10.65 ± 4.19 and 7.76 ± 2.89 µg/ml respectively, P<0.001). There was also a tendency towards lower adiponectin concentration in offspring of diabetic patients compared to control subjects (ANOVA, p=0.053). However, the difference between normoglycemic offspring and control subjects was not statistically significant (p=0.064), and the difference between subjects with IGT and control subjects (p=0.017) was no longer significant if adjusted for gender and BMI (p=0.059) or waist (p=0.162). Adiponectin levels were associated with all components of the MetS (inversely with BMI, fasting plasma glucose, fasting plasma insulin, 2-hour plasma insulin in an OGTT and triglycerides, and positively with HDL cholesterol), and except for BMI the differences remained statistically significant after the adjustment for

gender and waist circumference, and except for fasting glucose the differences remained significant also after the adjustment for gender and visceral fat.

Table 4. Clinical and laboratory characteristics of the offspring of T2D patients according to the tertiles of adiponectin concentration.

	Tertiles of adiponectin concentration				
	I	II	III	P value	P* value
Men/Women	35/17	23/31	12/40	<0.001	
Age	34.4 ± 5.5	35.4 ± 6.8	34.9 ± 6.6	NS	NS
Adiponectin (μg/ml)	5.74 ± 0.95	8.60 ± 1.00	13.79 ± 3.43	<0.001	<0.001†
Body mass index (kg/m2)	27.2 ± 4.8	26.6 ± 5.0	24.7 ± 4.5	0.029	NS
Waist (cm)	92.6 ± 10.6	90.2 ± 12.8	82.3± 10.5	<0.001	0.001
Systolic blood pressure (mmHg)	129.6 ± 14.3	127.0 ± 12.2	124.8 ± 10.1	NS	NS
Diastolic blood pressure (mmHg)	85.1 ± 10.0	85.0 ± 9.3	81.5 ± 8.3	NS	NS
Fasting glucose (mmol/L)	5.33 ± 0.42	5.11 ± 0.42	4.98 ± 0.32	<0.001	0.046†
120 min glucose (mmol/L)	6.46 ± 1.37	6.32 ± 1.44	6.06 ± 1.42	NS	NS
Fasting insulin (pmol/L)	60.8 ± 29.9	55.3 ± 29.3	36.7 ± 10.3	<0.001	<0.001††
120 min insulin (pmol/L)	319.7 ± 218.4	298.3 ± 223.8	181.9 ± 119.7	<0.001	<0.001†
Total cholesterol (mmol/L)	5.01 ± 0.84	4.90 ± 0.92	4.75 ± 0.84	NS	NS
HDL cholesterol (mmol/L)	1.09 ± 0.20	1.29 ± 0.30	1.45 ± 0.25	<0.001	<0.001††
Total triglycerides (mmol/L)	1.38 ± 0.55	1.18 ± 0.66	0.94 ± 0.50	<0.001	0.014††

Data are mean ± SD. * P value adjusted using univariate analyses of covariance (ANCOVA) or Mixed Linear Model analysis where appropriate; † Adjusted for gender and waist circumference; †† Adjusted for familiality, gender and waist circumference; NS = not significant. Cut-off points for adiponectin tertiles: 7.00 μg/ml between first and second tertile and 10.40 μg/ml between second and third tertile.

Although no differences were observed in the fasting state (data not shown), there were significant differences in glucose metabolism during hyperinsulinemia stratified by the adiponectin tertiles (Figure 9). The rates of WBGU/lean body mass (LBM) were significantly higher in the highest adiponectin tertile concentration than in the two other tertiles (P<0.001 adjusted for gender, Figure 9A) and the differences were observed in both nonoxidative (P=0.001 adjusted for gender) and oxidative glucose disposal (P<0.001 adjusted for gender). Thus, low insulin response in an IVGTT observed in subjects with high adiponectin concentration reflected better insulin sensitivity (P=0.003 adjusted for gender, and P=0.016

adjusted for gender and BMI). The amount of IAF was inversely associated with adiponectin concentration (P = 0.001 adjusted for gender, Figure 9 B). No significant differences in the amount of subcutaneous fat between the adiponectin tertiles were found (P = 0.456, adjusted for gender).

Energy expenditure/LBM during the hyperinsulinemic clamp was also linked to adiponectin levels (P<0.001; P=0.044 adjusted for gender and waist; P=0.019 adjusted for gender and total fat mass, Figure 9 C). Similarly, the change in energy expenditure from fasting to the hyperinsulinemic state during the clamp increased among the adiponectin tertiles (P=0.005, P=0.019 adjusted for gender and waist). Furthermore, respiratory quotient (RQ) increased with increasing adiponectin level (P<0.001 and P = 0.049 adjusted for gender and waist) suggesting differences in fuel preference. Accordingly, there was a trend for lower lipid oxidation during the clamp with increasing adiponectin tertiles (P=0.041, Figure 9 D), but the differences were not statistically significant after the adjustment for gender and waist.

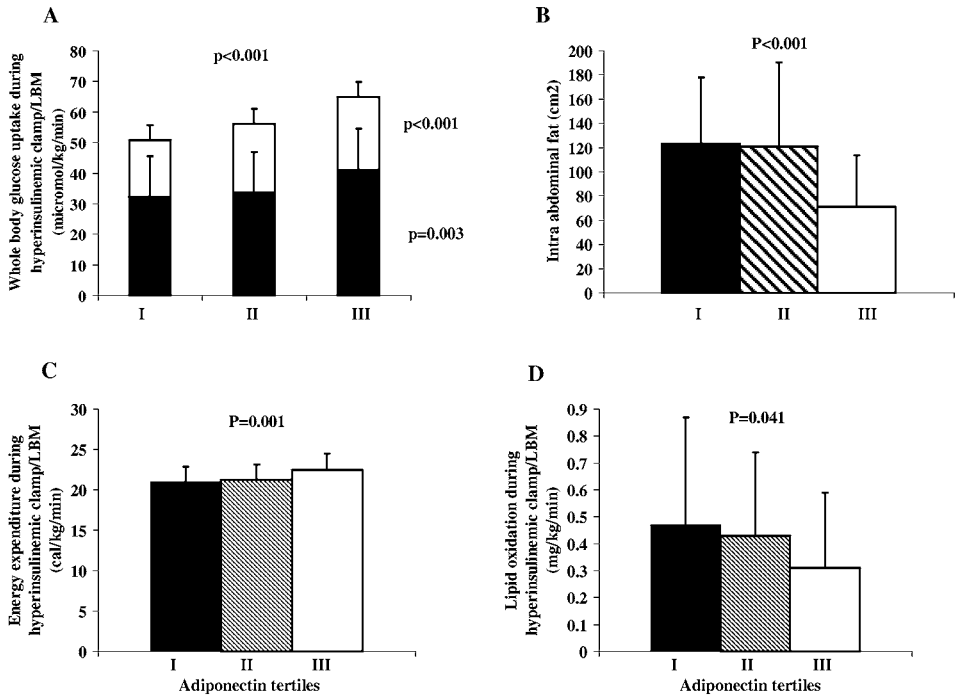

Figure 9. Rates of whole body glucose uptake (■ oxidative glucose disposal, non-oxidative glucose disposal) (**A**), intra-abdominal fat mass (**B**) Energy expenditure (**C**) and lipid oxidation (**B**) according to the adiponectin tertiles (I = lowest, II = middle, III = highest tertile). P values are unadjusted. In (A) P<0.001 stands for the differences in the rates of whole body glucose uptake and non-oxidative glucose disposal, and P=0.003 for the difference in oxidative glucose disposal.

5.4. Metabolic profile stratified by total amount and distribution of adipose tissue (Study III)

In order to investigate the relative contribution of general vs. abdominal obesity to metabolic consequences of obesity, subjects were grouped according to the gender-specific median of intra-abdominal fat mass (IAFM, 114.4 and 66.1 cm^2 for men and women, respectively) and total fat mass (TFM, 19.1 and 23.5 kg, respectively). These cut-off points were used to define the following four subgroups: subjects with low IAFM and TFM, subjects with high IAFM and low TFM, subjects with low IAFM and high TFM, and subjects with high IAFM and TFM.

In the entire study group the Pearson correlation coefficient between IAFM and TFM was 0.413 (P<0.001). Among the parameters measuring adiposity and fat distribution, TFM had

the highest correlation with subcutaneous fat mass (r=0.875, P<0.001), and IAFM with waist circumference (r=0.709, P<0.001). Within each subgroup the correlation between IAFM and TFM was no longer statistically significant, whereas the correlation between subcutaneous fat mass and TFM was statistically significant (r=0.675, P<0.001 in the group of low IAFM and low TFM; r=0.888, P<0.001 in the group of low IAFM and high TFM; r=0.610, P=0.007 in the group of high IAFM and low TFM; r=0.848, P<0.001 in the group of high IAFM and high TFM). The correlation between IAFM and waist circumference was significant in all groups (r=0.710, P<0.001 in the group of low IAFM and low TFM; r=0.587, P=0.010 in the group of high IAFM and low TFM; r=0.534, P<0.001 in the group of high IAFM and TFM) with the exception of subjects with low IAFM and high TFM (r=0.066, P=0.796).

Table 5 shows clinical and metabolic characteristics of the subgroups. Subjects with a similar amount of IAFM had similar metabolic characteristics, independently of total adiposity. High amount of IAFM was associated with adverse metabolic changes, such as high diastolic blood pressure, high fasting and 2-hour insulin, high 2-hour glucose, high triglycerides and low HDL cholesterol.

Similar differences between the groups according to the amount of IAFM were observed in substrate oxidation and energy expenditure during the hyperinsulinemic clamp. The rates of WBGU during the clamp were higher in subjects with low IAFM than in subjects with high IAFM (P<0.001, adjusted for gender and familiality, Figure 10 A). The differences were attributable to both oxidative and nonoxidative glucose disposal (P<0.001 and P=0.001, adjusted for gender and familiality, respectively, Figure 10 B-C). However, the differences between subjects with low IAFM and low TFM, and subjects with high IAFM but low TFM, did not reach statistical significance (P=0.053 for oxidative and P=0.098 for nonoxidative glucose disposal, adjusted for gender and familiality).

Table 5. Clinical and laboratory characteristics of the study subjects grouped according to the amount of total fatmass and intra-abdominal fat mass (IAFM). Group medians have been used as cut-off points separately for men and women.

	Low fatmass		**High fatmass**		
	low IAF	**high IAF**	**low IAF**	**high IAF**	**P value○**
	I	**II**	**III**	**IV**	
Number of cases	46	18	18	47	
Men/Women	21/25	8/10	8/10	22/25	ns
Age (years)	34.6 ± 6.45	37.2 ± 6.98	32.61 ± 4.69[2]	37.40 ± 5.96[3]	**0.015**
NGT/IGT	5	2	0	12	**0.042**
Body mass index (kg/m^2)	23.0 ± 2.24	24,5 ± 1.92[1]	26.6 ± 3.8[2]	29.71 ± 4.85[3]	**<0.001**
Waist (cm)	79.9 ± 7.13	87.58 ± 6.70[1]	88.36 ± 6.06	97.82 ± 11.41[3]	**<0.001**
Fat mass (kg)	16.6 ± 3.7	18.9 ± 2.0	26.0 ± 6.3	31.1 ± 9.2	**<0.001**
IAF (cm^2)	59.6 ± 24.2	126.7 ± 48.6	63.9 ± 19.7	152.3 ± 60.7	**<0.001**
Systolic BP (mmHg)	126.7 ± 12.5	129.4 ± 12.0	124.9 ± 8.4	127.4 ± 14.0	ns
Diastolic BP (mmHg)	81.4 ± 9.8	84.5 ± 9.5	81.6 ± 7.5	86.8 ± 9.6[3]	**0.025**
Fasting glucose (mmol/L)	5.07 ± 0.35	5.18 ± 0.38	5.06 ± 0.31	5.21 ± 0.48	ns
120min glucose (mmol/L)	6.12 ± 1.31	5.80 ± 1.39	5.67 ± 1.23	6.78 ± 1.49[3]	**0.008**
Fasting plasma insulin (pmol/L)	36.4 ± 12.7	50.3 ± 21.3[1]	40.1 ± 14.8	59.4 ± 27.2[3]	**<0.001**
120 min plasma insulin (pmol/L)	182.8 ± 125.8	248.8 ± 172.2	184.7 ± 129.7	349.0 ± 240.8[3]	**<0.001**
Total cholesterol (mmol/L)	4.71 ± 0.75	4.89 ± 1.01	4.84 ± 0.95	5.12 ± 0.90	ns
HDL cholesterol (mmol/L)	1.38 ± 0.26	1.14 ± 0.27[1]	1.37 ± 0.24[2]	1.15 ± 0.25[3]	**<0.001**
Total triglycerides (mmol/L)	0.96 ± 0.52	1.28 ± 0.79	1.03 ± 0.69	1.33 ± 0.56[3]	**0.005**

Data are mean ± SD. ○ adjusted for gender and familiality using Mixed Linear Model. [1] $P \leq 0.05$ for pairwise comparison between groups I and II, [2] $P \leq 0.05$ for pairwise comparison between groups II and III, [3] $P \leq 0.05$ for pairwise comparison between groups III and IV.
IAF = intra-abdominal fat, NGT=normal glucose tolerance, IGT=impaired glucose tolerance, BP= blood pressure, HDL = high density lipoprotein.

Energy expenditure was similar in subjects with high IAFM, independently of TFM, but significantly lower than in subjects with low IAFM (P<0.001, adjusted for gender and familiality, Figure 11 A).

Lipid oxidation during the clamp was highest in subjects with high IAFM and TFM (P<0.001, adjusted for gender and familiality, Figure 11 C). That was also seen in respiratory quotient which was lowest among subjects with high IAFM and TFM (P<0.001, adjusted for gender and familiality, Figure 11 B). No significant differences were observed in FFA levels in the fasting state or during the clamp (data not shown).

First-phase insulin secretion in an IVGTT was highest in subjects with high IAFM mass but low TFM (P=0.002, adjusted for gender and familiality) reflecting compensatory hyperinsulinemia to insulin resistance. However, this compensation was not observed in subjects with high IAFM and high TFM whose insulin level paralleled those of subjects with low IAFM and low TFM and subjects with low IAFM and high TFM (Figure 12 A).

There was a clear association between IAFM and adiponectin level independently of TFM (P=0.001, adjusted for gender and familiality, Figure 12 B). Similar findings were observed with respect to CRP level, although only the difference between subjects with low IAFM and high TFM, and subjects with high IAFM and TFM reached statistical significance (overall P=0.001, adjusted for gender and familiality, Figure 12 C).

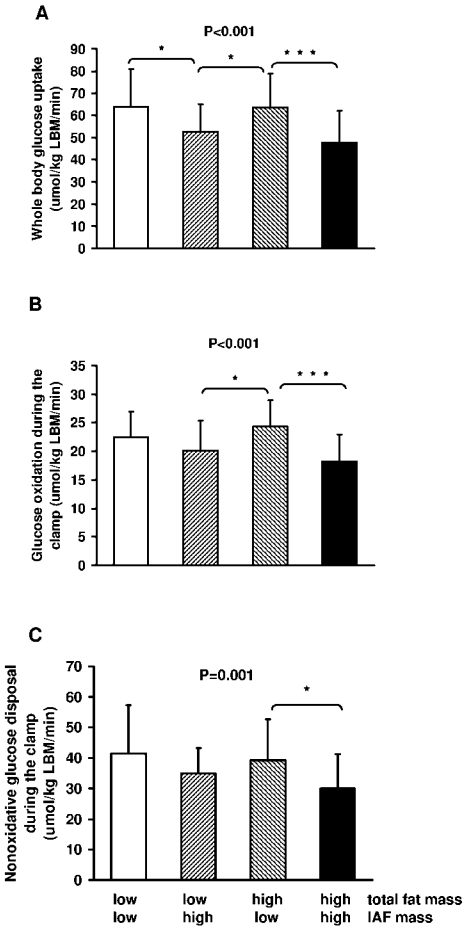

Figure 10. Rates of whole body glucose uptake (**A**) and oxidative (**B**) and nonoxidative (**C**) glucose disposal during the clamp in offspring of patients with T2DM according to the amount of total fat mass and intra-abdominal fat mass. P values are adjusted for gender and familiality. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 in pairwise comparison.

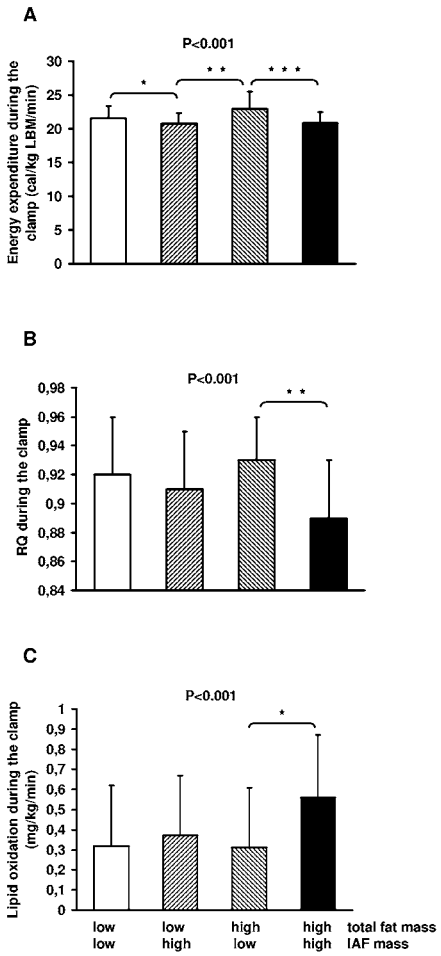

Figure 11. Rates of energy expenditure (**A**), respiratory quotient (**B**) and lipid oxidation (**C**) during the clamp in offspring of patients with T2DM according to the amount of total fat mass and intra-abdominal fat mass. P values are adjusted for gender and familiality. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 in pairwise comparison.

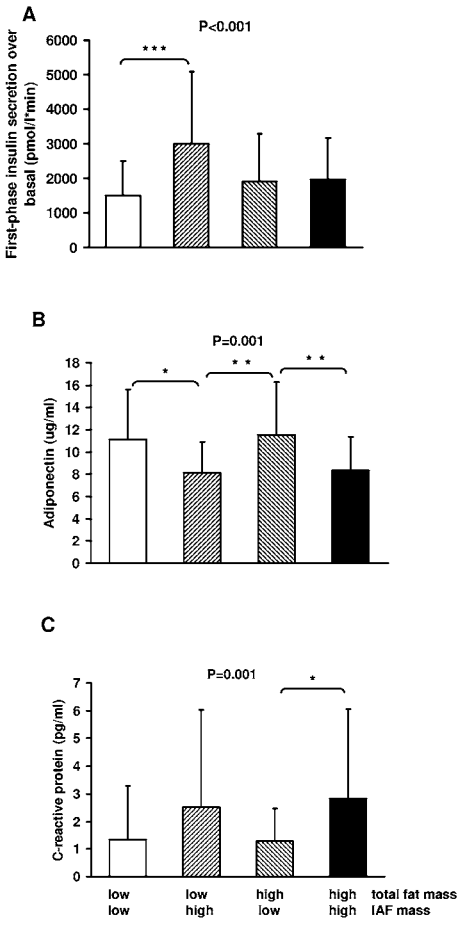

Figure 12. First-phase insulin secretion (**A**), and level of adiponectin (**B**) and C-reactive protein (**C**) in offspring of T2DM according to the amount of total fat mass and intra-abdominal fat mass. P values are adjusted for gender and familiality. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 in pairwise comparison.

5.5. Genotyping results (Studies II and IV)

Adiponectin gene (Study II)

Genotype distributions of *SNP +45* T/G and *SNP +276* G/T of adiponectin were in Hardy-Weinberg equilibrium, and SNPs were in linkage disequilibrium with each other (D′ = -0.997). Adiponectin level did not differ between the genotypes of *SNP +45* or *SNP +276*. Genotype combinations of *SNP +45* and *SNP +276* were formed as reported by Menzaghi et al (261), the risk genotype being TG/TG. Estimated haplotype frequencies of genotype combinations were 0.617 for TG, 0.340 for TT, 0.042 for GG and 0.001 for GT. No statistically significant differences in clinical or laboratory characteristics according to either *SNP +45/SNP +276* genotypes (data not shown) or their haplotypes were observed (Table 6).

Table 6. Clinical and laboratory characteristics of the offspring of patients with T2DM according to the genotype combinations of *SNP +45*(T/G) and *SNP +276*(G/T) of the adiponectin gene

	TG/TG	**TG/X**	**X/X**
	N=61	N=76	N=19
Men/Women	26/35	33/43	10/9
Adiponectin (μg/ml)	9.55 ± 4.16	9.28 ± 4.01	9.34 ± 3.10
Fasting glucose (mmol/l)	5.09 ± 0.38	5.15 ± 0.43	5.21 ± 0.41
120 min glucose (mmol/l)	6.13 ± 1.37	6.44 ± 1.51	6.13 ± 1.20
Fasting insulin (pmol/l)	8.54 ± 4.71	8.30 ± 4.13	8.39 ± 4.41
120 min insulin (pmol/l)	40.9 ± 28.3	47.7 ± 36.8	37.3 ± 23.7
First-phase insulin secretion (pmol/l*min)	2204.2 ± 1614.8	1977.9 ± 1463.7	1635.3 ± 970.7
WBGU /LBM (μmol/kg/min)	55.11 ± 16.35	57.68 ± 18.11	53.77 ± 15.27
Intra abdominal fat (cm^2)	101.0 ± 56.4	102.8 ± 68.5	112.9 ± 40.7
Subcutaneus fat (cm^2)	251.8 ± 117.9	252.1 ± 132.3	258.1 ± 86.8
Fasting energy expenditure /LBM (cal/kg/min)	20.7 ± 2.4	20.7 ± 1.9	20.2 ± 1.4
Energy expenditure during the clamp /LBM (cal/kg/min)	21.6 ± 2.2	21.7 ± 2.0	20.9 ± 1.53

No differences were statistically significant. WBGU= whole body glucose uptake, LBM= lean body mass.

Calpain-10 gene (Study IV)

The genotypes of *SNP-44*, *SNP-43*, *Indel-19* and *SNP-63* of *CAPN10* were in Hardy-Weinberg equilibrium. *SNP-43, Indel-19* and *SNP-63* were in linkage disequilibrium (LD) with each other (all pairwise D'>0.92). In addition, *SNP-44* was in linkage disequilibrium with SNP-43 and *Indel-19* (D'>0.80). Five haplotypes could be formed from the 4 polymorphisms (Table 7).

In QTDT analysis only the 1221 haplotype was significantly associated with the measures of glucose metabolism or visceral obesity. Subjects with this haplotype had higher levels of 2-h insulin in the OGTT (p=0.023, adjusted for age, gender and BMI), higher levels of fasting triglycerides (p=0.016), lower rates of WBGU (p=0.010) and higher amount of IAF (p=0.004) than subjects without the haplotype. No effect of this haplotype on BMI, total or HDL cholesterol, serum FFAs, insulin secretion (measured as insulin area under curve during the first 10 min of the IVGTT) or on the rates of energy expenditure and substrate oxidation was observed (data not shown). Combined haplogenotypes of *SNP-43*, *Indel-19* and *SNP-63* had no statistically significant effect on insulin sensitivity or visceral obesity (data not shown).

The effect of the 1221 haplotype was attributable to the effect of *SNP-43* only. *SNP-43* was associated with a high degree of visceral obesity (IAF; p=0.009, adjusted for age, gender and BMI) and tended to be associated with high 2-h insulin in an OGTT (p=0.059), high fasting triglycerides (p=0.071) and low rates of WBGU/LBM (p=0.062, adjusted for age and gender). The association of the A allele of *SNP-43* with low rates of WBGU was not significant if adjusted for IAF (p=0.449). However, the association with high amount of IAF (p=0.015), and high ratio of IAF to total abdominal fat (p=0.005) remained significant after the adjustment for the rates of WBGU.

Table 7. The effect of the CAPN10 haplotypes on glucose tolerance and hyperinsulinemia in an oral glucose tolerance test, fasting serum triglycerides, the rates of whole body glucose uptake (WBGU) and intra-abdominal fat (IAF) in 158 offspring of patients with type 2 diabetes (QTDT adjusted for age, sex and body mass index).

Haplotype SNP-44 – SNP-43 – Indel-19 – SNP-63	Freq.	Subjects (n)	Fasting glucose (mmol/L)	2h glucose (mmol/L)	Fasting insulin (pmol/L)	2h insulin (pmol/L)	Total triglycerides (mmol/L)	WBGU/LBM	IAF (cm^2)
1121	0.33	86	5.2±0.4	6.3±1.0	48.6±22.3	249.2±169.0	1.17±0.57	55.7±18.4	108±63
1221	0.23	65	5.2±0.4	6.5±1.5	55.5±30.3	311.5±225.5[a]	1.29±0.70[a]	52.6±16.2[a]	115±74[b]
2111	0.20	56	5.1±0.4	6.0±1.5	47.1±26.1	243.4±181.3	1.10±0.42	60.0±16.5	100±48
1111	0.13	40	5.1±0.4	6.1±1.0	50.7±24.8	224.6±155.0	1.23±0.59	54.8±14.8	104±60
1112	0.11	33	5.1±0.4	6.1±1.2	51.7±21.0	283.4±227.0	0.99±0.55	59.3±14.3	79±48

[a] $p<0.05$ and [b] $p<0.01$ in QTDT analysis against subjects without the studied haplotype. For coding of the genotypes, see Methods. HDL= high density lipoprotein. WBGU/LBM=whole body glucose uptake per lean body mass (µmol/kg of lean body mass/min). Mean±SD.

6. DISCUSSION

6.1. Study population

The probands were recruited from the Kuopio University Hospital District. Letters of invitation were sent to subjects with T2DM that had participated in our previous studies. Furthermore, probands were recruited by advertisements in local newspapers and health centers. The probands had to have drug treatment for T2DM or their diagnosis had to be confirmed according to the WHO criteria (283). Spouses of the diabetic patients underwent an OGTT and only offspring of diabetic probands whose spouses had normal glucose tolerance were invited to participate. Altogether 158 subjects (1-3 offspring from each family) were included in the study. Since hyperglycemia per se impairs both insulin secretion and insulin action (129), the study group was limited to nondiabetic subjects without chronic diseases or medication that could potentially disturb glucose metabolism. Thus, the study group was carefully selected for studies of metabolic and genetic variations associated with abdominal obesity and the MetS, and as such may not be representative of offspring of T2DM patients in general. The control subjects were healthy volunteers who had normal glucose tolerance (based on an OGTT) without a family history of T2DM. The size of the study group was large taking into account the laborious protocol.

6.2. Study design and methods

An evident strength of this study was a detailed phenotypic characterization of study subjects. Insulin sensitivity was assessed with the euglycemic hyperinsulinemic clamp, a method that is well validated and reproducible and can be considered to be the "gold standard" in the measurement of insulin sensitivity (27). Hyperinsulinemia during the clamp (insulin infusion of 40 mU/m^2) was adequate to completely suppress hepatic glucose output at least in normal weight subjects (284), and the duration of the clamp (120 min) was adequate to achieve the steady state of glucose disposal in nondiabetic subjects. Impairment of the first phase insulin secretion in an IVGTT has been considered to be the first detectable finding of defective β-cell function, although the hyperbolic association between insulin secretion and insulin sensitivity may lead to overestimation of insulin secretion capacity in insulin resistant subjects (285). Carrying out the euglycemic clamp immediately after an IVGTT has no significant effect on metabolic indices as

has been previously shown (286). CT, together with MRI, is the most accurate method available for the assessment of abdominal fat. CT was chosen because of better availability. An evident weakness of the study design is its cross-sectional nature.

Laboratory and genetic analyses were performed with standardized methods in the research laboratory of clinical chemistry and medicine. In statistical analysis the varying number of subjects from each family was taken into account by using the linear mixed model to adjust for familiality and other confounding factors. Factor analysis has many advantages compared to conventional statistical methods in study of the MetS. The method is well suited when there are complex intercorrelations between the variables included as is the case with the MetS. Instead of using different cut-off points factor analysis allows to include variables comprising the MetS as continuous variables. Furthermore, instead of having or not having the syndrome, the use of factor score allowed us to analyze the MetS as a continuous variable. Despite the advantages factor analysis has limitations. 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 leads to a two-factor solution). Furthermore, the number of variables has often impact on the number of factors. However, irrespective of these limitations factor analysis is a method which helps us to understand the clustering of cardiovascular risk factors belonging to the MetS.

6.3. Metabolic abnormalities associated with the MetS (Study I)

We identified the MetS factor by performing the factor analysis with the variables that have generally been included in the diagnostic criteria of the MetS. By grouping the study subjects into the tertiles according to the factor score and by identifying the subjects in the highest factor score tertile of the MetS, we observed a whole spectrum of metabolic abnormalities associated with the MetS. These individuals were insulin resistant, had increased amount of IAF, low levels of adiponectin, and high levels of inflammatory cytokines and adhesion molecules. A novel finding was that the MetS was also associated with low energy expenditure during hyperinsulinemia.

Subjects with the MetS have insulin resistance both in skeletal muscle and adipose tissue, as reflected by low rates of WBGU and high levels of FFAs (as a sign of impaired suppression of lipolysis) during hyperinsulinemia. Impaired suppression of lipolysis contributes to the dyslipidemia typical of insulin resistance (160). Furthermore, lipid oxidation during hyperinsulinemia was higher in subjects with the MetS than without the MetS. This finding may

be attributed to "metabolic inflexibility" which is a typical feature of insulin resistance, i.e. impaired ability to switch from predominantly lipid oxidation and high rates of lipid uptake during fasting conditions to suppression of lipid oxidation and increased glucose uptake and oxidation under insulin-stimulated conditions (287). Such a situation can contribute to lipid accumulation within skeletal muscle tissue and thus interference of the insulin signaling pathway (135;136;287). Metabolic inflexibility may also be viewed as a "thrifty" trait that provides a selective advantage in the setting of intermittent starvation, since the use of lipids by skeletal muscle tissue makes glucose more available for the brain thus reducing the need for gluconeogenesis from muscle proteins (288).

Our study also confirmed that low-grade inflammation and endothelial dysfunction are essential features of the MetS, as reflected by increased levels of CRP, inflammatory cytokines and adhesion molecules. These observations emphasize the atherogenic metabolic milieu of the MetS. Importantly, TNF-α, the most frequently studied cytokine that has previously been associated with insulin resistance (137;194;195) and endothelial dysfunction (198;199), was not associated with the MetS in our study. Instead, levels of IL-1β, IL-1RA, and IL-8 were linearly associated with the MetS factor score. Therefore, the commonly determined cytokines TNF-α and IL-6 may not be the best markers of low-grade inflammation and cytokine response in the MetS.

6.4. Abdominal obesity as a central feature of the MetS (Studies I, III and IV)

IAF, either measured by the waist circumference or CT, had the highest loading on the MetS factor. This suggests that of all the components of the syndrome, IAF has the highest correlation with the MetS (Study I). Although abdominal obesity has generally been recognized harmful, some controversy exists on the relative contribution of IAF and subcutaneous abdominal fat to metabolic disturbances (12-16). Our results clearly show that the adverse metabolic consequences of obesity are attributable to an increased amount of IAF. When study subjects were grouped according to the total amount and distribution of body fat tissue, subjects with similar amount of IAF had similar metabolic profile independently of the total amount of adipose tissue (Study III). Disadvantageous metabolic changes were observed only in subjects with increased amount of intra-abdominal fat. Similarly, when study subjects were grouped according to adiponectin level, no association was observed between adiponectin and subcutaneous fat mass despite a significant correlation between adiponectin and IAF (Study II). Our results imply that there are indeed

metabolically obese normal weight subjects attributable to high amount of IAF. Similarly, few previous studies have suggested an increase in total and IAF mass of these subjects despite of body weight in the normal range (289-291). Furthermore, the clustering of sedentary lifestyle, low birth weight and genetic factors have been associated with the phenotype (289;291). Conversely, our results also indicate that there are overweight subjects who have a low amount of IAF mass and metabolic profile similar to subjects with a low total and IAF mass. Probably these overweight subjects also have a lower risk for CVD than metabolically obese normal weight subjects. (Study III)

Our results are in accordance with previous findings that IAFM is closely associated with insulin resistance. Based on our study it is impossible to conclude which one of these two abnormalities is the primary defect. However, increased delivery of FFAs and inflammatory cytokines from expanded IAF depots with associated hypoadiponectinemia may be sufficient to bring about the whole spectrum of metabolic disturbances associated with insulin resistance and contribute to atherosclerosis. In agreement with this notion, a recent prospective study showed that the reduction of visceral fat mass resulted in a greater beneficial effect on insulin resistance than reduction of subcutaneous fat mass (292).

Genetic factors contributing to body weight and fat distribution are largely unknown. We observed that variants in *CAPN10* (A allele of SNP-43) are associated with IAF mass. Furthermore, this association remained significant even after the adjustment for the rates of WBGU. This is a novel finding suggesting that the previously observed association of *CAPN10* with DM2 (268) may be related to an increase in IAF. (Study IV)

Variants in *CAPN10* are likely to affect the levels of calpain-10 expression found in human tissues (275). Because the physiological function of calpain-10 is not yet fully known, potential mechanisms by which calpain-10 could affect adipose tissue metabolism also remain unclear. There are few previous studies that have found associations of the variants of *CAPN10* with lipid metabolism. In a Swedish study no association between *SNP-43* of *CAPN10* and obesity was seen, but obese subjects with the GG genotype of *SNP-43* had significantly elevated triglyceride levels compared with subjects with the A allele (275). Furthermore, *CAPN10* expression in fat tissue was reduced in subjects with the GG genotype (275). In another study the G allele of *SNP-43* of *CAPN10* was associated with increased levels of FFAs (272). Since the risk of T2DM has also been associated with the G allele of *SNP-43* (271) or a haplotype carrying this allele

(268;270;272), there is an evident discrepancy between the results of different studies. Reasons for these differences are unknown, but may be partly relate to the cross-sectional study design which increases the likelihood of confounding.

Thus, while many studies support the notion that calpain-10 plays a role in T2DM and related phenotypes, the results have been variable and further studies are needed, especially to investigate the physiological function of calpain-10.

6.5. Hypoadiponectinemia as a single marker of the MetS (Study II)

Adiponectin is the most abundantly produced adipokine having insulin-sensitizing and anti-atherosclerotic properties (207). Most currently available data on adiponectin has been derived from animal and in vitro studies, but the association of adiponectin with insulin resistance, DM2 and CVD has been constantly observed also in human studies (293-295). Similarly, in our study adiponectin was positively associated with the rates of WBGU, independently of confounding factors, including the amount of IAF. Consequently, the levels of adiponectin were negatively associated with lipid oxidation and FFA levels during the hyperinsulinemic euglycemic clamp. The novel finding was that adiponectin was also associated with energy expenditure during the clamp (see below). Furthermore, hypoadiponectinemia was in our study related to high fasting levels of plasma glucose, triglycerides, low levels of HDL cholesterol, increased amount of IAF and high levels of inflammatory cytokines and CRP, suggesting that adiponectin could be reliably used as a single marker of the MetS.

The signaling pathways of adiponectin are poorly known. However, the activation of 5'-AMP-activated protein kinase (AMPK) has been suggested (128;296). AMPK plays a central role in the regulation of energy metabolism and phosphorylates key target proteins that control the flux through various metabolic pathways (297). The net effect of AMPK activation is the stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle fatty acid oxidation and glucose uptake (297). Our findings are in accordance with the postulated mechanism of action of adiponectin through AMPK activity.

Because adiponectin has a central role in the regulation of insulin sensitivity, it is also a good candidate when considering genetic factors contributing to decreased insulin sensitivity in FDR of T2DM patients. However, there are only few previous studies that have assessed the levels of

adiponectin in FDRs. Lihn et al. (266) found reduced expression of adiponectin in adipose tissue of FDRs compared with controls, whereas serum adiponectin levels were comparable in the two groups. In contrast Pellme et al (298) found lower levels of adiponectin in FDRs than control subjects, and the difference remained significant after adjustment for obesity (298). Also in our study the levels of adiponectin were lower in offspring of DM2, although the difference between controls and offspring with normal glucose tolerance was not statistically significant.

Genome-wide scans have mapped the susceptibility locus of the MetS and T2DM to chromosome 3 q27, where the *AMP1* is located. Several SNPs of the *AMP1* have been suggested to account the association. However, the results have so far been rather inconsistent: the T allele of *SNP 45* (261) has been associated with insulin resistance, whereas the G allele has been associated with increased T2DM risk (262). Furthermore, whereas the G allele of *SNP 276* has been associated with DM2 risk and low adiponectin levels (262;299), the T allele in the same locus has been associated with insulin resistance (300). In our study, no association of these SNPs or their haplotype with adiponectin levels or any other metabolic variable were observed. These conflicting results could be attributable to the possibility that these SNPs are in linkage disequilibrium with some yet unknown polymorphisms that affect plasma adiponectin levels and contribute to the risk of DM2. Our negative findings may also be due to a rather small sample size.

6.6. Altered energy metabolism as a feature of the metabolic syndrome (Studies I, II, III)

No differences between study subjects were observed in total energy expenditure during fasting. However, significant differences emerged during hyperinsulinemia. Low energy expenditure/LBM was observed in subjects with the MetS. This novel finding suggests that a defect in meal-induced increase in energy expenditure is an integral part of the MetS. Thus subjects with this syndrome may have an increased tendency to gain weight.

Acute feeding increases metabolic rate by 25-40%, a phenomenon referred to as diet-induced thermogenesis (301). Sympathetic nervous system is the main regulator of this response via innervations of thermogenic targets such as brown adipose tissue and skeletal muscle (301). Since insulin is known to increase the sympathetic activity regulated by the CNS, insulin resistance in CNS could lead to blunted sympathetic activation and thereby to a decrease in thermogenesis (302). Alternatively, dysfunction of adrenergic β_3-receptors in adipose tissue (303)

or uncoupling proteins that mediate the energy dissipation as heat (301), could lead to reduced thermogenesis. Administration of adiponectin to Agouti yellow obese mice has also induced UCP-1 mRNA expression and sympathetic nerve activity in brown adipose tissue, accompanied by attenuated weight gain and reduced visceral fat (304). Although originally the effect on energy expenditure was observed only when adiponectin was administered peripherally (304) adiponectin may also act directly in the CNS to increase energy expenditure (212). However, none of these hypotheses has yet been established in humans. Furthermore, our observation that a decrease of energy expenditure was particularly accentuated in subjects with high intra-abdominal fat mass suggests that some other mechanisms characteristic to abdominal obesity, in addition to insulin resistance and hypoadiponectinemia, may contribute to decreased thermogenesis.

6.7. Implications for clinical practice (Studies I, II and III)

The diagnosis of the MetS has been recently criticized (53). The justification for the diagnosis of the syndrome has been questioned, as well as the common etiology of the components of the syndrome. Furthermore, it has been claimed that the predictive value of the MetS for future adverse events is not greater than sum of its components. The criticism has also pointed out that the best predictive combination of the clustering of cardiovascular risk factors and their cut-off points have not yet been established.

However, identification of subjects with high risk for CVD and T2DM is of importance. Tools for the identification of high-risk subjects in clinical practice should be easily available. Our study gives support for the use of the diagnosis of the MetS in the identification of high-risk individuals. Results of the factor analysis show that the combination of easily measured variables included in the diagnosis of MetS carry important information on individual's insulin sensitivity, amount of IAF, energy expenditure and levels of adiponectin and inflammatory cytokines. Our data also indicate that the waist circumference and fasting insulin levels are reliable estimates of IAF and insulin sensitivity, respectively. However, there are also subjects with normal weight and waist circumference, but who have a large amount of IAF and an adverse metabolic profile. This finding further highlights the importance of considering the risk factors as a whole and thus justifies the diagnosis of the MetS. Furthermore, if the focus of screening is on overweight/obese

subjects the presence of CVD risk factor cluster of metabolically obese normal weight subjects may go undetected. (Studies I and III)

The MetS factor explained about half of the variation of the variables included in the factor analysis model. Therefore, the inclusion of some additional variables in the diagnosis of the MetS could increase the variation explained. One of the variables could be CRP, because prospective studies have shown that CRP increases the predictive value of the MetS for CVD (59). On the other hand, our data show that the variation in the levels of adiponectin was associated with very similar metabolic changes to those of the MetS. Therefore, adiponectin levels are likely to be a good single marker for the MetS. Blood pressure was not associated with the levels of adiponectin and only diastolic blood pressure was associated with IAF mass suggesting that the etiology of high blood pressure differs from that of the other components of the MetS. Because hypertension is an important risk factor for CVD and because there are also pathophysiological mechanisms linking hypertension to intra-abdominal obesity and insulin resistance, the inclusion of blood pressure in the MetS diagnosis is well justified. (Studies I and II)

6.8. Concluding remarks

This study was undertaken to investigate metabolic abnormalities associated with abdominal obesity and the MetS in first-degree relatives of T2DM. Furthermore, the contribution of variants in *AMP1* and *CAPN10* to the MetS and related traits were assessed.

Our results show that the MetS is characterized with an excess of IAF, insulin resistance, hypoadiponectinemia, low-grade inflammation and endothelial dysfunction. Furthermore, our results suggest that subjects with the MetS have an impairment in diet-induced thermogenesis, possibly predisposing subjects to further weight gain. IAF mass was closely associated with all metabolic abnormalities observed in the MetS and seems to be responsible for adverse metabolic consequences of obesity. Variation in *CAPN10* was associated with an increase in IAF mass. In addition, hypoadiponectinemia seems a good single marker of the MetS.

In conclusion, this study gave important information on metabolic abnormalities associating with the MetS emphasizing the central role of intra-abdominal adipose tissue in the pathogenesis of the syndrome.

7. SUMMARY

Study I: The central feature of the MetS is an excess of intra-abdominal fat that is coupled with multiple metabolic abnormalities such as insulin resistance, hypoadiponectinemia, low-grade inflammation, endothelial dysfunction and low maximal oxygen uptake. Low energy expenditure during hyperinsulinemia suggests that impaired diet-induced thermogenesis is an integral part of the MetS that may predispose subjects to further weight gain.

Study II: Adiponectin is an adipose specific protein that has multiple insulin-sensitizing properties. Subjects with hypoadiponectinemia had excess of IAF mass and metabolic abnormalities typical of subjects with the MetS, suggesting that adiponectin level may be used as a single marker of the MetS.

Study III: Adverse metabolic consequences of obesity are mostly attributable to increased amount of IAF. The results also implicate that there are metabolically obese normal weight subjects and metabolically healthy overweight subjects depending on the amount of IAF. This finding emphasizes the importance of evaluating the risk factors for CVD as a whole, and thus also gives justification for the diagnosis of the MetS.

Study IV: Subjects with the A allele of *SNP-43* of *CAPN10*, were insulin resistant and had increased amount of IAF. The association of this SNP with IAF remained statistically significant even after adjustment for insulin resistance. Thus, *SNP-43* of *CAPN10* may contribute to the risk of T2DM by regulating IAF.

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