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ANNA-MAIJA TOLPPANEN

Genetic Association of the Tenomodulin Gene (*TNMD*) with Obesity- and Inflammation-Related Phenotypes

Doctoral dissertation

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School of Public Health and Clinical Nutrition
Department of Clinical Nutrition and Food and Health Research Centre
University of Kuopio

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ABSTRACT

Obesity is associated with chronic low-grade inflammation and dysregulations in the endocrinological functions of peripheral tissues, including adipose tissue. It predisposes the individual to chronic diseases, including cardiovascular diseases and type 2 diabetes (T2D), but also to other conditions affecting the quality of life, such as age-related macular degeneration (AMD). Many of the obesity-related conditions exhibit abnormal angiogenesis as a part of the pathophysiology. Previous studies by our group have demonstrated that long-term weight reduction can change the gene expression profile of adipose tissue in overweight individuals with impaired fasting glucose or impaired glucose tolerance (IGT). One of the most downregulated genes was tenomodulin (*TNMD*). *TNMD* is located in the X-chromosome and has been shown to inhibit angiogenesis.

The role of *TNMD* as a susceptibility gene for obesity- and inflammation-related traits was investigated by studying the association of single nucleotide polymorphisms (SNPs) with obesity and indicators of glucose and lipid metabolism in 507 overweight individuals with IGT who participated in the Finnish Diabetes Prevention Study (DPS), and in a cross-sectional population-based cohort of middle-aged men (the METSIM study, n=5298). In addition, the association with proinflammatory markers was studied in DPS and the association with AMD in a separate sample of 475 non-diabetic individuals.

Three markers were associated with conversion from IGT to T2D in DPS, but not with the prevalence of T2D in METSIM. The same genotypes that had elevated risk for developing T2D were associated with elevated serum concentrations of inflammation markers in DPS and with higher serum cholesterol concentrations in the obese men of both study populations. In women, the sequence variation of *TNMD* was associated with serum concentrations of proinflammatory factors, central obesity and prevalence of AMD. The associations with inflammatory mediators were modified by central obesity and the status of glucose metabolism.

In conclusion, these results suggest that the genetic variation of *TNMD* might be related to the risk for components of metabolic syndrome, a constellation of dyslipidaemia, central obesity, insulin resistance and chronic low-grade inflammation, especially in the high-risk individuals.

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Medical Subject Headings: Cholesterol; Diabetes Mellitus, Type 2/genetics; Dyslipidemias; Finland; Genetic Variation; Genotype; Glucose Intolerance/genetics; Glucose/metabolism; Insulin Resistance/genetics; Lipid Metabolism; Metabolic Syndrome X; Middle Aged; Obesity/genetics; Polymorphism, Genetic; Polymorphism, Single Nucleotide/genetics; Quality of Life; TNMD protein, human; X Chromosome

To my nearest and dearest

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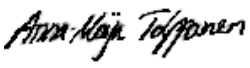
Kuopio, April 2009



Anna-Maija Tolppanen

ABBREVIATIONS

2h-PG	2-hour plasma glucose concentration in an oral glucose tolerance test
AACE	the Association of American Clinical Endocrinologists
ABCA*	adenosine tri-phosphate binding cassette A
ADAM30*	a disintegrin and metalloproteinase domain 30
ADAMTS9*	a disintegrin and metalloproteinase with thrombospondin type 1 motif, 9
AHA/NLBI	the American Heart Association/ National Heart, Lung and Blood Institute
AMD	age-related macular degeneration
aP2	adipocyte fatty acid binding protein
BMI	body mass index
C3*	complement component 3
C/EBP-α*	CCAAT/enhancer-binding protein α
CAMK1D *	calcium/calmodulin-dependent protein kinase 1D
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD36*	fatty acid translocase
CDC123*	cell division cycle 123 homolog (*S. cerevisiae*)
CDKN*	cyclin-dependent kinase inhibitor
CEU	the CEPH population of HapMap database (Utah residents with ancestry from northern and western Europe)
CFH*	complement factor H
CHM*	chondromodulin
CI	confidence interval
CRP	C-reactive protein
CTNNBL1*	catenin, beta- like 1
DGAT2*	diacylglycerol O-acyltransferase 2
DIAPH*	diaphanous 2 *Drosophila* homologue
DPS	the Finnish Diabetes Prevention Study
EGIR	the European Group for the Study of Insulin Resistance
ELISA	enzyme-linked immunosorbent assay
ELOVL*	elongation of very long chain fatty acids-like 4
ER	endoplasmic reticulum
ERK/MAPK	extracellular-signal regulated kinase/mitogen-activated protein kinase
FADS1*	fatty acid desaturase
FASN*	fatty acid synthase
FDR	false discovery rate
FPG	fasting plasma glucose concentration
FTO*	fat mass and obesity- associated gene
HDL	high-density lipoprotein
HHEX*	homeobox, hematopoietically expressed
HMGCR*	3-hydroxy-3- methyl-glutaryl- CoA reductase
HR	hazard ratio
HSL*	hormone-sensitive lipase
HWE	Hardy-Weinberg equilibrium
IFG	impaired fasting glucose
IDF	International Diabetes Federation
IGT	impaired glucose tolerance
IL	interleukin
IQ	interquartile
JAZF1*	juxtaposed with another zinc finger gene 1

KCNJ11*	potassium inwardly rectifying channel, subfamily J, member 11
KO	knock-out
LD	linkage disequilibrium
LDL	low-density lipoprotein
LGR5*	leucine-rich repeat-containing G protein coupled receptor 5
LPL*	lipoprotein lipase
MBTPS2*	membrane-bound transcription factor protease, site 2
METSIM	the Metabolic Syndrome in Men- Study
MIF	macrophage migration inhibitory factor
MSTN*	myostatin
NAFLD	non-alcoholic fatty liver disease
NCEP: ATP III	National Cholesterol Education Program's Adult Treatment Panel III
NOTCH2*	Notch homolog 2 (*Drosophila*)
OGTT	oral glucose tolerance test
OR	odds ratio
PEDF*	pigment epithelium-derived growth factor
PFKP*	phosphofructokinase, platelet type
PPAR*	peroxisome proliferator- activated receptor
RANTES	regulated upon activation, normally T-expressed, and presumably secreted
RT	receiving treatment
RT-PCR	reverse-transcriptase-polymerase chain reaction
SAA	serum amyloid A
SCD*	stearoyl coenzyme A desaturase
SCX*	scleraxis
SEM	standard error of the mean
sICAM	soluble intercellular adhesion molecule 1
SNP	single nucleotide polymorphism
SREBP*	sterol regulatory element binding protein
T2D	type 2 diabetes
TCF7L2*	transcription factor 7-like 2
TGF-β	transforming growth factor β
THADA*	thyroid adenoma associated gene
TNMD*	tenomodulin
TNF-α	tumour necrosis factor-α
TSP*	trombospondin
TSPAN8*	tetraspanin 8
UTR	untranslated region
VEGF*	vascular endothelial growth factor
VLDL	very low-density lipoprotein
WT	wild-type
WHR	waist to hip-ratio
WHO	World Health Organization
X^M	maternally inherited X- chromosome
X^P	paternally inherited X- chromosome

*the genes are indicated with *italic font* and proteins with normal font

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals (I-IV)

I. Tolppanen AM, Pulkkinen L, Kolehmainen M, Schwab U, Lindström J, Tuomilehto J, Uusitupa M; Finnish Diabetes Prevention Study Group. Tenomodulin is associated with obesity and diabetes risk: the Finnish Diabetes Prevention Study. Obesity 2007;15(5):1082-1088.

II. Tolppanen AM, Pulkkinen L, Herder C, Koenig W, Kolehmainen M, Lindström J, Tuomilehto J, Uusitupa M; Finnish Diabetes Prevention Study Group. The genetic variation of the tenomodulin gene (*TNMD*) is associated with serum levels of systemic immune mediators--the Finnish Diabetes Prevention Study. Genet Med. 2008;10(7):536-544.

III. Tolppanen AM, Pulkkinen L, Kuulasmaa T, Kolehmainen M, Schwab U, Lindström J, Tuomilehto J, Uusitupa M, Kuusisto J. The genetic variation in the tenomodulin gene is associated with serum total and LDL cholesterol in a body size-dependent manner. Int J Obes 2008. Published online at 4.11.2008.

IV. Tolppanen AM, Nevalainen T, Kolehmainen M, Seitsonen S, Immonen I, Uusitupa M, Kaarniranta K, Pulkkinen L. Single nucleotide polymorphisms of the tenomodulin gene (*TNMD*) in age-related macular degeneration. Molecular Vision: in press.

In addition, some unpublished data are presented.

TABLE OF CONTENTS

1 INTRODUCTION

Obesity, defined as body mass index (BMI) ≥30 has become a major public health problem, especially in the developed countries but also in the rapidly-developing countries. The aetiology of obesity is a complex interplay between environmental, genetic and behavioural factors even though the fundamental cause is known, i.e. the imbalance between energy expenditure and intake. The storage of this surplus energy into adipocytes evokes disturbances in the cellular organization and secretory functions of adipose tissue, thereby leading to various metabolic abnormalities and chronic low-grade inflammation.

Excess fat mass, especially in the abdominal region, is one key component of metabolic syndrome, a cluster of metabolic abnormalities including dyslipidaemia, insulin resistance, glucose intolerance, hypertension and inflammation. The obesity epidemic has also resulted in a higher prevalence and the incidence of obesity-related conditions, including diseases which can dramatically shorten the life span, for example, cardiovascular diseases, certain types of cancer and type 2 diabetes (T2D). In addition, obesity predisposes to other conditions with tremendous effect on the quality of life, such as osteoarthritis and age-related macular degeneration (AMD).

In addition to the inflammatory mediators, adipose tissue produces and secretes molecules that regulate angiogenesis. Interestingly, many of the related conditions, including cardiovascular diseases, AMD and microvascular complications of T2D exhibit vascular dysfunction and dysregulation as an essential part of their pathophysiology.

It is also known that alterations in body weight and fat mass influence the gene expression profile of adipose tissue. In a previous study, tenomodulin (*TNMD*), a putative angiogenesis inhibitor, was one of the most extensively downregulated genes during long-term weight reduction in overweight individuals with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). This finding provided the impetus to investigate whether *TNMD* could be a susceptibility gene for obesity and its related conditions.

The purpose of this work was to investigate the association of common sequence variation in the *TNMD* gene with obesity- and inflammation-related phenotypes, including 1) anthropometric measurements, 2) glucose metabolism and incidence or

prevalence of type 2 diabetes, 3) low-grade inflammation indicated by serum levels of systemic immune mediators, 4) serum levels of lipids and lipoproteins and 5) prevalence of age-related macular degeneration.

2 REVIEW OF THE LITERATURE

2.1 Obesity

Obesity is characterized by the excess accumulation of adipose tissue, often to an extent that endangers an individual's health. The adipose tissue mass can be measured by various methods such as bioelectrical impedance, underwater weighing, total body water or potassium content or by different imaging methods (1-3). Apart from bioelectrical impedance, these techniques are rather cumbersome and expensive and thus different surrogate measures are applied in the clinical settings. The most common surrogate marker for body fat content is BMI, calculated as weight in kilograms divided by height in meters squared. According to World Health Organization (WHO) guidelines (4), determined on the basis of mortality statistics from the United States (5), overweight is defined as BMI>25 kg/m^2 and obesity as BMI ≥30 kg/m^2. Since abdominal obesity is specifically associated with the metabolic risk factors (6), the measures of central obesity, such as waist circumference or waist to hip-ratio (WHR) are also feasible in the estimation of abdominal and general fat mass (1,2). The cut-offs for central obesity in European populations, based on the definitions of metabolic syndrome according to WHO (7) and the European Group for the Study of Insulin Resistance (EGIR) (8) are waist circumference ≥80 cm in women and ≥94 cm in men (8) and/or WHR ≥0.85 in women and ≥0.9 in men (7).

In the population-based FIN-D2D survey, which was conducted in Finland between October 2004 and January 2005, 24% of men and 29% of women were classified as obese, 50% of men and 38% of women were overweight and 69% of men and 76% of women fulfilled the criteria for central obesity (9). These numbers are in line with estimations from many other developed countries, as for example in the United States where 31.1% of men and 33.1% of women were obese in 2004 (10) while 17.8% of Australian men and 15.1% of women were obese and 61.9% of men and 45% of women were overweight in 2006 (11). In the majority of European countries, the prevalence of obesity increased by up to 40 % between 1989 and 1999 (2).

2.1.1 Lifestyle-related risk factors of obesity

The high and constantly increasing prevalence of obesity is due to two major environmental factors: changes in food intake and physical activity (6). During the last

decades, the energy intake has increased due to larger portion sizes and higher energy density of foods (12-15). In combination with decreased physical activity (15,16), these plentiful supplies of food in the developed countries have resulted in the mushrooming of obesity, which represents a major challenge for modern society. Accordingly, a multi-faceted approach including urban planning, lifestyle education and changes in the food policy is needed to overcome these factors (17).

2.1.2 Genetic risk factors of obesity

The obesity epidemic can be considered as having strong genetic determinants since 30-80% of the variation in body fat has been attributed to genetic factors (18-20). The inheritance of abdominal obesity is also high, e.g in a sample of post-menopausal women genetic factors were considered to explain 60% of the variance in abdominal fat (21). Many of the characterized genetic risk factors are related to regulation of food intake and metabolic pathways (22), but susceptibility genes with unknown functions have also been identified (23-27).

The genetic risk factors can be divided into variants that cause mono- or polygenic obesity. The human obesity gene map published in 2005 (22), lists a total of 11 genes in which mutations cause monogenic obesity, such as the leptin (28), leptin receptor (29) and melanocortin 4 receptor genes (30). However, since these mutations with high penetrance and a large effect are rare, they are not feasible markers at the population level.

The recent technological advancements which have made genome-wide scans easier and more affordable have facilitated the identification of common variants. For example, the association of the genes encoding fat mass and obesity- associated gene *(FTO)* (23-26), catenin, β-like 1 (*CTNNBL1*) and phosphofructokinase platelet type (*PFKP*) (25,27) with obesity have been replicated in more than one large study population, but as these variants have low penetrance and a relatively small effect size, they are currently not useful predictors for the propensity to obesity at the general population level. For example, the individuals who harbour the risk genotype (AA) of the marker rs9939609 within the gene encoding *FTO* weigh approximately 3 kg more than individuals without the risk allele (genotype rs9939609-TT) (24). The effect of the marker rs6013029, which is located in the *CTNNBL1* is stronger, since the individuals with the rs6013029-TT genotype have 2.67 units higher BMI and 5.96 kg higher fat mass than individuals with the rs6013029-GG genotype (27).

In addition to these genes and variants in which the associations have been replicated, there are a number of genes with conflicting results, such as peroxisome proliferator-activated receptor-γ (*PPAR-γ*) (31,32). The failure in replication can result from differences in the study populations, heterogeneity in the disease aetiology or from dissimilar ascertainment schemes, for example recruiting subjects with mild or severe obesity (33). The replication studies might also have been conducted in different ethnic groups with allele frequencies that differ from those observed in the original study population (34). Inadequate sample sizes, failure to attribute positive results to chance in the initial studies (35) or environmental differences can also account for the heterogeneity between different genetic association studies.

2.2 Obesity-related co-morbidities

In obese individuals, the mass of adipose tissue, a major endocrine organ with various para- and autocrine functions, is increased. Therefore it is not surprising that obesity is the main risk factor for a number of metabolic abnormalities (1,2,4). Almost all, 90%, of individuals who have type 2 diabetes (T2D) are overweight (36). Furthermore, obesity increases the risk for various other conditions, many of which are associated with vascular dysfunction and disturbances in neovascularization, such as cardiovascular disease, certain types of cancer and age-related macular degeneration (1,2,4). In addition, central obesity is a key component of the metabolic syndrome, a constellation of metabolic abnormalities and cardiovascular disease risk factors (6,7,37).

2.2.1 Metabolic syndrome

The concept of the metabolic syndrome has existed for at least 80 years and was originally defined as the clustering of hypertension, hyperglycaemia and gout but in 1940´s upper body adiposity was also included in the definition (38). In 1988, Reaven underlined the importance of insulin resistance in his description of the metabolic syndrome, or syndrome X, a combination of hyperinsulinaemia, glucose intolerance, hypertension and dyslipidaemia (39). It is notable that central obesity was not included in this definition.

Nowadays, the definition of metabolic syndrome as a constellation of metabolic abnormalities has been widely accepted, but the exact diagnostic criteria were defined for the first time in 1998 when WHO (7), EGIR (8) and the National Cholesterol

Education Program's Adult Treatment Panel III (NCEP: ATP III) (40) formulated their consensus statements. Subsequently, various other criteria, including those of International Diabetes Federation (IDF) (41), American Heart Association/National Heart, Lung and Blood Institute (42) and Association of American Clinical Endocrinologists (43) were introduced (Table 1).

All of these six definitions include central obesity, hyperglycaemia, hypertension and dyslipidaemia as indicated by elevated serum triglycerides and/or decreased high-density lipoprotein (HDL) concentration, but the cut-off points and the amount of criteria that need to be fulfilled vary to some extent. This discrepancy between criteria naturally affects the absolute prevalence estimates of the metabolic syndrome, but regardless of the applied criteria, the explosion in the numbers of individuals with these metabolic abnormalities is a growing burden to health care systems (41).

Visceral, rather than the subcutaneous fat depot is generally believed to be the main culprit of the metabolic syndrome (44), as it is considered to be more metabolically active and it is able to deliver endocrinal factors to the portal veins and can thus directly impact on the liver (45). The amount of the subcutaneous depot can exceed that of visceral by 3-4 times (46), and thus it should not be ignored. However, a recent study in the Framingham Heart Study population showed that while abdominal adiposity in general was related to a higher risk of metabolic and cardiovascular disease, subcutaneous abdominal fat was not associated with a linear increase in the prevalence of components of metabolic syndrome, including low HDL, high triglycerides and hypertension among obese individuals (47).

It has been suggested that especially the visceral adipose depot has a central role in the development and maintenance of a proinflammatory state, as reflected in the elevated serum C-reactive protein (CRP) concentration and prothrombotic state, evident as increased plasma concentrations of plasminogen activator inhibitor and fibrinogen (44,45). These two states are also characteristics of the metabolic syndrome, but they are not included in the diagnostic criteria (7,8,40-42). Both features are likely caused by multiple mechanisms, but there is a growing body of evidence suggesting that these states are metabolically interconnected and result from the dysregulation in the expanding adipose tissue (6,48-51).

Table 1. The definition of metabolic syndrome according to the WHO, EGIR, NCEP:ATPIII, IDF, American Heart Association/National Heart, Lung and Blood Institute, and Association of American Clinical Endocrinologists. HDL high-density lipoprotein cholesterol, IFG impaired fasting glucose, IGT impaired glucose tolerance, RT receiving treatment, WHR waist to hip-ratio

Criteria	Definition of metabolic syndrome	Reference
World Health Organization (WHO 1999)	Diabetes, insulin resistance*, IFG** or IGT*** and two of the following features: - WHR >0.9 for men and 0.85 for women or BMI>30 - blood pressure ≥140/90 mmHg - microalbuminuria (urinary albumin excretion rate ≥20 µg/min^{-1} or albumin:creatinine ratio ≥30 mg/g^{-1}) - serum triglycerides ≥1.7mmol/l - serum HDL <0.9 mmol/l for men and <1.0 mmol/l for women	(7)
The European Group for the Study of Insulin Resistance (EGIR 1999)	Insulin resistance* and at least two of the following: - IFG** - waist circumference ≥94 cm for men and ≥80 cm for women - blood pressure ≥140/90 mmHg - serum triglycerides >2.0 mmol/l - serum HDL<1.0 mmol/l	(8)
The National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATPIII 2001)	At least three of the following: -IFG** - waist circumference ≥ 102 cm for men and ≥88 cm for women - blood pressure ≥130/85 mmHg - serum triglycerides ≥1.7mmol/l -serum HDL <1.04 mmol/l for men and <1.29 mmol/l for women	(40)
The International Diabetes Federation (IDF 2006)	- Waist circumference ≥ 94 cm for men and ≥80 cm for women and two of the following: - blood pressure ≥130/85 mmHg or RT - serum triglycerides ≥1.7mmol/l or RT - serum HDL <1.03 mmol/l for men and <1.29 mmol/l for women or RT - IFG** or previously diagnosed type 2 diabetes	(41)

The American Heart Association/ National Heart, Lung and Blood Institute (AHA/NLBI 2004)	At least three of the following: -IFG** or RT - waist circumference ≥ 120 cm for men and ≥88 cm for women - blood pressure ≥130/85 mmHg or RT - serum triglycerides ≥1.7mmol/l or RT - serum HDL <0.9 mmol/l for men and <1.1 mmol/l for women or RT	(42)
The Association of American Clinical Endocrinologists (AACE 2003)	Diagnosis depends on the clinical judgement based on the following risk factors: - IFG** or IGT*** but not type 2 diabetes - BMI≥25 - blood pressure ≥130/85 mmHg - serum triglycerides ≥1.7mmol/l - serum HDL <1.04 mmol/l for men and <1.29 mmol/l for women - family history of type 2 diabetes - cardiovascular disease - polycystic ovary syndrome - sedentary lifestyle - age - ethnicity	(43)

*defined by sex- and cohort-specific top 25% distribution of fasting serum insulin concentration in the non-diabetic population
** fasting plasma glucose concentration ≥ 6.1 mmol/l in WHO, EGIR, NCEP:ATPIII and AACE, ≥ 5.6 mmol/l in IDF and AHA/NLBI
*** 2-hour plasma glucose concentration ≥ 7.8 mmol/l

2.2.1.1 Genetic risk factors for metabolic syndrome

In addition to obesity, many of the other individual components of metabolic syndrome have genetic background, although they also are strongly influenced by environmental factors. Insulin resistance clusters in families, since 45% of first-degree relatives of patients with T2D are insulin resistant on the basis of euglycaemic insulin clamp technique, compared with 20% of people without a family history of T2D (52,53). The heritability estimates for other components of the metabolic syndrome range from 0.3 to 0.92 (Table 2). Findings from twin and family studies suggest that in addition to the individual components, the clustering of metabolic syndrome factors is also heritable (54-56).

Table 2. The heritability estimates for the components of metabolic syndrome.

Component	Heritability	Reference
Glycaemic disturbances	0.57-0.92	(55)
Blood pressure	0.4-0.5	(57)
Dyslipidaemia	0.3	(55)
Albumin excretion	0.3	(58)
Abdominal visceral fat	0.42-0.6	(21,59)
Body fat	0.3-0.8	(18-20)

2.2.2 Type 2 Diabetes

T2D is a heterogeneous group of diseases, characterized by hyperglycaemia resulting from defects in insulin secretion and insulin responses (60,61). Prolonged hyperglycaemia is associated with dysfunction, damage to and even failure of different tissues and organ systems, including eyes, kidneys, heart, nerves and blood vessels (61,62). The related conditions include microvascular complications such as diabetic nephropathy, retinopathy and neuropathy and macrovascular complications, including cardiovascular, cerebrovascular and peripheral vascular diseases (62,63). The WHO 1985 and 1999 diagnostic criteria for impaired glucose regulation which are based on the determination of fasting plasma glucose concentration (FPG) and 2-hour venous plasma glucose concentration (2h-PG) in an oral glucose tolerance test (OGTT) are presented in Table 3.

Table 3. The WHO 1985 and 1999 diagnostic criteria of impaired glucose regulation (60,62).

	1985 criteria		1999 criteria	
	FPG (mmol/l)	2h-PG (mmol/l)	FPG (mmol/l)	2h-PG (mmol/l)
Normoglycaemia	<7.8 implied	<7.8 implied	<6.1	<7.8 implied
IFG	Not defined		≥6.1, <7	<7.8
IGT	<7.8	≥7.8, <11.1	≥6.1, <7	≥7.8, <11.1
T2D	≥7.8	≥11.1	≥7.0	≥11.1

The category of IFG was introduced in the WHO criteria in 1999, with the main aim of creating a fasting category which would be analogous to IGT. The suitable lower cut-off for this glucose tolerance class has been disputed. In 2003, the American Diabetes Association recommended that it should be lowered to 5.6 mmol/l (64), while the cut-off proposed by WHO 1999 criteria is 6.1 mmol/l (62). The rationale was to identify similar proportions of the population with IFG and IGT, and to produce equivalent predictive power for progression to diabetes from the IGT and IFG categories (64). The European Diabetes Epidemiology Group estimated that the change in cut-off would have resulted in two-to five-fold increase in the prevalence of IFG across the world and since the total benefits or costs of designating individual as at risk for diabetes were not known, they did not recommend the lower threshold (65).

In parallel with the obesity epidemic, the prevalence of T2D has increased during the last decades (66). According to the FIN-2D2 survey of 2004-2005, 16 % of Finnish men and 11 % of women had T2D, while 42% of men and 33 % of women had abnormal glucose regulation (IFG, IGT or T2D) (9). The global prevalence approximation of T2D in 2000 was 2.8%, which is estimated to increase to 4.4% in 2030 (67). The highest increases in T2D prevalence are predicted to take place in the Middle Eastern Crescent (163%), Sub-Saharan Africa (161%), Latin America and the Caribbean and in Asia (regionwise estimates ranging from 104 to 151%).

2.2.2.1 Environmental risk factors of type 2 diabetes

Obesity, especially in the abdominal region, increases the risk of T2D and accordingly, the main environmental risk factors of T2D are related to lifestyle (68,69). Several studies have indicated that metabolic syndrome predicts future diabetes (70,71). However, as hyperglycaemia and insulin resistance are the key components of EGIR´s (8) and WHO´s (7) diagnostic criteria for metabolic syndrome and they also belong to the other definitions of metabolic syndrome (40-43), this is not unexpected. Other non-

genetic risk factors include age (69), low physical activity (68,69) and intrauterine exposure to hyperglycaemia and malnutrition (72,73). The nutritional risk factors include a high fat diet rich in saturated fatty acids and low intake of dietary fibre (74). In addition, consumption of foods with a high glycaemic index has been linked to an increased risk of T2D (75-79), but these findings are controversial (80,81).

The successfulness of lifestyle intervention on preventing the onset of T2D in high-risk individuals has been demonstrated in different study populations, including Finnish (82,83), Swedish (84), Chinese (85) and American (86) individuals. In the Finnish Diabetes Study (DPS) (83), 522 middle-aged overweight individuals with IGT were randomized into two groups. The intervention group received intensive, individualized diet and exercise counselling while the control group received general information about diet and exercise instructions. During the actual study period which had a median follow-up time of four years, the risk of T2D was reduced by 58% in the intervention group (82). This reduction was directly associated with lifestyle changes (82) and the reduction in the incidence of T2D was sustained when the participants were further followed up for a median of three years (87). In the 6-year Malmö feasibility study which examined Swedish middle-aged men, a 50% risk reduction in the incidence of T2D was observed among those who volunteered to participate in the diet and exercise intervention in comparison to those who refused to participate (84). The Chinese Da Qing- Study investigated the efficacy of diet, exercise or their combination in reducing the incidence of T2D during six years of follow-up (85). All three approaches were almost equally effective, since the incidence of T2D was 67.7% in the control group, 41.1% in the exercise group, 43.8% in the diet group and 46% in the group that combined diet and exercise. The Diabetes Prevention Program, conducted in the US, compared the efficacy of lifestyle modification and oral administration of metformin in preventing or delaying the onset of T2D among high-risk individuals (86). Similar to the DPS, the participants were overweight and had IGT. Metformin treatment reduced the risk of T2D by 31%, while the risk reduction achieved by lifestyle modification was identical to that observed in the DPS (58%).

2.2.2.2 Genetic risk factors for type 2 diabetes

The genetic determinants of T2D are indicated by familial clustering (52,53), marked differences in the prevalence among various ethnic and racial groups (88-91) and different concordance rates between monozygotic and dizygotic twins (55,92). The

general pattern of inheritance of T2D in families is consistent with it being a complex, multifactorial disease with polygenic background (93,94). Accordingly, only a few monogenic forms have been described and they are estimated to account for only approximately 5% of the total T2D in most populations (95). The genetic risk factors are estimated to account for 40-85% of total disease susceptibility (96).

Many genes with a modest effect size have been identified with the candidate gene approach (93,94,97,98), the best-established being *PPAR-γ* (99-102) and potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*) (26,101,103-107). The associations of these two genes have been replicated in the genome-wide scans (25,26,108), which have also revealed many new, promising candidates, including the genes encoding transcription factor 7-like 2 (*TCF7L2*), *FTO*, homeobox hematopoietically expressed (*HHEX*) and cyclin-dependent kinase inhibitor-2A/B (*CDKN-2A/B*). The most consistent associations have been observed with *TCF7L2* (26,107,109-111). A meta-analysis of 29195 controls and 17202 cases provided a pooled odds ratio (OR) of 1.46 for the rs7903146-TT genotype (112). The variants of *TCF7L2* increase the risk of T2D independently of BMI (26,107,113) and have been linked to impaired insulin secretion (113). In most of the studies, the variants of *FTO* have been shown to increase the risk of T2D by affecting the body size (24-26,114), but in a German cohort a BMI-independent effect was observed (107). The OR for the risk genotype rs9939609-AA ranges between 1.22-1.27 (24,26,114). The associations of *HHEX* and *CDKN-2A/B* have been replicated in populations of Asian and Caucasian origin, with the ORs for risk genotypes being between 1.1-1.4 (26,107,108,114). In addition, in a recent meta-analysis of three genome-wide scans for T2D, six new loci were identified, including juxtaposed with another zinc finger gene 1 (*JAZF1*), thyroid adenoma associated gene (*THADA*) and a disintegrin and metalloproteinase with thrombospondin type 1 motif, 9 (*ADAMTS9*) and the intergenic regions between the genes encoding cell division cycle 123 homolog (*S. cerevisiae*) (*CDC123)* and calcium/calmodulin-dependent protein kinase 1D (*CAMK1D*), tetraspanin 8 (*TSPAN8)* and leucine-rich repeat-containing G protein coupled receptor 5 (*LGR5*), and between Notch homolog 2 (*Drosophila*) (*NOTCH2*) and a disintegrin and metalloproteinase domain 30 (*ADAM30*) (115). The OR for the individual risk alleles range between 1.05 and 1.11.

2.2.3 Age-related macular degeneration

Age-related macular degeneration is a progressive, chronic disease with a multifactorial background (116). According to the prevalence estimates from WHO, it is the most common cause of blindness in the developed countries (117) as it has been estimated to be the cause of half of all cases of blindness in Western populations over 65 years of age (118). AMD is associated with aging and it gradually destroys sharp, central vision (116) as degenerative tissue alterations occur at the interface between the neural retina and underlying choroid (119,120).

AMD can be divided into atrophic (dry) and exudative (wet) subforms, with the former being more common and accounting for approximately 80% of AMD cases (121). Drusens are one of the most common early manifestations, followed by geographic atrophy in the atrophic form of AMD, or by neovascularization in the exudative form. The atrophic form involves modifications in pigment distribution, loss of retinal pigment epithelium cells and photoreceptors, and reduced retinal function due to an overall atrophy of the cells (116,122). Together these changes gradually blur the central vision. The hallmark feature of the exudative form is the proliferation of abnormal, fragile choroidal blood vessels, which enter into the subretinal space thereby resulting into retinal detachment, hemorrhages, exudates and glial proliferation with scarring (116,122).

Although the exact pathogenic process is still unclear, the roles of oxidative stress (119) and dysregulated angiogenesis (123) are now well established. The expression levels of inhibitors and stimulators of neovascularization are known to be altered during the development of AMD (123-125). For example, vascular endothelial growth factor (VEGF), is strongly involved in choroidal neovascularization (125) and accordingly, the VEGF-blocking compounds are emerging as the most successful treatment for exudative AMD (126-129).

2.2.3.1 Environmental risk factors for age-related macular degeneration

In addition to age (116), gender and smoking, obesity and its related conditions such as hypertension and hypercholesterolemia predispose to AMD (130-135). Interestingly, many of these environmental risk factors, such as smoking status, dietary habits, obesity, high serum cholesterol, gender and age are associated with the amount of macular pigment (136,137), which seems to be a protective factor from photo-oxidative damage (138).

2.2.3.2 Genetic risk factors for age-related macular degeneration

Family and twin studies have underlined the presence of genetic risk factors. First-degree relatives of patients with AMD have a higher risk of AMD than those without a family history (139,140). They are also affected at a younger age and have an increased lifetime risk of late AMD (141,142). Accordingly, the heritability estimates are relatively high, 0.46-0.71 for AMD (139), 0.67-0.85 for macular pigment density (143) and 0.63 for the amount of small hard drusens (144).

The importance of genetic risk factors, specifically of those related to the complement system, has been demonstrated with genome-wide scans and the candidate gene approach. Recently, an association between the rs1061170 (also known as Y402H) of the complement factor H gene *CFH* and AMD was revealed in several different populations (145-151) with ORs generally ranging between 2.45 and 5.57 for the homozygotes of the risk allele rs1061170-C. An association between the *LOC387715/HTRA1* locus and AMD in both Caucasian and Japanese and Chinese populations has been documented (152-159). The odds ratios range between 1.69 and 2.61 for heterozygotes and between 2.20 and 9.90 for homozygotes of the risk genotypes. A common polymorphism (rs2230199) in the complement component 3 gene (*C3*) has also been associated with AMD (160,161). Other suggested candidates include genes related to fatty acid metabolism, such as apolipoprotein E (162-164), ATP-binding cassette, subfamily A, member 4 (*ABCA4*) (165,166) and elongation of very long chain fatty acids-like 4 (*ELOVL*) (150,167), but their roles in AMD pathogenesis are controversial.

The role of angiogenesis regulators as susceptibility genes for AMD has also been studied, but the genetic association studies on the role of VEGF polymorphisms in the exudative AMD have resulted into conflicting results (168-171). However, there is some evidence on the association between polymorphisms of the gene encoding the antiangiogenic pigment epithelial growth factor (*PEDF*) and AMD (172,173).

2.3 Pathophysiological changes in obesity

A long-term imbalance between energy expenditure and intake has harmful systemic effects (Figure 1), many of which are attributable to adipose tissue dysfunction (48,174,175). In addition to increased adipocyte size which itself is an independent marker for metabolic abnormalities (176), other adverse events take place in the adipose

tissue. The number of preadipocytes and mature adipocytes is in a dynamic equilibrium, which is regulated by various stimuli, including nutritional status (177) and exposure to medication and cytokines and other signalling molecules (178,179). In obesity, this equilibrium is disrupted, as obese individuals have approximately three-fold higher necrosis rate of adipocytes in comparison to lean persons (180). Impaired adipocyte differentiation has also been demonstrated in insulin resistant states (181-183) and this probably accounts, at least in part, for both the increased serum free fatty acids (FFAs) and the altered pattern of adipokine secretion observed in obesity. One of the crucial events is the activation of the Wnt-signaling pathway which, in turn, impairs normal adipocyte differentiation as well as the secretion of adipokines (182-184).

The connection between inflammation and adipocyte differentiation is highlighted by the negative correlation between the degree of adipocyte differentiation and activation of proinflammatory molecules. For example, undifferentiated human preadipocytes express high levels of many proinflammatory genes, which are then downregulated as the cells differentiate (48) and the classic proinflammatory factor, tumour necrosis factor α (TNF-α) has been shown to inhibit normal adipogenesis by inhibiting the Wnt pathway (184). Inflammation, together with the other consequences of adipocyte hypertrophy causes metabolic stress in the endoplasmic reticulum (ER) and mitochondria (185,186), which can have detrimental effects on lipid and cholesterol metabolism (185,187).

Normally, adipocytes have a large capacity to synthesize and store triglycerides during feeding and to hydrolyse and release triglycerides as FFAs and glycerol during the fasting state (188,189). During the early stages of excess energy intake, the adipocytes continue to actively store additional triglycerides and maintain a nearly normal rate of lipolysis during fasting (190). Circulating FFA levels can become elevated, but skeletal muscle maintains high insulin sensitivity (191). As the energy imbalance continues, the enlarged adipocytes develop a diminished capacity to store fat and their endocrine functions change so that they produce excessive amounts of cytokines that promote inflammation, atherosclerosis and insulin resistance (48,175). When adipocytes become insulin-resistant, they fail to secrete normal amounts of insulin-sensitizing adipokines. This sets off a vicious cycle further promoting insulin resistance and evoking chronic low-grade inflammation which further disposes to other metabolic diseases such as metabolic syndrome and T2D (48,175,192). These changes in adipocyte function and lipid metabolism can ultimately result in ectopic fat

accumulation and lipotoxicity in various tissues when the fatty acid spillover exceeds the needs of oxidative metabolism and enhances metabolic flux into harmful nonoxidative metabolism pathways (193). One of the complications of obesity that seems to be related to these processes is non-alcoholic fatty liver disease (NAFLD), a spectrum of liver damage including steatosis and fibrosis (194-196). NAFLD is defined as an excess of fat in the liver in which at least 5% of hepatocytes display lipid droplets (197).

In addition to these inflammatory and insulin-sensitizing effects, the secreted compounds are involved in many diverse processes, including the regulation of neovascularization and the extracellular matrix (198-200). For example, monobutyrin has been shown to act as an adipose tissue-specific promoter of angiogenesis (201). Other well-known adipose tissue derived angiogenesis regulators include VEGF, transforming growth factor β (TGF-β) and leptin (198-200,202-204). Angiogenetic changes have been described, both in obese (202) and hyperglycaemic states (205,206).

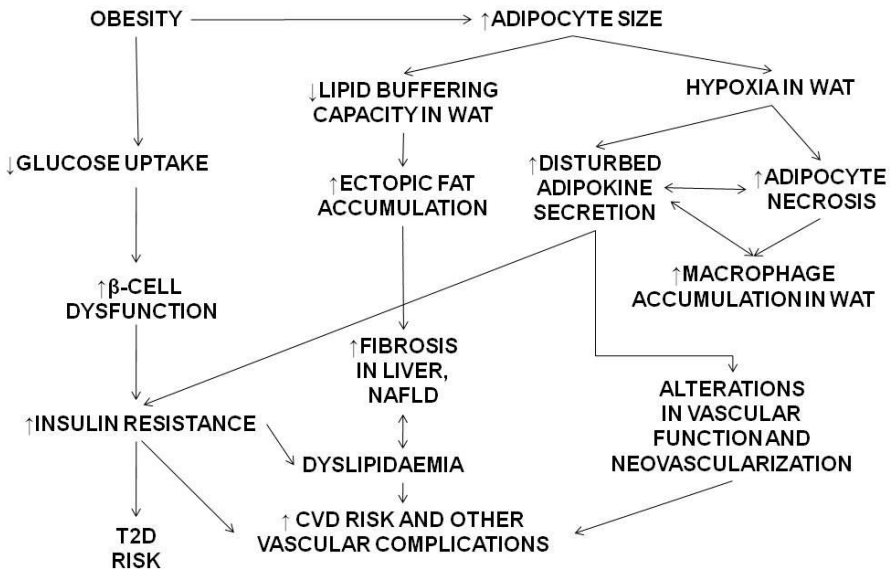


Figure 1. A simplied diagram showing the pathophysiological changes in obesity. NAFLD non-alcoholic fatty liver disease, T2D type 2 diabetes, WAT white adipose tissue

2.3.1 Glucose homeostasis in obesity

Insulin resistance that accompanies obesity is related to a deterioration in glucose disposal in peripheral tissues, including skeletal muscle and adipose tissue, but also in liver (207,208). Obesity contributes to alterations in glucose metabolism in different ways, including, but not exclusively due to, enhanced lipolysis, lipotoxicity, elevated serum FFA concentrations and dysregulation in fat accumulation, mitochondrial function and cytokine production in peripheral tissues (174,193,209,210).

Increased lipolysis results in elevated levels of circulating FFAs and triglycerides, thereby contributing to lipid overload and the flow of fatty acids into skeletal muscle and liver and interfering with the insulin signalling pathways in the skeletal muscle (174,210-212). The hypothesis that FFAs are the mediators of insulin resistance is consistent with the strong association between obesity, insulin resistance and high circulating FFA levels (213) and the observation that elevated levels of circulating FFAs can cause peripheral insulin resistance in both animals and humans (214,215). Moreover, acute lowering of FFAs with an antilipolytic drug (Acipimox; 6-methyl-1-oxido-pyrazine-2-carboxylic acid) has been shown to enhance the ability of insulin to promote glucose uptake in peripheral tissues (216). It has been shown that FFAs compete with glucose as fuel for skeletal muscle and can thereby cause impaired glucose uptake and failure of insulin to suppress hepatic gluconeogenesis (214,217,218).

In addition to the distribution of lipids, the proliferation and differentiation capacity of adipocytes have been suggested to contribute to the altered glucose metabolism occurring in obesity. Enlarged abdominal adipocytes have been shown to predict the development of type 2 diabetes independently from insulin resistance and insulin secretion (176). Impaired fat oxidation has also been suggested to cause ectopic fat accumulation, since the inhibition of fat oxidation was shown to increase intracellular lipid content and to decrease insulin action in rats (219). In humans, decreased postabsorptive fat oxidation was shown to predict weight gain and to be associated with reduced insulin sensitivity (220,221). This "inadequate fat oxidizing machinery" as proposed by Heilbronn *et al* (209) may result from decreased mitochondrial capacity (222) and lower mitochondrial DNA copy number among obese individuals (223), although these hypotheses have been challenged by data from mouse studies (224-226). In addition, changes in sympathetic nervous system activity have been proposed to affect the fat oxidation capacity (227,228). Interestingly, in the study

of Perseghin *et al* (229) with nonobese and obese individuals with similar insulin sensitivity and intramyocellular lipid content, the obese individuals were shown to have higher fasting lipid oxidation rates. This indicates that increased fat oxidation might be an adaptative mechanism that is aimed at maintaining normal intramyocellular lipid concentrations and insulin sensitivity despite the increase in the amount of body fat.

The role of adipose tissue in glucose homeostasis is further illustrated by the observation that an insufficient mass of adipose tissue is associated with elevated circulating triglyceride and fatty acid concentrations and leads to insulin resistance, both in mice (230-232) and humans (233-235). The observations from humans with lipodystrophies have demonstrated that inadequate adipose tissue mass leads to ectopic fat storage in liver, pancreas and skeletal muscle, which then may trigger insulin resistance and other metabolic alterations (235,236). Lipodystrophies are linked to insulin resistance also *via* impaired adipokine secretion (237-239). For example, individuals with lipodystrophies have low circulating levels of leptin and adiponectin (238) and the administration of leptin has been shown to improve the glycaemic control and to decrease serum triglyceride levels in lipodystrophic patients (237).

In contrast to individuals with lipodystrophy, obese persons have a large mass of adipose tissue, although they have similar metabolic perturbations. Therefore, it has been suggested that obesity is another ectopic fat accumulation syndrome because the adipose tissue is not sufficient to store the excess energy (209). This is in line with the increased content of triglycerides within skeletal muscle in obesity and T2D (215), the strong association between the increased intramyocellular lipid content and insulin resistance (209,210,235,240) and the concept that fatty acid overload in pancreas results in β-cell dysfunction and apoptosis (193). The association of hepatic fat content with insulin resistance (241) and impaired suppression of hepatic glucose production by insulin (242) also support this hypothesis.

2.3.2 Lipid metabolism in obesity

The connection between obesity and serum lipid and lipoprotein levels has been established in many large epidemiological studies, including the Framingham Heart Study (243,244). Changes in body weight have also been shown to result in alterations in serum lipoprotein concentrations and thereby to affect the risk of atherogenic traits (245). The serum concentrations of total and low-density lipoprotein (LDL) cholesterol are generally increased and the concentrations of HDL cholesterol, an acceptor of

cholesterol efflux, are decreased in obese individuals (244,246), and these are believed to account for at least some of the increased risk of cardiovascular events (245,247).

During constant positive energy balance both the triglyceride pools of adipose tissue and triglyceride synthesis in the liver are increased. This promotes the overproduction of very low density lipoprotein (VLDL)-triglycerides in obese people (248,249). The excess production is stimulated by constant, increased influx of nutrients into the liver as excess energy is derived from the diet in the postprandial state (190,250), and the plasma concentration of FFAs is increased in the fasting state (191,192). The increased input of fatty acids into the liver may be accentuated by central obesity, because visceral adipose tissue directly releases fatty acids into the portal circulation (45).

Obese individuals have lower cholesterol absorption rates (251), but their cholesterol synthesis is increased in comparison to individuals with normal body size (252). Weight reduction has been shown to increase the absorption of cholesterol (253). Restriction of caloric and dietary sterols has been shown to decrease cholesterol synthesis and improve glycaemic control in obese individuals with T2D, linking glucose and lipid metabolism (254). The production of VLDL-triglycerides and consequently, VLDL-apolipoprotein B and LDL-apolipoprotein B are increased in obese individuals (249,255-258). The elevated concentrations of VLDL- and LDL-particles suppress LDL-receptor activity and thereby raise the serum LDL levels (248,249,258). Excessive intake of saturated fatty acids and cholesterol have been suggested to contribute to overproduction of VLDL, and consequently, to the higher production of LDL (250,259). However, the high-fat diet-induced elevations of total and LDL cholesterol levels result mainly from suppressed LDL receptor activity (260,261).

The reduced HDL concentrations in obesity (246) have been suggested to result from the increased synthesis of LDL which drains away HDL-cholesteryl esters and HDL-apoA-I thereby limiting the HDL synthesis (248,262). Another hypothesis is that the excess adipose tissue simply removes HDL from the circulation (246).

Many of these changes are identical to the characteristic disturbances of lipid metabolism occurring during the acute-phase response to infection as well as inflammation (263), and the major acute-phase reactants CRP and serum amyloid A (SAA) have been shown to be involved in the rapid recycling of cholesterol (264). During the acute-phase response, the cytokine-mediated changes in lipid metabolism are aimed at decreasing the toxicity of harmful biological and chemical agents by redistributing nutrients to cells which are important in host defense (264-267). The

inflammatory cascade induces a decrease in HDL, impaired reverse cholesterol transport, elevated serum triglycerides, changes in serum apolipoproteins, related enzymes, antioxidant capacity and adenosine tri-phosphate binding cassette A1 (ABCA1)-transporter dependent cholesterol efflux (263,266-268). Thus, increased serum triglycerides and decreased HDL, the classic lipid changes associated with the metabolic syndrome and T2D, could be regarded also as a highly conserved evolutionary response aimed at tissue repair (264).

2.3.3 Angiogenesis in obesity

Adipogenesis and angiogenesis are temporally and spatially coupled processes during embryogenesis and their reciprocal crosstalk *via* paracrine signaling systems continues throughout adult life (202). Each adipocyte is nourished by a well-organized capillary network in normal weight individuals (203,204,269). Adipocytes and other cells in the adipose tissue produce many angiogenic factors including angiopoietins, hepatocyte growth factor, VEGF and TGF-β, but also traditional adipokines such as leptin and adiponectin have been suggested to be involved in the regulation of angiogenesis (Figure 2) (203,204,270,271).

The common ground between adipogenesis and angiogenesis is highlighted in several ways: human adipose tissue–derived stem cells can differentiate into endothelial cells and improve postnatal neovascularization (270), and adipocytes and their accompanying endothelial cells seem to share a common progenitor that can differentiate into adipocytes or endothelial lineages depending on the type of exposure in different environments (272). Accumulating evidence shows that capillary endothelial cells communicate with adipocytes *via* paracrine signaling pathways, extracellular components, and direct cell-cell interactions (204,273,274).

These two processes also share some common molecular factors such as PPAR-γ and VEGF. PPAR-γ, an essential mediator of preadipocyte differentiation, is involved in the regulation of adipose tissue angiogenesis and inhibition of adipocyte differentiation by overexpression of a dominant-negative PPAR-γ construct (Leu^{468} and Glu^{471} →Ala) has been shown to impair both adipogenesis and angiogenesis (275). It has also been shown that rosiglitazone, a PPAR-γ agonist, stimulates angiogenic sprouting in adipose tissue fragments (276). The inhibition of the VEGF signalling system also inhibits angiogenesis and preadipocyte differentiation (275). The role of adipose tissue-derived VEGF in angiogenesis was recently highlighted by Ledoux *et al*

(277), who also demonstrated that both subcutaneous and visceral depots have equivalent angiogenic potencies.

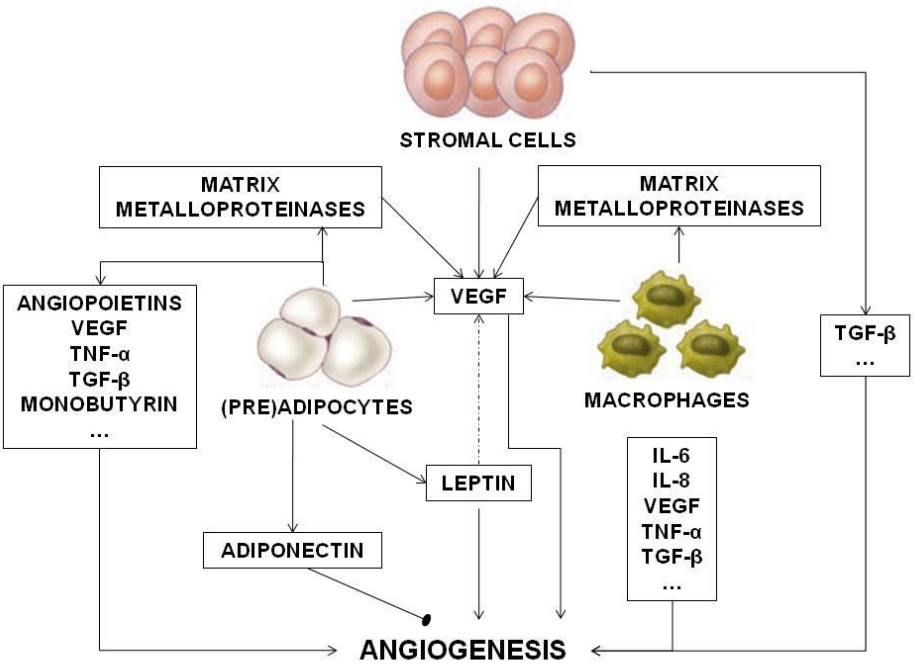


Figure 2. Angiogenetic factors secreted by adipose tissue (modified from Cao 2007 (202)). Different cell types of adipose tissue contribute to the production of pro- and antiangiogenic factors. In addition, leptin indirectly stimulates the secretion of VEGF. IL interleukin, TGF-β transforming growth factor-β, TNF-α tumour necrosis factor-α, VEGF vascular endothelial growth factor

Recently, it was reported that also macrophages may stimulate angiogenesis in adipose tissue by secreting platelet-derived growth factor and that way they can regulate the tube formation of endothelial cells (278). The secreted compounds stimulate neovascularization during fat mass expansion, either acting alone or in co-operation with other angiogenic factors (202-204,270). Since the secretion of these factors is often induced by hypoxia, it has been suggested that expansion of adipose tissue is associated with local hypoxia. In agreement with this hypothesis, the tip region of epididymal adipose tissue in adult mice is extremely hypoxic and expresses high levels of angiogenesis-promoting factors (279).

It has been speculated that when the growth rate of adipose tissue becomes stabilized, high expression levels of angiogenesis inhibitors are required to restrict further vessel growth (202). In agreement with this hypothesis, expression of

thrombospondin-1 (TSP-1), a well-known angiogenesis inhibitor is downregulated in preadipocytes and upregulated in differentiated adipocytes (280,281). Administration of angiostatin, endostatin, and TNP-470 (5-Methoxy-4-(2-methyl-3-(3-methyl-2-butenyl)-1-oxaspiro(2,5)oct-6-yl(chloroacetyl)carbamate), a compound that arrests the endothelial cell cycle, results in a dose-dependent and reversible weight reduction and a loss of adipose tissue in both genetic and diet-induced obesity in mice. Since angiostatin and endostatin specifically target endothelial cells, these effects are solely due to the antiangiogenic properties of these molecules (282,283).

The altered vascularization in obesity has been demonstrated in animal models: the fat pads of obese mice have increased vascularization (284) and fat pads of obese rats have increased perfusion and decreased vascular resistance (285). Voros *et al* (284) showed that the increased blood content of adipose tissue in obese animals was not only the consequence of functional modulations but also resulted from the growth of the vascular network. In the same study, the protein expression of angiogenesis-promoting angiopoietin-1 was lower, and the expression of TSP-1 was higher in the adipose tissue of ob/ob mice when compared to the corresponding expression levels in the wild-type mice. Recently, Varma *et al* (286) confirmed the previous observations on the TSP-1 expression in intra-abdominal adipose tissue in humans (287) and showed that TSP-1 is a true adipokine, preferentially expressed in the adipocyte fraction and with higher expression levels in obese, insulin-resistant individuals.

2.3.4 Chronic low-grade inflammation in obesity

Adipocytes and macrophages have the same evolutionary origin: the fat body which is still present in insects has diverged to liver and adipose tissue during vertebrate evolution (288). The common origin of these organs is still visible in the similar organization of metabolic cells, i.e. adipocytes or hepatocytes, in the close proximity to inflammatory cells (macrophages or Kupffer cells). This has been proposed to account for the links between adiposity, inflammation and metabolic disorders (289). Adipocytes and macrophages share several functional similarities: under appropriate stimulation, preadipocytes can achieve phagocytotic capacity (290) while macrophages can also take up and store lipids. The gene expression profiles of these cells also resemble each other: for example the transcription factor *PPAR-γ*, fatty acid binding protein *aP2*, interleukin (*IL)-6* and matrix metalloproteinases are expressed in both macrophages and adipocytes (291-294).

Consistent with these findings, a gain in weight can evoke many inflammation-related changes in the adipose tissue (Figure 3) (289,295). Furthermore, obesity is associated with a chronic low-grade inflammation state, characterized by abnormal cytokine production, increased serum concentrations of acute phase reactants and other inflammatory mediators and activation of inflammatory signalling pathways (289,295-297). The obesity-related inflammation seems to be one of the common links between defects in fatty acid metabolism and insulin resistance (298). The elevated levels of proinflammatory substances including TNF-α, SAA and IL-6 alter the lipid-storing capabilities and affect insulin sensitivity by increasing lipolysis and decreasing triglyceride synthesis (174,299-304). This results in elevated circulating FFA concentrations, higher availability of triglycerides and accumulation of fatty acid derivatives in the skeletal muscle, liver and β-cells, disrupting the normal metabolic and secretory functions in these tissues (210,211).

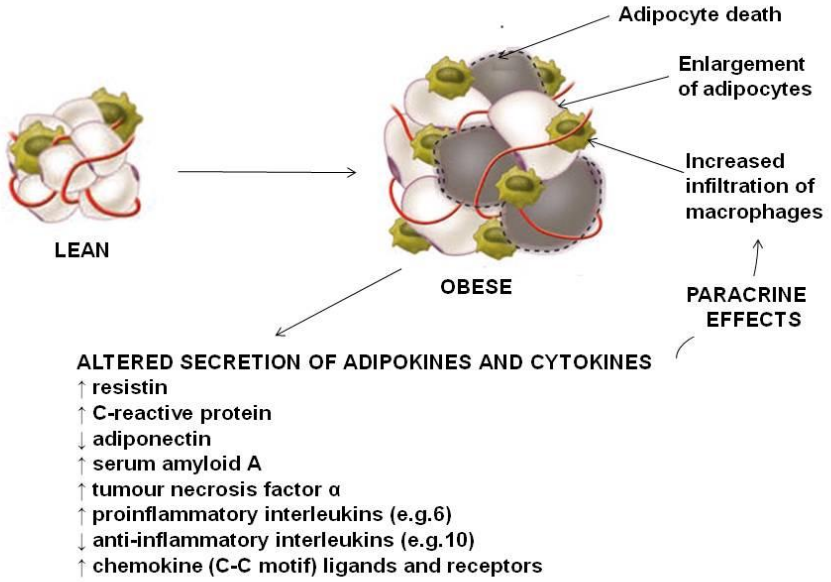


Figure 3. Obesity-induced inflammation-related changes in adipose tissue (modified from Schenk *et al* 2008 (295)). Weight gain results to increased necrosis rate of adipocytes and thereby the characteristic inflammatory response is evoked. This includes the increased production and release of proinflammatory cytokines and chemokines and the recruitment of macrophages. The increased secretion of proinflammatory substances further stimulates the chronic low-grade inflammation.

The obesity-stimulated inflammatory response has been suggested to be mainly triggered by adipose tissue, although other metabolic sites, such as liver are also likely to be involved (293,305). In obesity, the secretion of proinflammatory factors is upregulated and the secretion of anti-inflammatory factors such as adiponectin is downregulated (200,297,306). Specifically, increased visceral fat is associated with a shift in the normal balance of these adipokines resulting in a pro-inflammatory state (44). Macrophages are the main targets for many of the secreted proinflammatory substances and accordingly, obesity is associated with an increased accumulation of macrophages in adipose tissue (307). The macrophages in obese individuals are in a proinflammatory state, which is reflected in the high levels of secreted TNF-α (308). One of the factors contributing to macrophage infiltration in adipose tissue is the monocyte chemoattractant protein-1, a chemokine (C-C motif) receptor (CCR)-2 ligand (309) which is upregulated in obesity (310). Recently also CCR-5 receptor and its ligand chemokine (C-C motif) ligand (CCL)5, also known as regulated upon activation, normally T-expressed, and presumably secreted (RANTES), have been shown to be upregulated in the adipose tissue of obese human and rodents (306).

Inflammatory and metabolic processes are coordinately regulated by many transcription factors, such as PPARs and liver X receptors (311,312). Ligands of all three PPARs suppress production of proinflammatory mediators, mainly by inhibiting nuclear factor κB (311,312). Reciprocally, TNF-α decreases the expression of adipocyte-specific genes and transcription factors which are necessary for adipocyte differentiation, including PPAR-γ and CCAAT/enhancer-binding protein α (C/EBP-α) (313,314). Liver X receptor is also able to suppress the production of inflammatory mediators (315). Interestingly, the activation of liver X receptor improves glucose tolerance by regulating glucose metabolism in liver and adipose tissue (316). This, together with the anti-inflammatory properties of insulin (317) and the insulin-sensitizing actions of adiponectin (318) represents a bridge between inflammation and the characteristics of obesity and impaired glucose metabolism.

2.3.5 Effect of weight change on gene expression in peripheral tissues

It has been shown that weight loss can induce changes in the gene expression in human adipose tissue (319-325), but also in other tissues such as skeletal muscle (326,327) and peripheral blood mononuclear cells (328). In humans, the effects of overfeeding in controlled clinical settings have been studied to a lesser extent, but also a positive

energy balance has been shown to affect transcription levels (329,330). In addition to the energy content, the composition of diet, such as the amount (331) and quality of fat (332,333) and the amount (319,332-334) and glycaemic index of consumed carbohydrates (335) of isocaloric diets all are factors which can influence the transcription of genes in different tissues.

Meugnier *et al* (330) have reported that overfeeding alters the gene expression profile of skeletal muscle. At the same time, these changes stimulate triacylglycerol synthesis and the development of adipocytes, inhibit lipolysis and reduce fatty acid oxidation. Promoter analysis of the regulated genes showed that sterol regulatory element binding proteins (SREBPs) might be important players in the short-term adaptation to fat overfeeding in human skeletal muscle. Accordingly, excess energy intake has been shown to increase the mRNA expression of *SREBP-1c* in both overweight and lean individuals (329).

As expected, weight reduction downregulates the mRNA expression of leptin, IL-6 (319,331) and other proinflammatory factors and upregulates the expression of mRNAs encoding anti-inflammatory factors (323). Many of the genes involved in fatty acid and cholesterol metabolism, such as hormone-sensitive lipase (*HSL*) (331,336), fatty acid synthase (*FASN*), fatty acid translocase (*CD36*), lipoprotein lipase (*LPL*) (331), diacylglycerol O-acyltransferase 2 (*DGAT2*) (333), fatty acid desaturase (*FADS1*), stearoyl conezyme A desaturase (*SCD*) (333,334), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGCR*) and LDL-receptor (332) are also downregulated by weight loss, both in adipose tissue (331,333,334) and mononuclear cells (332).

In a recent study, the genes defined by gene ontology groups of the extracellular matrix and cell death were differentially regulated in adipose tissue by long-term weight loss in persons with the features of metabolic syndrome (325). One of the most extensively downregulated genes was tenomodulin (fold change 0.67). In that study, the participants underwent a 12-week intensive weight reduction and were expected to maintain their reduced weight for the following 20 weeks. A change of similar magnitude in *TNMD* expression (0.75) was observed by Dahlman *et al* (334) when they compared the effects of two different diets during a 10-week weight loss period.

2.4 Tenomodulin

2.4.1 Structure and function of the tenomodulin gene and protein

Tenomodulin was identified in 2001 by Cros *et al* (337), who demonstrated that a novel gene which they named myodulin, was two-fold downregulated in muscle atrophy in mice. Simultaneously, other groups cloned the same gene and named it chondromodulin-I-like (338), tenomodulin (339), and tendin (340). The tenomodulin gene spans approximately 15 kb in chromosomal locus Xq22. *TNMD* has at least three splice variants. In addition to the variant containing seven exons (341), a five-exon splice variant is described in the UCSC Genome Browser (342), March 2006 Assembly (http://genome.ucsc.edu/) and a three-exon variant in Ensembl´s Vega Transcript Report ((343), v.31 April 2008; http://vega.sanger.ac.uk/). The functions and tissue distribution of these shorter transcripts have not been characterized.

TNMD belongs to the BRICHOS protein family (344). In a similar manner to the other members of this family, TNMD is an integral type 2 transmembrane protein with cytoplasmic N-terminal and extracellular C-terminal, from which the C-terminal part is cleaved proteolytically (344,345). While two other members of the BRICHOS-family, chondromodulin (CHM) and familial dementia BRI2 have a furin cleavage site, TNMD contains an RXXR-cleavage motif (amino acids 233-236) that has been shown to be functional (345). The TNMD protein is composed of short N-terminal cytoplasmic domain (residues 1-30), a transmembrane domain (residues 31-51), BRICHOS-domain, which has been suggested to function as an intramolecular chaperone for the cleaved part (344,346) (residues 93-186) and a cysteine-rich C-terminal antiangiogenic domain (residues 202-317) (337). Similar structural components are found in the CHM (Figure 4) (341).

TNMD does not have any close homologs, but it exhibits overall 33% amino acid sequence identity with CHM, which is a chondrocyte growth factor (347) and angiogenesis inhibitor (348). The similarities in the structural organization between these two proteins are rather apparent (Figure 4).

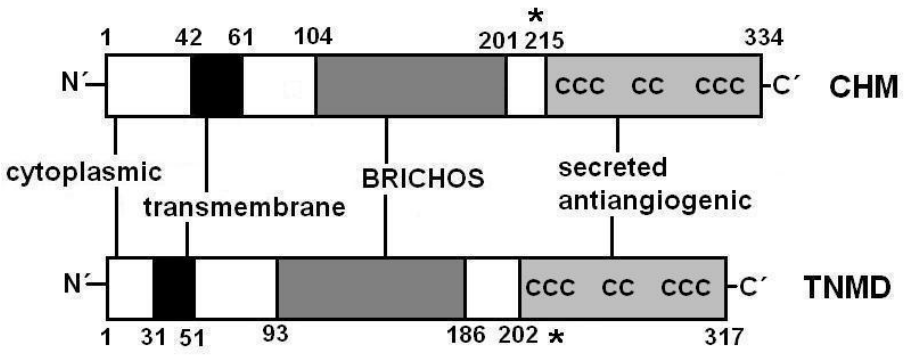


Figure 4. The domain architecture of human chondromodulin (CHM) and tenomodulin (TNMD) proteins (according to Oshima *et al* 2004 (349)). Different domains are indicated with greyscale. The furin cleavage site in the amino acid position 211-214 in CHM and RXXR-cleavage site in the position 233-236 in TNMD are denoted by asterisks (*) and the eight conserved cysteine residues by C.

The sizes of unprocessed CHM and TNMD precursors are almost identical: CHM is composed of 334 amino acid residues while TNMD consists of 317 amino acid residues (341). Although the sequence similarity of this domain is quite high (65%), and the cysteine residues that are needed for the correct folding are identically spaced in both CHM and TNMD (345,347,348), the molecular weight of secreted part is different. The secreted part of TNMD is only 16kDa (345), while that of CHM is 25 kDa (347,348). The exact targets of this domain are unknown.

Functional studies performed *in vitro* have shown that the secreted parts of both CHM and TNMD inhibit angiogenesis by preventing endothelial proliferation and tube formation (349,350). However, *TNMD*-deficient mice did not exhibit any vascular abnormalities (345), though this could be due to compensatory mechanisms which maintain normal vasculature in the absence of tenomodulin. In addition, *in vivo* studies performed in mice (345,351) and chicks (352) have demonstrated the necessity of TNMD for tenocyte proliferation and tendon maturation.

2.4.2 Expression profile and tissue distribution

TNMD is mainly expressed in hypovascular connective tissues such as tendons, ligaments and eye. The main results from mouse studies are summarized in Table 4.

Table 4. The expression of tenomodulin mRNA and protein in mouse tissues. RT-PCR reverse-transcriptase-polymerase chain reaction

Tissue	Expression level	Method	Author
Skeletal muscle (whole)	High	Northern blot, RT-PCR	Cros *et al* (337) Yamana *et al* (338) Shukunami *et al* (339) Brandau *et al* (340)
Skeletal muscle (epimysium envelope and tendon)	High	*In situ* hybridisation	Shukunami *et al* (339)
Skeletal muscle (ligament and tendon)	High	*In situ* hybridisation	Brandau *et al* (340)
Whole rib	High	RT-PCR	Yamana *et al* (338)
Thymus and brain	High	*In situ* hybridisation	Brandau *et al* (340)
Eye (whole)	High	Northern blot	Yamana *et al* (338) Brandau *et al* (340) Oshima *et al* (350)
Eye (cornea, sensory retina, lens fiber and sclera)	High	*In situ* hybridisation	Oshima *et al* (350)
Eye (choroidal tissues, e.g. retinal pigment epithelium)	Low	*In situ* hybridisation	Oshima *et al* (350)

Tenomodulin has not been reported to be expressed in the adipose tissue of mice, but it has been shown to be expressed in human adipose tissue (325,334). It is not known which actual cell types in the adipose tissue express tenomodulin, but according to Gene Atlas database ((353) http://symatlas.gnf.org/SymAtlas/), *TNMD* expression is detected in the adipocytes, albeit at a modest level. In addition, our preliminary studies have shown that TNMD is expressed in adipocytes and blood vessels of adipose tissue (unpublished observation). Other human tissues that exhibit relatively high mRNA expression of *TNMD* are cardiac myocytes, tongue and certain regions of brain, such as temporal lobe and globus pallidus (353).

2.4.3 Regulators of tenomodulin expression

The regulators of *TNMD* expression are not very well known. Scleraxis (SCX), a transcription factor and a tendon-specific marker (354), has been shown to upregulate TNMD expression in chick embryo and tenocyte cultures (352). Recently, Mendias *et al* (351) reported that myostatin (MSTN) upregulates *TNMD* expression in tendon fibroblasts. Myostatin, also known as growth and differentiation factor 8 is a member of TGF-β superfamily, a large group of secreted growth and differentiation factors that are essential for regulation of tissue development and homeostasis. The members of this family are involved in myogenesis, angiogenesis and adipogenesis (202,355-357).

Myostatin deficiency also influences the mechanical properties of tendons, as *MSTN*-deficient mice have been shown to have stiff and brittle tendons with significantly lower *TNMD* expression than their wild-type counterparts (351).

The *MSTN* gene has been widely studied, since the alterations in its expression affect the body composition. *MSTN*-deficiency causes muscle hypertrophy (356-361,361,362) and overexpression leads to decreased adipogenesis (363,364). The importance of myostatin in adipose tissue development has been proven both *in vitro* and *in vivo*. Rebbapragada *et al* (365) first demonstrated that MSTN blocks adipogenesis in both mesenchymal precursor cells and preadipocytes. Subsequently, Feldman *et al* (364) showed that myostatin modulates adipogenesis so that the generated adipocytes had favourable metabolic characteristics including reduced lipid accumulation, diminished incorporation of exogenous fatty acid into cellular lipids and high insulin sensitivity. The cells resembled immature adipocytes, since they were smaller than normal adipocytes and displayed low expression levels of *LPL*, *PPAR-γ,* leptin, adiponectin, TNF-α and resistin. In the study of Zimmers *et al* (366), the pharmacological administration of myostatin in adult mice reduced fat mass by up to 50% without affecting muscle mass, but these results have not been successfully duplicated (367). MSTN has not been studied in the context of human obesity, but it has been claimed that weight loss significantly downregulates the expression of *MSTN* in the skeletal muscle of morbidly obese persons (368).

2.4.4 Tenomodulin knock-out mouse

Docheva *et al*. (345) have reported that the ablation of *TNMD* expression by gene-targeting did not affect the viability or life span of mice. The body size, weight, basic histology of the main organs (muscle, thymus, heart, liver, spleen, and lung) or skeletal development were not affected by *TNMD* deficiency.

The *TNMD*-knock-out (KO) mice had a reduced tenocyte density due to impaired proliferation and an altered structure of collagen fibrils. However, despite the lower cell numbers, the tendons of KO mice were of the same size as those measured in the wild-type (WT) mice, suggesting that either the remaining tenocytes were able to compensate for the loss of cells or that the turnover of extracellular matrix was delayed in the *TNMD*-deficient tendons. The tendons of *TNMD*-null mice also exhibited greater variation in collagen fibril diameters and an increase in the maximal fibril diameters in comparison to WT mice. Interestingly, knock-out of *TSP-2* gene, a close homolog of the

adipokine *TSP-1* (369), results in similar phenotype in the tendons of these knock-out mice (370).

At odds with the previously reported antiangiogenic activity (349), a loss of *TNMD* expression did not affect tendon vessel density and mice lacking both *TNMD* and *CHM* had normal retinal vascularization and neovascularization after oxygen-induced retinopathy (345). Similarly, the deletion of the *TSP-1* gene in mice did not result in severe vasculature-related abnormalities (371).

3 AIMS OF THE STUDY

Two independent studies have shown that weight loss decreases the expression of *TNMD* in the adipose tissue (325,334). TNMD mediates antiangiogenic effects (349,350), and recently another angiogenesis inhibitor, TSP-1, was confirmed to be an adipokine (286). Therefore the research hypothesis was that *TNMD* might be a susceptibility gene for obesity and related conditions.

The purpose of the study was to investigate the association of a common sequence variation in the *TNMD* gene with obesity and related phenotypes. These association studies were performed in three different study populations, both in longitudinal and cross-sectional settings. The specific research questions were whether the common single nucleotide polymorphisms (SNPs) in the *TNMD* gene would be associated with:

1. Obesity and anthropometric measurements (*Studies I* and *III*)
2. Glucose metabolism and incidence or prevalence of type 2 diabetes (*Studies I* and *III*)
3. Chronic low-grade inflammation status indicated by serum levels of systemic immune mediators (*Study II*)
4. Serum levels of lipids and lipoproteins (*Study III*)
5. Prevalence of age-related macular degeneration (*Study IV*)

4 SUBJECTS AND METHODS

4.1 Study populations

4.1.1 The Finnish Diabetes Prevention Study (*Studies I-III*)

The Finnish Diabetes Prevention Study (DPS) is a randomized, controlled lifestyle intervention study conducted in the cities of Helsinki, Kuopio, Tampere Turku and Oulu in Finland (82). The main aim of DPS was to investigate whether the onset of T2D could be prevented or delayed among high-risk individuals by lifestyle modification. The main inclusion criteria were BMI over 25 kg/m^2, age 40 to 64 years and impaired glucose tolerance tolerance (2h-PG 7.8–11.0 mmol/l and FPG<7.8 mmol/l) on the basis of the mean value of two consecutive OGTTs. It should be noted that the glucose tolerance status was diagnosed on the basis of WHO 1985 criteria (60) and according to the current criteria (62), some of the participants would have been diagnosed with T2D in the beginning of the study

Altogether 522 individuals were randomized into two groups according to the centre, gender, and mean 2h-PG in OGTT. The intervention group (n=265) received intensive individualized diet and exercise counselling and were given detailed advice on how to achieve the objectives of the intervention, while the control group (n=257) received general written and oral information about diet and exercise at baseline and annual visits (82). Medical history questionnaires, anthropometric and laboratory measurements were obtained at baseline and at the annual visits. DNA was available from 507 individuals (166 men and 341 women).

The study protocol was approved by the Ethics Committee of the National Public Health Institute in Helsinki, Finland and the participants received both oral and written information of the study and provided written informed consent.

4.1.2 Metabolic Syndrome in Men (*Study III*)

The Metabolic Syndrome in Men- study (METSIM) is a random population-based sample of 5298 Finnish 50-70 years old men living in the city of Kuopio in Eastern Finland (113). The primary aim of this ongoing study is to investigate the genetic risk factors of T2D and cardiovascular diseases. According to WHO´s 1999 criteria (62),

3020 individuals were normoglycaemic, 984 had impaired fasting glucose, 436 had impaired glucose tolerance and 811 had known or newly diagnosed T2D.

The Ethics Committee of the District Hospital Region of Northern Savo and Kuopio University Hospital approved the study plan. The participants received both oral and written information of the study and gave their written informed consent.

4.1.3 Study population for age-related macular degeneration (*Study IV*)

Altogether 475 persons (162 men, 313 women) from the regions of Kuopio and Helsinki were included in this study. Eighty-nine men and 175 women had exudative AMD and 18 men and 25 women had atrophic AMD. The control group consisted of 55 men and 113 women. All participants were over 65 years old. Diabetes mellitus, based on medical history and patient records was considered as an exclusion criterion. The study was approved by the Ethics Committees of the Kuopio University Hospital and Helsinki University Eye and Ear Hospital. All participants signed an informed consent. The controls were patients with other ophthalmologic conditions (e.g. cataract) who had no signs of AMD in biomicroscopy examination. Age-related macular degeneration was diagnosed on the basis of choroidal neovascularization in fundus photographs and fluorescein angiography in the Department of Ophthalmology at Kuopio University Hospital or Helsinki University Hospital.

4.2 Methods

4.2.1 Anthropometric measurements (*Studies I-III*)

Weight and height were measured in light clothing and BMI was calculated as the weight in kilograms divided by the square of the height in meters in both DPS and METSIM studies. In the DPS, the waist circumference was measured midway between the lowest rib and iliac crest and hip circumference over the great trochanters in the standing position. Sagittal and horizontal diameters were measured with the person in supine position on a hard surface as the distance from the surface to the highest point of the abdomen (sagittal diameter) and the maximum width of the abdomen (horizontal diameter) at the level of the iliac crest using especially built equipment. The exact description for methdology in DPS is described in (83).

4.2.2 Biochemical and diagnostics measurements (*Studies I-IV*)

In both METSIM and DPS, the glucose tolerance was determined by 2h OGTT with 75 g glucose dose after an overnight fast. Samples for plasma glucose and insulin concentrations were drawn at 0, 30 and 120 min.

DPS. The plasma glucose concentrations were determined locally according to standard guidelines while all other biochemical determinations were performed in the central laboratory of the Department of Biochemistry, National Public Health Institute, Helsinki. Serum insulin was determined with radioimmunoassay (Pharmacia, Uppsala, Sweden). Serum total cholesterol, HDL-cholesterol and triglycerides were determined with an enzymatic assay method (CHOD-PAP, Boehringer Mannheim, Germany, Monotest). LDL-cholesterol was calculated with the Friedewald formula (372) and applied only when triglyceride levels were <4.5mmol/l (83).

The serum concentrations of CRP and SAA were assessed by a high-sensitivity latex-enhanced nephelometric assay and immunonephelometry, respectively with BN II analyzer (Dade Behring, Marburg, Germany). Enzyme-linked immunosorbent assay (ELISA) was used for determining the serum concentrations of IL-6 (Sanguin, Amsterdam, Netherlands), soluble intercellular adhesion molecule-1 (sICAM-1; Diaclone, Besancon, France), CCL3, CCL5 and macrophage migration inhibitory factor (MIF; R&D Systems, Wiesbaden Germany for all three).

METSIM. The biochemical analyses were performed at the Clinical Research Unit in the University of Kuopio. Serum insulin was determined with an immunoluminometric method (Avidia Centaur IRI) on Advia Centaur Immunoassay System (both from Siemens Medical Solution Diagnostics, Tarrytown, NY, USA). The plasma glucose concentration was determined by the hexokinase method (Thermo Fisher Scientific, Vantaa, Finland). Serum total cholesterol and triglycerides were analyzed with an enzymatic method and serum HDL and LDL were determined with direct enzymatic assays (KoneLab Systems Reagents). KoneLab 20XTi Clinical Chemistry Analyzer was used for both glucose and lipoprotein analyses.

4.2.3 Genetic association studies

4.2.3.1 The selection and genotyping of single nucleotide polymorphisms

Studies I-II. The HapMap- (373) and the National Center for Biotechnology Information databases were used for selection of *TNMD* SNPs for genotype analysis. Specifically, two of two tag-SNPs of haploblock 1 (rs5966709 and rs4828037) and three from five tag-SNPs of haploblock 2 (rs2073162, rs2076163, and rs4828038) were selected from the HapMap database. The CEPH (Utah residents with ancestry from northern and western Europe (CEU) was used as a reference population. In addition, two SNPs were selected from the National Center for Biotechnology Information database to cover the 5' and 3' ends of the gene (rs11798018 and rs1155974). The selected markers cover 63% of the common sequence variation with r^2>0.8 in the coding region of *TNMD*.

Study III. Markers rs2073162, rs2073163 and rs1155974 that were associated with T2D risk in the DPS (*Study I*) were genotyped from 2045 participants of the METSIM study, but as the three markers were in complete linkage disequilibrium (LD), genotyping was continued only for rs2073162 for the remaining 3253 individuals.

Study IV. Six markers covering 75% of the common sequence variation with r^2>0.8 in the coding region of *TNMD* (15kb) and 10 kb up-and downstream from the coding region (35kb) were selected with the Tagger algorithm (374). The markers rs2073163 and rs1155974 were forced in the selection procedure.

Genotyping for all studies was carried out using TaqMan Allelic Discrimination Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The genotyping reactions were amplified using GeneAmp PCR system 2700 and allelic discrimination according to the fluorescent labels was performed with ABI Prism 7000 sequence detector (both from Applied Biosystems). The error rate for genotyping was estimated by repeating a random sample of 3.5% of the METSIM study population and 6.3% of the DPS- and AMD-study populations.

4.2.3.2 Statistical analyses

Haploview software (375) was used for LD and Hardy-Weinberg equilibrium (HWE) calculations. Statistical analyses were performed with SPSS14.0 for Windows (SPSS, Chicago, IL, USA). The data are presented as median (interquartile range) (tables) or means±standard error of the mean (SEM; figures) and $p<0.05$ was considered statistically significant. Due to the X-chromosomal location of the *TNMD* gene, men and women were analyzed separately. Data from women was analyzed with the additive model in *Studies I-IV*, and with dominant (major allele homozygotes vs. other genotypes) and recessive (minor allele homozygotes vs. other genotypes) models in *Studies II-IV*.

The distribution of genotypes among genders and study groups was assessed with Pearson´s χ^2-test. Normal distribution was tested with Lilliefors-corrected Kolmogorov–Smirnov test (*Studies I-III*) and by plotting the residuals of each statistical model (*Studies II-III*). Appropriate transformations were performed to achieve normal distribution when necessary. If the distribution of continuous variables could not be normalized, Kruskal-Wallis or Mann-Whitney´s U tests were used. The association of *TNMD* SNPs with continuous variables in *Studies I-III* was analyzed with a general linear models using univariate analysis of variance for baseline data and repeated measurements for follow-up data from baseline and three annual visits. Adjustments for age, BMI and intervention group were done when necessary, i.e. their contribution to the model was $p<0.1$. Bonferroni correction was used in pairwise comparisons between the three genotypes in women. In addition, the effects of genotype and intervention on the changes in weight and waist circumference at year 1 (calculated as $\text{measurement}_{\text{baseline}}$-$\text{measurement}_{\text{year1}}$) were assessed in *Study I*.

In *Study II*, three different models with either BMI, waist circumference or 2h-PG as covariates were constructed on the basis of correlations between systemic immune mediators and potential adjustment factors in this population (376). Both the main effects and covariate*genotype-interactions were studied. Due to the observed interactions, the data was stratified according to median 2-hour plasma glucose concentration (8.72mmol/l) and genderwise median BMI (29.43 for men, 31.21 for women) and waist circumference (98.5 for men, 103.25 for women) to study whether the genotype effect is modified by body size or the status of glucose metabolism. The overlapping of median categories was analysed by Pearson´s χ^2-test.

In *Study III*, the results were adjusted for age, BMI, use of statin and/or use of reimbursed cholesterol-lowering medication. Due to observed genotype*BMI-interactions, additional stratified analyses were performed according to the quartiles of BMI in METSIM and according to the medians of BMI in DPS due to smaller number of participants in the DPS. In the METSIM study, the ranges for the quartiles were 16.18-24.58 kg/m^2, 24.59-26.72 kg/m^2, 26.73-29.40 kg/m^2 and 29.41-52.11 kg/m^2. In the DPS, the range for the lower median was 23.50-29.40 kg/m^2 and 29.45-44.80 kg/m^2 for the upper median.

The association of SNPs with conversion of IGT to T2D was analyzed with Cox regression using appropriate covariates (*Study I*). The association of *TNMD* SNPs with the prevalence of T2D in the METSIM study population *Study III* was analyzed with logistic regression (adjusted for age and and BMI). The associations of *TNMD* SNPs with the prevalence of total AMD and exudative and atrophic subforms were tested with unadjusted logistic regression (*Study IV*).

THESIAS 3 (*Study I*) and THESIAS 3.1 (*Studies II* and *IV*) (377) were used for haplotype analysis of LD-based haplotypes. In *studies II* and *IV*, the correction for multiple hypothesis testing was performed with the false discovery rate (FDR) using Q-value 1.0 software (378). π_0 was estimated with a bootstrap method using λ range from 0 to 0.9 by 0.05.

5 RESULTS

5.1 Genotype frequencies and success and error rates

The genotype frequencies in DPS and AMD study populations are shown in Table 5. In the METSIM population, the frequencies were 65.8% for rs2073162-G and 34.2% for rs2073162-A. In DPS, all markers except rs4828038 (p=0.01) were in HWE, when the cut-off of $p \leq 0.01$ was applied. In the AMD study population, the markers rs7890586 and rs2073163 were not in HWE (p=0.002 and 0.009, correspondingly).

In DPS, the markers belonged to two haploblocks on the basis of their LD pattern (Figure 5a), the first consisting of markers rs11798018, rs5966709 and rs4828037 and the second of rs2073162, rs2073163, rs4828038 and rs1155974. In the AMD data set, two markers, rs11798018 and rs5966709, formed the first LD-based haploblock (Figure 5b) and rs2073163 and rs1155974 made up the second. The frequencies of major haplotypes of these two blocks in both study populations are represented in Table 6.

In the DPS and METSIM studies, the genotyping success rate was 100% for all markers. In the AMD study population, the genotyping success rate was 98.5% for rs7890586, 99.6% for rs1204384, and 100% for markers rs11798018, rs5966709, rs2073163 and rs1155974. The genotyping error rate was 0% in all study populations.

Table 5. Genderwise genotype frequencies of the *TNMD* SNPs in the Finnish Diabetes Prevention Study (DPS) and age-related macular degeneration study (AMD) populations. HWE for genotype frequency is estimated for women only.

Marker	Genotype	DPS			AMD		
		Frequency in men (n)	Frequency in women (n)	*p* for HWE	Frequency in men (n)	Frequency in women (n)	*p* for HWE
rs7890586	GG	Not genotyped in this population.			81.5 (132)	78.6 (242)	0.002
	GA				0	16.9 (52)	
	AA				17.3 (28)	4.5 (14)	
rs11798018	CC	66.3 (110)	38.1 (130)	0.469	60.5 (98)	31.0 (97)	1
	CA	0	48.7 (166)		0	49.5 (155)	
	AA	33.7 (56)	13.2 (45)		39.5 (64)	19.5 (61)	
rs5966709	GG	66.9 (111)	42.2 (144)	1	61.7 (100)	44.4 (139)	1
	GT	0	45.5 (155)		0	44.7 (140)	
	TT	33.1 (55)	12.3 (42)		38.3 (62)	10.9 (34)	
rs4828037	TT	65.1 (108)	37.8 (129)	0.721	Not genotyped in this population.		
	TC	0	48.4 (165)				
	CC	34.9 (58)	13.8 (47)				
rs2073162*	GG	64.5 (107)	39.0 (133)	0.012	Not genotyped in this population.		
	GA	0	41.3 (141)				
	AA	35.5 (59)	19.6 (67)				
rs2073163**	TT	69.9 (116)	43.1 (147)	0.065	68.5 (111)	43.8 (137)	0.009
	TC	0	41.3 (141)		0	39.3 (123)	
	CC	30.1 (50)	15.5 (53)		31.5 (51)	16.9 (53)	
rs4828038	TT	56.0 (93)	37.5 (128)	0.010	Not genotyped in this population.		
	TC	0	40.6 (142)				
	CC	44.0 (73)	20.8 (71)				
rs1155974**	CC	71.7 (119)	42.8(146)	0.107	65.4 (106)	41.2 (129)	0.962
	CT	0	41.9 (143)		0	45.7 (143)	
	TT	28.3 (47)	15.2 (52)		34.6 (56)	13.1 (41)	
rs1204384	AA	Not genotyped in this population.			69.1 (112)	45.5 (142)	0.806
	AT				0	43.3 (135)	
	TT				30.4 (49)	11.2 (35)	

*Genotyped from 5298 participants of the METSIM study. Frequency of rs2073162-G =65.8 and frequency of rs2073162-A =34.2.
** Genotyped from 2045 participants of the METSIM study. Frequency of both rs2073163-T and rs1155974-C=66.0 and frequency of both rs2073163-C and rs1155974-T=34.0.

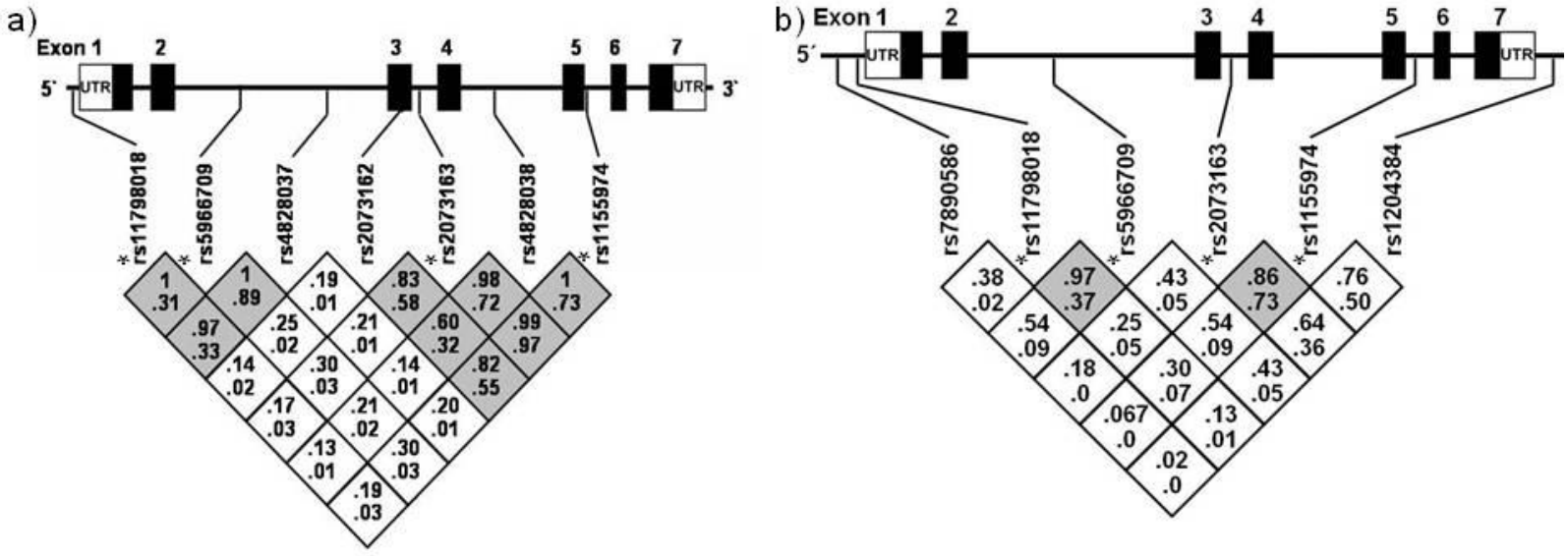


Figure 5. The location of selected markers in the *TNMD* gene and their pairwise D'- (upper) and r^2-values in the (a) DPS- and (b) AMD-study populations. The gray toning indicates the two LD-based haploblocks. The SNPs that were genotyped in bith populations are denoted with asterisks (*). UTR untranslated region

Table 6. Frequencies of the major (frequency >0.05) LD-based haplotypes in men and women of the DPS- and AMD- study populations.

DPS						AMD			
Markers				Men (n=166)	Women (n=341)	Markers		Men (n=161)	Women (n=312)
Haploblock 1						Haploblock 1			
rs11798018	rs5966709	rs4828037				rs11798018	rs5966709		
A	G	T		0.33	0.37	A	G	0.39	0.44
C	T	C		0.33	0.35	C	T	0.38	0.33
C	G	T		0.32	0.25	C	G	0.23	0.23
Haploblock 2						Haploblock 2			
rs2073162	rs2073163	rs4828038	rs1155974			rs2073163	rs1155974		
G	T	T	C	0.43	0.51	T	C	0.64	0.61
A	C	C	T	0.22	0.33	C	T	0.30	0.33
G	T	C	C	0.16	0.05				
A	T	T	C	0.11	0.07				
G	C	C	T	0.06	<0.05				

5.2 *TNMD*, obesity and anthropometric measurements *(Study I)*

The observed associations between the *TNMD* and anthropometric measurements in the DPS are summarized in Table 7. In the follow-up data analysis, the intervention and control groups of the DPS were analyzed together, because the allele distribution of each marker was similar in both groups, and the genotype-randomization group interaction was statistically non-significant in all analyses.

The SNP rs11798018 was associated with BMI and weight (p=0.038 and p=0.029, respectively) during the 3-year follow-up in men, but no significant associations were observed at baseline. The persons with the A-allele had lower BMI than individuals with the C-allele (Table 7).

Among the women of the DPS, rs2073162 was associated with horizontal diameter at baseline. The individuals with the rs2073162-AA genotype had the smallest values. Furthermore, rs4828037 was associated with the sagittal diameter such that the individuals with rs4828037-TT genotype had the highest values (Table 7).

Table 7. The observed associations of the *TNMD* SNP*s* with anthropometric measurements in the DPS. Numeric data are given as median (interquartile range).

Gender	Marker	Genotype	Parameter	Median (IQ range)	*p* for baseline	*p* for follow-up
Men	rs11798018	CC AA	BMI (kg/m^2)	29.70 (4.56) 29.01 (4.70)	0.810*	0.038*
	rs11798018	CC AA	Weight (kg)	90.30 (16.40) 86.60 (15.80)	0.064*	0.029*
Women	rs2073162	GG GA AA	Horizontal diameter (cm)	39.40 (4.25) 39.20 (5.00) 38.70 (6.70)	0.038**	0.266**
	rs5966709	GG GT TT	Waist to hip-ratio	0.89 (0.08) 0.89 (0.09) 0.87 (0.07)	0.316**	0.028**
	rs5966709	GG GT TT	Sagittal diameter (cm)	24.40 (4.68) 24.30 (4.30) 23.00 (3.58)	0.080**	0.014**
	rs5966709	GG GT TT	Horizontal diameter (cm)	39.20 (5.48) 39.50 (4.40) 37.95 (5.53)	0.055**	0.043**
	rs5966709	GG GT TT	Waist circumference (cm)	99.00 (16.35) 99.50 (14.50) 94.35 (19.00)	0.068**	0.036**
	rs4828037	TT TC CC	Sagittal diameter (cm)	24.40 (4.80) 24.30 (4.40) 23.20 (4.10)	0.015**	0.026**
	rs4828037	TT TC CC	Waist to hip-ratio	0.89 (0.08) 0.89 (0.09) 0.87 (0.07)	0.479**	0.056**

*adjusted for age
***p* for additive model adjusted for age and baseline BMI

During the 3-year follow-up, two markers, rs5966709 and rs4828037, were associated with central obesity in women. In both cases, participants homozygous for the minor alleles (5966709-TT and 4828037-CC) had lower values than the individuals with other genotypes (Figure 6). The associations, apart from that of rs5966709 with horizontal diameter, were considerably stronger when analyzed with the recessive model (p=0.007 for waist circumference, p=0.009 for waist to hip-ratio and p=0.003 for sagittal diameter with rs5966709 and p=0.007 for sagittal diameter with rs4828037).

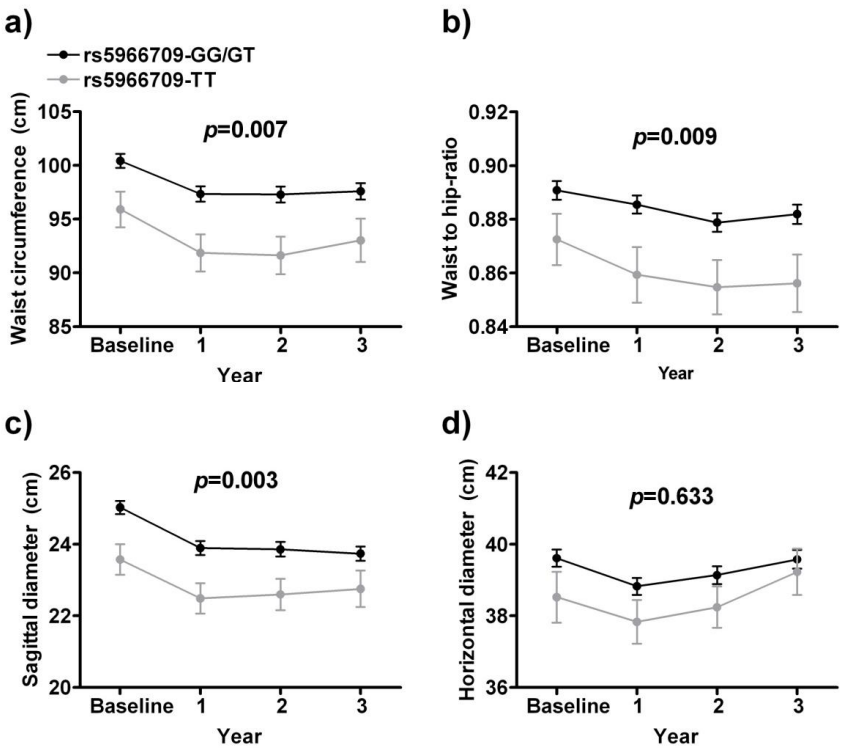


Figure 6. The mean±SEM values of waist circumference (a), WHR (b), horizontal diameter (c), and sagittal diameter (d) in women according to the genotypes of rs5966709 during the 3-year follow-up according to recessive model.

To study haplotype effects of haploblock 1 (Figure 5a) on body size measurements at baseline in women, three-marker haplotypes (markers rs11798018, rs5966709, and rs4828037) were constructed. Three major haplotypes with frequencies of >0.05 were observed (Table 6). The results were consistent with the baseline analysis of individual markers, since in comparison to the carriers of the reference haplotype AGT, the individuals with the CTC haplotype had a lower sagittal diameter (p=0.044). The former haplotype includes the rs4828037-C-allele, which was *per se* associated with a smaller sagittal diameter.

The genotypes of rs2073162 were not associated with BMI (*Study III*), weight or indicators of central obesity (p>0.1) in the men of METSIM study population.

5.3 *TNMD*, glucose metabolism and type 2 diabetes (*Studies I and III*)

In DPS, no genotype differences in insulin and glucose levels were observed at baseline, except for rs2073162 in women. Specifically, the rs2073162-GG genotype was associated with lower fasting plasma glucose levels than the other genotypes (p=0.021). The median (interquartile range) FPG was 5.89 (0.88) mmol/l for the carriers of GG genotype, 6.21 (1.03) mmol/l for GA and 6.16 (0.87)mmol/ for the AA genotype. In women, the same marker was associated with 2h-PG concentrations during the 3-year follow-up, but contradictory to the baseline results, the lowest levels were observed with the AA-genotype.

Among the men of DPS, the markers rs2073163 and rs1155974 were associated with 2h-PG during the 3-year follow-up. The marker rs2073163 was also associated with conversion of IGT to T2D in men in DPS and a borderline association was observed with rs1155974. The individuals with the minor alleles (rs2073163-C or rs1155974-T), which were associated with higher 2-hour plasma glucose concentration, were approximately two times more likely to develop T2D during the 5-year follow-up than the major allele carriers. Asimilar association was also observed with rs2073162, which was not associated with 2h-PG. In women, none of the SNPs contributed to the risk of T2D. The observed associations with 2H-PG and T2D risk are summarized in Table 8. The marker rs2073162 was not associated with plasma glucose or serum insulin concentrations during OGTT in the METSIM study and the prevalence of T2D was also similar between the genotypes (15.2 vs 15.1%).

Table 8. The observed associations of the *TNMD* SNP*s* with 2H-PG during the 3-year follow-up and T2D risk in the DPS. HR hazard ratio (reported only for statistically significant associations), CI confidence interval

Marker	Gender	2H-PG p*	Risk genotype	T2D risk HR (95%CI)	p**
rs2073162	Men	0.249	A	2.193 (1.105- 4.354)	0.025
rs2073162	Women	0.013	GG	0.755 (0.473-1.204)	0.238
rs2073163	Men	0.038	C	2.191 (1.092-4.394)	0.027
	Women	0.056	TC/CC	1.012 (0.546-1.875)	0.971
rs1155974	Men	0.011	T	1.998 (0.989-4.036)	0.054
	Women	0.065	CT/TT	1.082 (0.678-1.728)	0.741

*adjusted for age and baseline BMI
**adjusted for baseline BMI, waist-to hip-ratio, fasting plasma glucose and intervention

To estimate the haplotype effect of these SNPs in DPS, three-marker haplotypes of SNPs rs2073162, rs2073163, and rs1155974 were constructed and four major haplotypes (frequencies >0.05) were observed (Table 6). The most common haplotype (frequency=0.595) contained the rs2073162-G-, rs2073163-T- and rs1155974-C-alleles, which were also individually associated with a lower risk of T2D. Interestingly, the complement haplotype ACT, containing all individual risk alleles, was associated with a 2.3-fold risk for developing T2D (p=0.041; 95% CI, 1.034 to 5.175). Although this analysis did not uncover a haplotype combination that would explain the results substantially more than individual markers, the results support those obtained from single-marker analysis.

5.4 *TNMD* and low-grade inflammation indicated by the serum levels of systemic immune mediators (*Study II*)

The association of the common sequence variation in the *TNMD* gene with acute phase reactants (SAA and CRP), proinflammatory cytokines (MIF and IL-6), ligands of CCR-5, which induce the production of proinflammatory cytokines (CCL3 and CCL5) and sICAM were addressed. In addition, the effect modification by the status of glucose tolerance, central obesity and general body size (indicated by 2h-PG, waist circumference and BMI, respectively) was assessed. As BMI and waist circumference had almost identical effects, and the low-grade inflammation is more related to the central obesity than body size in general, only the results in the medians of waist circumference and 2h-PG are reported.

The three markers, rs2073162, rs2073163 and rs1155974, which were associated with the risk of T2D in men in *Study I* were associated with serum concentrations of CRP and SAA so that the individuals harbouring the genotypes (rs2073162-A, rs2073163-C and rs1155974-T) related to higher T2D incidence had higher serum levels of the three inflammatory markers (Table 9). The markers rs2073163 and rs1155974 were also associated with the serum levels of sICAM so that the men with the rs2073163-C or rs1155974-T genotypes had higher concentrations than the individuals with the other genotypes. In addition, two markers, rs5966709 and rs4828037, were associated with serum levels of CCL5 in men so that the rs5966709-G and rs4828037-T genotypes had higher serum concentrations (Table 9). In women, the same genotypes were associated with elevated serum concentrations of CCL3 and the rs5966709-GG, but not rs4828037-TT genotype was associated with higher CCL5 concentrations. Both

of these markers were associated with central obesity in women (*Study I*). Furthermore, all four markers from the second haploblock (Table 6) were associated with serum concentrations of MIF and the genotype of rs11798018 was associated with serum concentrations of IL-6 in women (Table 10).

The genotype effects were modified by the status of glucose metabolism so that the effect was generally clearer in the individuals who had 2h-PG>median (Tables 9-10). In addition, central obesity, as reflected in the waist circumference, modified the effect in a similar manner, i.e., the genotype effect was more pronounced in the upper medians. This was observed particularly in men. CCL5 was the only exception to this, as the genotype effects were observed in the lower medians of the obesity parameters. In general, central obesity modified the association of *TNMD* with acute-phase reactants, while the association with CCR-5 ligands were more dependent on the status of glucose metabolism (Tables 9-10).

As this was an explorative analysis, the multiple comparisons were necessary, and some of the findings might be false positives. We applied FDR to control for the multiple hypothesis testing and the FDR for association of rs2073163 and rs1155974 with acute phase proteins and sICAM and those of rs5966709 and rs4828037 with CCL5 in men was less than 5%. The FDR was below 5% also for the association of rs5966709 with CCL3 and CCL5 and those of rs2073163 and rs1155974 with CCL5 and MIF in women.

The single-marker associations were mostly haploblock-specific. The markers from the second haploblock (rs2073162, rs2073163, rs4828038 and rs1155974) were associated with serum concentrations of acute-phase reactants in men and with serum concentrations of MIF in women, while the markers from the first haploblock (rs5966709 and rs4828037) were associated with CCL3-concentrations in women and CCL5-concentrations in men. Markers from both haploblocks were associated with CCL5-concentrations in women. However, the LD-based haplotype analysis did not reveal a haplotype that explained the association substantially more than any individual SNP, although the results of the analyses were in line with the single marker analyses (data not shown).

Table 9. The observed associations of the SNPs in the *TNMD* gene with systemic immune mediators in the men of DPS. Data are given as median (interquartile range). Only *p<0.1* (adjusted for BMI and waist circumference) is reported.

Marker	Immune mediator	All men		2H-PG<median	2H-PG>median		Waist<median		Waist>median	
		Median (IQ range)	*p* *q*	Median (IQ range)	Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*
rs5966709-G	CCL5	50.6 (39.7)	0.023	49.1 (34.4)	52.0 (50.5)	0.006	52.8 (42.7)	0.048	43.8 (34.3)	
rs5966709-T	ng/ml	40.1 (32.6)	0.033	41.8 (30.1)	37.7 (29.7)	0.082	40.1 (24.9)	0.568	40.5 (33.7)	
rs4828037-T	CCL5	50.7 (38.0)	0.020	49.1 (34.4)	52.1 (48.3)	0.006	53.5 (39.3)	0.041	43.8 (34.3)	
rs4828037-C	ng/ml	39.8 (32.7)	0.030	41.9 (30.1)	35.6 (29.3)	0.082	39.7 (29.3)	0.568	40.5 (33.7)	
rs4828038-T	CCL5	50.6 (36.3)	0.097	50.5 (50.4)	50.6 (50.4)		59.0 (39.5)	0.004	38.7 (30.1)	
rs4828038-C	ng/ml	41.5 (38.2)	0.244	40.8 (35.8)	44.0 (43.5)		40.8 (38.1)	0.100	43.8 (38.8)	
rs2073612-G	CRP	1.1 (1.3)	0.056	1.1 (1.2)	1.4 (1.9)		1.0 (1.0)		1.5 (2.2)	0.022
rs2073612-A	mg/l	1.4 (2.7)	0.051	1.5 (1.9)	1.3 (4.4)		1.1 (1.1)		1.9 (4.7)	0.092
rs2073612-G	SAA	3.0 (1.7)	0.057	2.6 (2.3)	3.1 (1.6)	0.046	3.0 (1.7)		3.0 (2.4)	0.004
rs2073612-A	mg/l	3.9 (3.0)	0.051	3.8 (2.8)	4.0 (4.9)	0.210	3.2 (2.1)		4.3 (3.3)	0.031
rs2073163-T	CRP	1.1 (1.3)	0.051	1.1 (1.3)	1.1 (1.7)	0.052	0.9 (1.1)		1.5 (2.2)	0.01
rs2073163-C	mg/l	1.5 (2.9)	0.048	1.3 (1.8)	1.6 (4.5)	0.210	1.1 (1.1)		3.0 (5.2)	0.062
rs2073163-T	SAA	2.9 (1.8)	0.016	2.6 (2.2)	3.0 (1.6)	0.021	2.7 (1.6)		2.9 (2.5)	0.01
rs2073163-C	mg/l	4.1 (2.6)	0.029	4.1 (2.1)	4.1 (5.4)	0.164	3.6 (1.9)		4.6 (2.2)	0.062
rs2073163-T	sICAM	883 (329)	0.028	875 (363)	888 (312)		875 (330)		894 (347)	
rs2073163-C	ng/ml	923 (413)	0.032	947 (486)	900 (323)		910 (341)		1009 (492)	
rs1155974-C	CRP	1.1 (1.2)	0.011	1.1 (1.3)	1.1 (1.7)	0.052	0.9 (1.1)		1.4 (2.3)	0.001
rs1155974-T	mg/l	1.6 (3.1)	0.022	1.7 (1.8)	1.6 (4.5)	0.210	1.1 (1.1)		3.3 (5.4)	0.031
rs1155974-C	SAA	2.9 (1.8)	0.010	2.6 (2.2)	3.0 (1.6)	0.021	2.6 (1.6)		3.0 (2.3)	0.003
rs1155974-T	mg/l	4.1 (2.7)	0.022	4.1 (2.4)	4.1 (5.4)	0.164	3.6 (1.9)		4.9 (2.3)	0.031
rs1155974-C	sICAM	885 (332)	0.019	880 (364)	888 (312)		875 (351)		907 (344)	
rs1155974-T	ng/ml	931 (451)	0.030	971 (514)	900 (323)		917 (329)		1009 (536)	

Table 10. The observed associations of the SNPs in the *TNMD* gene with systemic immune mediators in the women of DPS. Data are given as median (interquartile range). *p<0.1* (adjusted for waist circumference and BMI) is reported. *p* is calculated for additive model, unless indicated otherwise.

Marker	Immune mediator	All women		2H-PG<median		2H-PG>median		Waist<median		Waist>median	
		Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*	Median (IQ range)	*p* *q*
rs11798018-CC	IL-6 pg/ml	1.7 (1.7)	0.032	2.2 (2.1)		1.6 (1.2)	0.095	1.7 (1.6)		1.8 (2.0)	0.053
rs11798018-CA		2.0 (1.6)	0.068	2.0 (1.4)		2.0 (2.0)	0.229	1.7 (1.5)		2.4 (1.3)	0.343
rs11798018-AA		1.5 (1.1)		1.4 (1.1)		1.6 (1.1)		1.3 (0.8)		2.0 (1.4)	
rs5966709-GG	CCL5 ng/ml	61.7 (44.2)	0.013	59.0 (45.9)	0.038	64.2 (43.8)	0.089	57.0 (45.4)	0.033	68.0 (64.3)	0.083
rs5966709-GT		53.9 (47.6)	0.033	50.5 (37.6)	0.049*	59.2 (63.1)	0.229*	50.3 (37.1)	0.617*	58.6 (55.0)	0.568*
rs5966709-TT		53.7 (36.3)		48.8 (38.1)		59.9 (33.9)		59.9 (42.3)		49.0 (33.6)	
rs5966709-GG	CCL3 pg/ml	14.3 (32.0)	0.028	14.3 (30.4)		12.3 (33.7)	0.009	15.6 (33.7)	0.059	11.9 (30.8)	
rs5966709-GT		9.4 (23.8)	0.066	13.7 (38.1)		5.8 (19.0)	0.026*	6.2 (25.8)	0.617*	11.4 (22.6)	
rs5966709-TT		18.9 (27.5)		13.3 (30.9)		19.3 (21.6)		19.3 (31.8)		16.3 (20.8)	
rs4828037-TT	CCL3 pg/ml	14.9 (33.7)	0.052	15.0 (38.6)		12.8 (33.8)	0.011	16.2 (39.7)	0.038	11.9 (31.6)	
rs4828037-TC		10.0 (25.8)	0.07	13.7 (36.8)		6.2 (21.8)	0.026*	8.2 (27.8)	0.617*	11.4 (22.6)	
rs4828037-CC		13.3 (29.2)		12.0 (29.2)		16.3 (25.1)		12.4 (29.9)		13.3 (21.3)	
rs2073162-GG	MIF ng/ml	5.5 (4.4)	0.032	5.4 (4.1)		5.5 (4.7)		5.3 (4.6)		5.6 (3.7)	
rs2073162-GA		6.0 (5.6)	0.068	6.4 (6.0)		6.0 (5.5)		6.2 (6.1)		6.0 (5.2)	
rs2073162-AA		6.2 (5.7)		6.3 (6.6)		5.9 (3.7)		5.8 (5.2)		7.3 (7.2)	
rs2073163-TT	CCL5 ng/ml	54.2 (45.7)	0.023	48.2 (34.2)		59.9 (54.7)	0.005	51.4 (36.4)	0.059	61.2 (53.3)	
rs2073163-TC		61.1 (42.1)	0.043	61.8 (52.5)		61.1 (37.6)	0.026**	59.9 (37.9)	0.617**	61.6 (49.5)	
rs2073163-CC		60.3 (74.0)		54.0 (44.7)		84.4 (87.2)		57.9 (75.6)		73.3 (89.5)	
rs2073163-TT	MIF ng/ml	5.7 (4.5)	0.027	5.6 (4.4)		5.7 (4.7)		5.5 (4.9)		5.8 (3.5)	0.007
rs2073163-TC		6.0 (5.5)	0.066	6.4 (6.0)		6.0 (5.4)		6.2 (6.1)		5.9 (5.2)	0.135**
rs2073163-CC		6.2 (6.9)		7.2 (7.1)		5.8 (6.7)		5.8 (5.5)		9.4 (14.1)	
rs4828038-TT	MIF ng/ml	6.2 (6.4)	0.014	6.2 (6.7)		5.9 (5.7)		5.8 (5.6)		8.1 (10.5)	0.07
rs4828038-TC		6.0 (5.5)	0.046	6.3 (6.0)		5.9 (5.5)		6.1 (6.4)		5.9 (5.2)	0.380
rs4828038-CC		5.7 (4.2)		5.6 (3.6)		5.7 (4.7)		5.6 (4.6)		5.8 (3.3)	
rs1155974-CC	CCL5 ng/ml	54.5 (46.0)	0.025	48.2 (34.2)		60.4 (56.7)	0.005	51.8 (37.0)	0.059	61.2 (53.3)	
rs1155974-CT		61.1 (41.2)	0.044	62.4 (50.5)		60.5 (36.3)	0.026**	59.8 (37.8)	0.617**	62.3 (48.9)	
rs1155974-TT		59.9 (76.0)		52.3 (45.1)		84.4 (87.2)		57.9 (75.6)		71.7 (103.1)	
		5.7 (4.4)		5.6 (4.4)		5.7 (4.7)		5.6 (4.9)		5.8 (3.6)	

rs1155974-CC	MIF	6.0 (5.5)	0.013	6.43 (6.0)	6.0 (5.5)	6.1 (6.4)	6.0 (5.2)	0.006
rs1155974-CT	ng/ml	6.2 (7.1)	0.046	7.4 (7.3)	5.8 (6.7)	5.8 (5.5)	10.3 (14.6)	0.135**
rs1155974-TT								

*dominant model
**recessive model

5.5 *TNMD* and serum lipids and lipoproteins (*Study III*)

The genotype of rs2073162 was not associated with the serum lipid or lipoprotein concentrations in the unstratified METSIM population, but genotype*BMI interactions were observed. In subsequent analyses when the data were subdivided according to the quartiles of BMI, no associations were evident in the three lowest quartiles, but in the highest quartile, the carriers of the rs2073162-A-allele had higher concentrations of serum total, LDL and HDL cholesterol than carriers of the rs2073162-G-allele (Figure 7a-c). These differences remained statistically significant after additional adjustment for cholesterol-lowering medication (p=0.016 for total cholesterol, p=0.033 for LDL and p=0.038 for HDL). The median (interquartile range) for serum total, LDL and HDL cholesterol were 5.17 (1.45), 3.23 (1.28) and 1.24 (0.39) mmol/l for the G allele carriers and 5.25 (1.47), 3.37 (1.29) and 1.27 (0.40) mmol/l for the A allele carriers. No differences were observed in the triglyceride levels.

In DPS, genotype differences were not observed in either gender when all individuals were included in the analysis, but again genotype*BMI interactions were observed. In the data stratified by the median of BMI, the genotype was not associated with the serum lipoproteins or lipids in either median in women or in the lower median in men. In the upper median of BMI in men, the genotype of rs2073162 was associated with serum levels of total and LDL cholesterol (Figure 7d-f). The median (interquartile range) for serum total and LDL cholesterol were 5.26 (1.16) and 3.30 (1.13) mmol/l for the G allele carriers and 5.49 (0.97) and 3.60 (1.06 mmol/l for the A allele carriers. No associations with serum HDL cholesterol or triglyceride levels were observed. The lower cut-off was very similar in both studies (29.41kg/m^2 for the 4th quartile in METSIM, 29.45 kg/m^2 for the upper median in DPS).

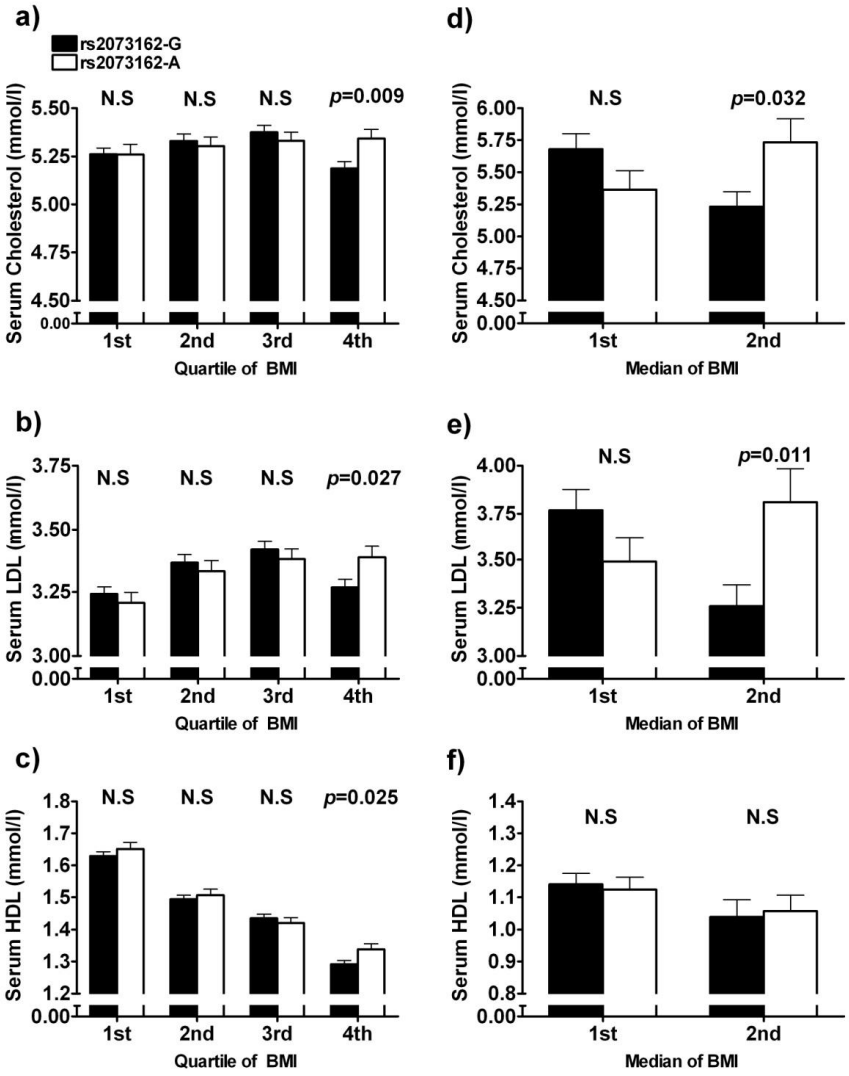


Figure 7. The serum concentrations of total, LDL and HDL cholesterol in the quartiles of BMI (ranges 13.18-24.58 kg/m^2 for 1st, 24.59-26.72 kg/m^2 for 2nd, 26.73-29.40 kg/m^2 for 3rd and 29.41-52.11 kg/m^2 for the 4th quartile) in the METSIM study population (a-c) and in the medians of BMI (ranges 23.50-29.40 kg/m2 for the 1st and 29.45-44.80 kg/m2 for the 2nd median) in the DPS study population (d-f) according to the genotypes of rs2073162. *p* is adjusted for the use of cholesterol-lowering medication.

5.6 *TNMD* and age-related macular degeneration (*Study IV*)

In women, markers rs7890586 and 1155974 were associated with total prevalence (atrophic or exudative form) of AMD and a trend was observed with rs2073163. Specifically, these differences were due to an unequal prevalence of exudative AMD in the genotype groups (Table 11). None of the markers were associated with prevalence of AMD among men.

Haplotype analyses were performed according to the two LD-based haploblocks (Table 6), one consisting of rs11798018 and rs5966709 and the other of rs2073163, rs1155974 and rs1204384. Additional analyses were performed for combinations of individual markers that were associated with AMD (rs7890586, rs2073163 and rs1155974). Neither of these approaches revealed a haplotype that would explain the results substantially more than the individual markers (data not shown).

Table 11. The association of the selected markers of the *TNMD* gene with the prevalence of exudative AMD in women according to the recessive inheritance model. False discovery rate *q* is reported only for $p<0.1$. OR odds ratio, CI confidence interval

Marker	Genotype	Atrophic or exudative AMD			Exudative AMD		
		Prevalence	Recessive model		Prevalence	Recessive model	
			OR (95%CI	*p (q)*		OR (95%CI	*p (q)*
rs7890586	GG/GA	196/294	1 (reference)	0.001 (0.004)	172/270	1 (reference)	0.002 (0.007)
	AA	2/14	0.083 (0.018-0.380)		2/14	0.095 (0.021-0.443)	
rs11798018	CC/CA	159/252	1 (reference)	0.548	141/234	1 (reference)	0.714
	AA	41/61	1.199 (0.663-2.169)		34/54	1.121 (0.608-2.066)	
rs5966709	GG/GT	179/279	1 (reference)	0.784	158/258	1 (reference)	0.628
	TT	21/34	0.902 (0.433-1.880)		17/30	0.828 (0.385-1.777)	
rs2073163	TT/TC	160/260	1 (reference)	0.057 (0.067)	88/192	1 (reference)	0.038 (0.042)
	CC	40/53	1.923 (0.980-3.772)		37/50	2.062 (1.042-4.080)	
rs1155974	CC/CT	167/272	1 (reference)	0.021 (0.037)	146/251	1 (reference)	0.022 (0.037)
	TT	33/41	2.594 (1.154-5.830)		29/37	2.607 (1.146-5.931)	
rs1204384	AA/AT	176/277	1 (reference)	0.801	154/255	1 (reference)	0.720
	TT	23/35	1.100 (0.525-2.304)		21/33	1.148 (0.541-2.435)	

6 DISCUSSION

6.1 Methodological issues

These association studies were performed in a carefully phenotyped and selected set of individuals who participated in a lifestyle intervention and were followed up for approximately four years (*Studies I-III*), a large, cross-sectional extensively phenotyped population-based sample of men (*Study III*) and finally in a smaller set of individuals for which only limited background information was available (*Study IV*). In general, the requirements for genetic association studies have changed during the last years and especially in the field of complex diseases, large-scale genotyping of large sample sizes are becoming a pre-requisite for studies that are considered to be of good quality (379).

6.1.1 Candidate gene approach

Genome-wide scans are potential tools for identifying common susceptibility variants and they can be considered as a new approach for discovering candidate genes. In comparison to the hypothesis-free genome-wide scans, the traditional candidate gene studies are hypothesis-driven with the assumption that genes with functions relevant to the phenotype of interest would represent putative susceptibility genes (380). In our group, this approach is applied by studying genes whose expression in adipose tissue is differentially regulated by weight loss. *TNMD* was selected as a potential candidate gene since it was one of the most extensively downregulated genes in adipose tissue during moderate weight loss in overweight individuals with features of the metabolic syndrome (325). Recently, we have shown that TNMD is expressed in adipocytes and blood vessels in adipose tissue (Figure 8, unpublished data) and therefore it might be relevant for the adipose tissue biology. Investigation of the differences in mRNA expression in adipose tissue of obese and lean subjects has identified candidate genes for obesity and related traits (381) and led to discovery of novel adipokines (286,382). Furthermore, combining gene expression profiles from the tissue of interest together with genetic linkage information has been proposed as a strategy to identify susceptibility genes for complex traits (383).

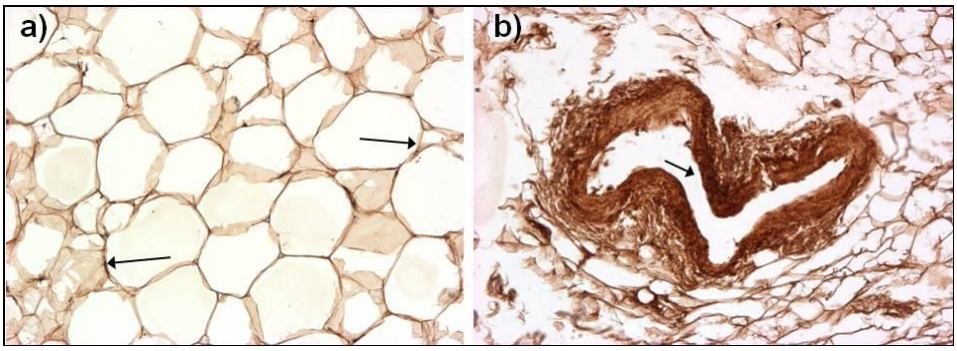


Figure 8. TNMD expression in human formalin-fixed paraffin-embedded adipose tissue. TNMD was detected with Novolink^{TM} polymer detection system (Novocastra Laboratories Ltd, Newcastle, UK) using 1:500 diluted TNMD primary antibody kindly provided by Prof. Reinhardt Fässler (Max-Planck Institute, Martinsried, Germany). TNMD is expressed in a) adipocytes and b) blood vessels (Unpublished data).

6.1.2 Study populations

The DPS (*Studies I-III*) is nowadays considered as a small sample for genetic association studies (n=507), although one of the original study aims was to investigate the impact of genetic factors on T2D risk (384). The stratification of a relatively small population into different groups by randomization and gender further weakens the statistical power. We handled this issue by applying the mandatory stratification by gender and used the randomization group as an adjustment factor, because this was justifiable based on the lack of interaction between the genotypes and randomization group and the similar allele frequencies in both groups. In *Studies II-III,* we stratified the data into medians instead of other quantiles, which would have resulted in smaller number of individuals in each strata and would have increased in the number of groups thereby causing power loss. Despite these weaknesses, DPS is a representative sample of Finnish middle-aged persons who had a high risk for developing T2D, as BMI>25 and IGT were part of the inclusion criteria. It is notable that the study enables the investigation of long-term gene*environment-interactions, either with regard to lifestyle modification in general or to specific components of dietary habits or physical activity. The follow-up data of various quantitative and categorical traits also provides more information than a single measurement of certain surrogate variable. The comparability of measurements from different centres is good, as this was addressed already in the study design by standardizing the measurement methodologies between the laboratories.

The METSIM study (*Study III*) is currently cross-sectional, so the research setting is very different from that applied in DPS. The participants are middle-aged men from the

region of Kuopio and while the DPS is representative of a population in high risk for T2D, the METSIM is a population-based sample thereby representing the general population with the whole range of BMI and glucose metabolism. The advantage of this cohort is that it is specifically collected for the purpose of genetic association studies and the sample size is therefore appropriate.

The AMD study population (*Study IV*) is small, and especially the number of controls would need to be increased. This is highlighted in the analysis of studying X-chromosomal gene which makes the stratification by gender mandatory and further decreases the size of groups. Moreover, no further background data apart from age and gender were available from this study population. The number of individuals with atrophic AMD is too small for investigating the risk factors specifically accounting for this subtype, but the original aim of the study was to investigate the association with AMD in general.

Since all of the study participants were Finnish, the observed associations are unlikely to result from population stratification. The Finnish population originates mainly from a small group of founders and relatively little immigration has occurred during the last 80-100 generations (385,386). The founder effects are more recent in the Northern and Eastern Finland, as these regions can be viewed as being "founded" only in the 1500s (385). The Finnish population has been referred to as a model population for human genetic studies, especially in the context of linkage mapping (387), and it has been claimed to offer substantial advantages for genetic studies (388).

6.1.3 Genotyping accuracy

The test for HWE is generally applied as a diagnostic test for genotyping errors (389). However, HWE of genotype frequencies is a population-based characteristic assuming discrete generations, random mating in an infinite-sized population and the absence of selection, mutation or migration. Therefore, the deviations observed in these studies can be a result of population properties or random chance rather than genotyping error, as the inspection of discrimination plots did not reveal any unequivocal genotyping mistakes. In addition, when a marker is associated with the disease, the corresponding genotypes may no longer actually be a random sample and therefore it can lead to deviation from HWE (390). As the associations with multiple continuous phenotypes

were investigated in *Studies I-II*, the HWE was calculated from all individuals. Interestingly, in *Study IV* two of the three markers that were associated with AMD risk were not in HWE, which is in line with the theory of Li and Li (390).

The genotyping accuracy was also confirmed by re-genotyping a representative random subsample from each population. The error rate for each studied marker in all study populations was 0%, suggesting that factors other than genotyping errors must account for the deviation from HWE.

6.1.4 Statistical issues

Investigating the association of multiple markers with different phenotypes raises the issue of multiple hypothesis testing. Traditionally this has been addressed with Bonferroni-correction or its derivatives, which are considered to be too conservative for genetic association studies (391).

It should be noted that the results of *Study I* are not adjusted for testing of multiple hypotheses, apart from pairwise comparisons in women. In *Studies II* and I*V,* we applied the false discovery rate (378), indicated as *q*-values. This approach simultaneously corrects for the number of phenotypes and markers that are tested. Statistically significant results (i.e. small *p*-value) with a high *q*-value are likely to be false positive findings. Instead of applying an arbitrary threshold, we chose to report the exact *q*-value for each *p*.

In *Study II,* the FDR for the main results, i.e. for the associations with acute phase reactants and sICAM in men and for CCR5 ligands and MIF among women were low, suggesting that these findings are likely to be "true" and that *TNMD* might be related to inflammatory status. Due to the limited power caused by relatively low number of study participants and the further stratification into different groups, there may be some undetected associations that would be significant if we had had access to larger study populations. In addition, some of the observed associations that were no longer statistically significant after correcting for multiple hypothesis testing (i.e., had FDR>0.05), might remain statistically significant in a more powerful study.

In *Study III,* associations of a single marker were studied so the results were not adjusted for testing of multiple hypotheses testing, although this might be justifiable because multiple phenotypes were tested. In general, the FDR-computations in Q-value work better with large number of *p*-values, although there are no recommendations for the minimum amount of *p*-values. Our study contained data for only 4 phenotypes,

meaning that the number is very low for applying FDR according to Storey *et al* (378). Another possibility would have been to utilize a permutation-based method, for example a modification of the procedure introduced by Kimmel *et al* (392).

In *Study IV*, the FDR for the associations of markers rs7890586, rs2073163 and rs1155974 with AMD was less than 5%, apart from that of rs2073163 with total prevalence of AMD (q=0.067; Table 11). This suggests that the results are unlikely to be false positives. However, they should be replicated in a larger study and with adjustment for possible confounders such as smoking and body size. Due to the low genotype frequencies, the number of individuals was small in some of the analysis (e.g. the number of persons with the rs7890586-AA-genotype) meaning that these results are tentative and should be interpreted cautiously.

6.2 General discussion

6.2.1 Gender differences

Almost all of the observed associations were gender-specific. These differences can arise from the genetic locus. X-chromosomal genes often display a significant variation in expression and function between men and women, partly because of variation in gene dosages and random inactivation of one of the X-chromosomes in women (393). Accordingly, women had two times higher tenomodulin expression in adipose tissue than men (325), evidence of dosage-specific expression levels. *TNMD* is located approximately 27000 kb away from the X-inactivation centre (locus Xq13.2-q21-1) (394), suggesting that the *TNMD* locus might avoid X-inactivation. The cellular microenvironment can also differ in men and women because of differences in hormone levels and gene expression (395).

The X-chromosome is an interesting locus for genetic association studies regarding serum lipids, since the monosomy of the X-chromosome has been shown to be specifically related to fat accumulation and serum lipid profile in women. In the study of Van *et al* (396) women with Turner´s syndrome (45, X) had higher LDL-cholesterol and triglyceride levels and smaller LDL and HDL particle sizes than women with 46, XX. These effects were independent of lifestyle, body composition, insulin sensitivity or hormonal status. As the only characterized difference was the presence of the second X-chromosome, the authors speculated that the differences in lipid metabolism could be

due to previously unrecognized disparity in X-chromosome gene dosage (396). Similar results have been reported by Cooley *et al* (397). In both studies, the degree of difference in lipid levels and particle size observed between women with one and two copies of X-chromosome was similar to the genderwise differences (398,399). One potential explanation for these observations is that X-chromosome gene or genes are involved in lipid metabolism or transport. According to this hypothesis, membrane-bound transcription factor protease, site 2 (*MBTPS2,* locus Xp22*),* encoding the site 2 protease that cleaves SREBP, is a key regulator of cholesterol metabolism (400).

Genomic imprinting of X-linked genes could lead to different gene expression in men and women, since women are normally mosaic for maternally and paternally inherited active X-chromosomes (X^M and X^P, correspondingly), while men are monosomic for the maternally inherited X^M. Interestingly, in women with Turner´s syndrome, the monosomy for X^M is associated with greater visceral fat accumulation and a more atherogenic lipid profile than monosomy for the paternal chromosome (401). These differences between 45,X^P and 45,X^M women parallel the usual metabolic and adiposity differences seen between women and men (398,399).

6.2.2 *TNMD* and obesity (*Studies I* and *III*)

The sequence variation of *TNMD* was associated with central obesity in women and with the general body size, indicated by BMI and weight in men of the DPS (*Study I*). We did not detect any association with indicators of body size in the larger cross-sectional sample of METSIM (*Study III*), as the sample consisted only of men and only the markers that associated with T2D risk in men were genotyped. These markers did not associate with body size in the DPS and since the association with T2D was considered the main result in men, the SNP that associated with body size was not selected for replication in the DPS. Furthermore, the association with obesity measures in men was observed in the longitudinal data, which was not available from the METSIM.

With regard to the genetic association studies in general, without functional assays it is difficult to know whether the causative variant truly is one of the studied markers or simply a SNP that is in complete linkage disequilibrium with them, or whether the associations truly arise from the *TNMD* locus. However, there are no known obesity or T2D loci nearby (22,24-27,107,108). Apart from rs2073162, which is a nonsynonymous

SNP located in the third exon, all of the selected markers were intronic. Therefore, if the causative variant is indeed one of the selected SNPs, the effect does not result from altered structural and/or functional properties secondary to the amino acid change. However, the synonymous SNPs can affect transcription of the protein by modifying transcription factor binding or the extent of the genemethylation or splicing.

Although tenomodulin is believed to mediate anti-angiogenic effects, its specific function in adipose tissue is still unknown. In our further studies we have shown that *TNMD* expression is induced during adipocyte differentiation (unpublished data). The genes involved in angiogenesis are an interesting new group of susceptibility genes for obesity and related traits. It has been shown that targeted induction of apoptosis in the vasculature of adipose tissue can reverse obesity and normalize metabolism in *ob/ob* mice (402), and the administration of angiogenesis inhibitors reverses both genetic and diet-induced obesity in mice (282,283). It has also been suggested that changes in adipose tissue blood flow may modulate the β-cell dysfunction in T2D in a rat model (403). However, this data is solely based on animal studies. Still, one possibility is that tenomodulin could regulate vasculature formation in adipose tissue and thereby also modulate adiposity, glucose metabolism, and T2D risk. Interestingly, TSP-1 an angiogenesis inhibitor with a similar knock-out mouse phenotype as *TNMD*, was recently shown to be an adipokine that was associated with obesity, adipose tissue inflammation and insulin resistance (286).

TNMD belongs to the family of BRICHOS-domain containing proteins, most of which are associated with chronic diseases. These include BRI2, which is linked to familial British and Danish dementia, chondromodulin-I related to chondrosarcoma, CA11 related to stomach cancer and surfactant protein C, involved in respiratory distress syndrome (344). TNMD, like other proteins of this family, is an integral transmembrane protein with a type 2 orientation, from which the extracellular part is cleaved proteolytically. The BRICHOS-domain has been suggested to function as an intramolecular chaperone for the cleaved part (344-346) and mutations in the BRICHOS region have been shown to cause endoplasmic reticulum (ER) stress and proteasome dysfunction (346), providing another interesting functional link to the chronic diseases caused by misfolded proteins. Chronic excessive nutrient intake has been shown to cause ER stress in adipose tissue of ob/ob mice and mice fed with a high-fat diet (404,405). Markers of ER stress are associated with obesity in non-diabetic individuals (406). It has also been shown that obesity increases ER stress in human subcutaneous

adipose tissue (407) and that the unfolded protein response, a mechanism aimed to alleviate ER stress, is activated in subcutaneous adipose tissue of obese humans (408). Data from cell culture and mouse studies suggest that leptin resistance might be one of the linking mechanisms (409).

6.2.3 *TNMD* and glucose regulation (*Studies I* and *III*)

The SNPs of *TNMD* were associated with 2h-PG, and consequently with risk of T2D during the follow-up of DPS. We did not detect any cross-sectional differences between the genotypes in men, either in the baseline data of DPS with respect to plasma glucose or serum insulin levels (*Study I*), or in METSIM regarding prevalence of T2D, plasma glucose or serum insulin levels (*Study III*).

The METSIM and DPS study populations are essentially different: Both men and women were included in the DPS and the sample was collected from five Finnish cities and their surroundings. The individuals were also relatively homogeneous as all of the study participants had BMI>25 kg/m^2 and IGT. The METSIM study population is a random sample of men aged from 45 to 70 years and living in the Kuopio area. Therefore, the range for BMI is considerably larger (16.18-52.11 kg/m^2) and all four glucose tolerance categories were included. Furthermore, in the METSIM study the prevalence of T2D was determined cross-sectionally, while in the DPS study the conversion of IGT to T2D was assessed. These differences between the studies might explain why the association with T2D could not be replicated. In future, when the longitudinal data from the METSIM becomes available, it will be interesting to see whether the association observed in the DPS can be replicated, despite the different baseline characteristics. It might also be that different genes operate in distinct phases of the development of T2D.

Interestingly, the markers rs2073162 and rs2073163 that were associated with an elevated risk of T2D in the DPS are located within the region that encodes the BRICHOS-domain, and also rs1155974 is in close vicinity. ER stress has been suggested to be one of the common links between obesity, T2D and insulin resistance in adipocytes and liver (186,404,405,410). Treatment of obese and diabetic mice with chemical chaperones has been shown to result into improved insulin sensitivity in skeletal muscle, adipose tissue and liver, resolution of fatty liver disease and normalized hyperglycaemia (411). Another interesting pharmacological link is provided by the PPAR-agonists, since the treatment of mice with rosiglitazone or macelignan has been

shown to alleviate ER stress in mouse liver and adipose tissue (412,413), although pioglitazone treatment did not reduce ER stress in human adipose tissue (407).

6.2.4 *TNMD* and inflammation (*Study II*)

The sequence variation of *TNMD* was consistently associated with serum concentrations of different systemic immune mediators in individuals with IGT, suggesting that the chronic low-grade inflammation could be the link between the observed associations of *TNMD* with obesity, dyslipidaemia, AMD and T2D. All these states are also strongly interconnected. In women, the same genotypes that were associated with elevated concentrations of CCL3 and CCL5 were associated with a larger waistline, as indicated by all four parameters for central obesity that were measured in *Study I*. In men, the genotypes that were associated with the higher risk of T2D, were associated with higher levels of CRP and SAA. In addition, the same genotype of rs2073162 that was related to higher acute phase reactant concentrations and T2D risk, was correlated with higher serum total and LDL cholesterol levels in the obese men and the markers rs2073163 and rs1155974 that were associated with serum MIF concentrations among women, were associated with the risk of exudative AMD in women.

Both angiogenesis and ER stress, and, therefore, both of the important functional motifs of *TNMD*, namely the angiogenesis inhibiting C-terminal domain and BRICHOS-domain can be connected to inflammation. Inflammation, together with other harmful consequences of the expansion of adipose tissue mass, such as hypoxia and oxidative stress causes dysfunctions in ER (185,186). ER stress is linked to major inflammatory and stress-signalling networks *via* a variety of mechanisms (185,186). Hypoxia stimulates both angiogenesis and inflammation, but angiogenesis has been suggested to also sustain inflammation, by providing oxygen and nutrients for the cells present at the inflammatory sites (414). Angiogenesis and inflammation have been connected in the pathogenesis of a number of chronic diseases and these two processes can be initiated by the same molecular events (414). For example, both angiogenesis and inflammation have actions at the endothelial cell-cell junctions and adhesion molecules are therefore essential for both processes (415,416). Long-term angiogenetic imbalance often accompanies inflammation (417) and inflammatory cells are known to promote (lymph)angiogenesis in tumours (418).

6.2.5 *TNMD* and serum lipoproteins (*Study III*)

The rs2073162-A genotype, which was associated with higher T2D incidence in *Study I* and elevated serum concentrations of CRP and SAA in *Study II*, was associated with elevated concentrations of serum lipoproteins in a BMI-dependent manner in two independent studies including Finnish men. Specifically, in the METSIM study differences in total, HDL and LDL cholesterol were observed, whereas in the DPS the differences were found only in total and LDL cholesterol levels.

The associations were observed in those individuals who belonged to the highest quantiles of BMI, and the results remained similar when the cut-off of 30kg/m^2 was used. It is difficult to determine whether these differences result from altered cholesterol absorption or synthesis, since the data on the indicators of cholesterol metabolism was not available. It has been established that serum total cholesterol is increased in obesity, partially because of increased cholesterol synthesis (252). In this study, the differences in serum total cholesterol between the genotypes were almost entirely due to the difference in LDL cholesterol levels, but as with the total cholesterol levels, one can only speculate if they are caused by increased LDL synthesis or by decreased catabolism. Since both SAA and CRP have been shown to be involved in cholesterol recycling (264), it is possible that the observed association could be attributable to the association with low-grade inflammation.

6.2.6 *TNMD* and age-related macular degeneration (*Study IV*)

The sequence variation of the *TNMD* gene was associated with the prevalence of AMD in women. Specifically, the genotypes rs2073163-CC and rs1155974-TT, which were associated with a higher risk of T2D and elevated serum acute phase reactant concentrations in men, and with higher serum concentrations of MIF and CCL5 in women, were linked with a higher prevalence of exudative AMD. We did not observe any association with atrophic AMD, which was likely due to the small number of cases. The same cytogenetic band, Xq22, has been linked to AMD previously by Zheng et al (419), who also observed gender-specific associations of the diaphanous *2 Drosophila* homologue- gene (*DIAPH*). However, *DIAPH* is located 3.5 Mb away from *TNMD,* and there is practically no LD between the *TNMD* markers that were associated with AMD and rs10521496 of *DIAPH*, which was associated with the risk of AMD in the previous study (419). The pairwise r^2 for rs10521496 and rs7890586 is 0.007, and for

rs10521496 and rs2073163 or rs1155974 it is 0.1 in the CEU population of HapMap-database (public release #26).

Abnormal neovascularization is an essential part of the pathophysiology of exudative AMD, and genetic associations of angiogenesis regulators such as *VEGF* (168-171) and *PEDF* (172,173) with AMD have been reported. In relation to these previous association studies, the relationship between *TNMD* sequence variation and exudative AMD raises an interesting possibility for a regulatory role of TNMD in choroidal neovascularization and exudative AMD, but like other hypothetical connections suggested in chapters 6.2.2-6.2.5, this will require replication in other study populations and above all, functional studies. Interestingly, the ER chaperones (420) and ER stress in general (421,422) are known to affect the expression of angiogenic factors such as VEGF, and therefore TNMD could, in theory, affect the vascularization *via* ER stress caused by the dysfunction of the BRICHOS-domain and the resulting accumulation of misfolded TNMD. Mutations in the BRICHOS-region of surfactant protein C have been shown to increase ER stress *via* this mechanism (346).

In addition to angiogenesis, inflammation is involved in the pathogenesis of AMD and the best established genetic risk factors are related to the complement system (145-161,167,423-432). Thus, it might be that also these associations of *TNMD* could be due to the association with the inflammatory status that was observed in DPS. Unfortunately, there is no inflammatory marker data available from the AMD study population.

7 CONCLUSIONS

The sequence variation of *TNMD* was associated with glucose metabolism, serum lipoprotein and inflammatory marker levels in men and with central obesity, serum levels of systemic immune mediators and exudative AMD in women. All these phenotypes are linked by inflammation and angiogenesis. As discussed in chapters 6.2.2-6.2.7, there are various parallels that provide an interesting basis for speculation. The hypotheses depicting the mechanisms of the observed associations, based on the available data from *Studies I-IV* are shown in Figure 9.

The effects of acute phase reactants and other inflammatory factors on lipolysis and cholesterol synthesis are well recognized (263-265,433). It is also known that lipid overload interferes with insulin signaling pathways, and that enhanced lipolysis and dyslipidaemias contribute to the deterioration in glucose metabolism in obesity (174,193,209,210). Therefore, it might be that the association of *TNMD* with inflammation could explain many of the observed associations in men (Figure 9a). This hypothesis is supported by the fact that the same genotype associated with elevated serum concentrations of acute phase reactants in DPS, was related to elevated serum LDL levels in obese individuals in DPS and METSIM, but controversially also to increased HDL levels in the METSIM. In addition to this marker (rs2073162), two other markers (rs2073163 and rs1155974) were associated with elevated serum concentrations of CRP and SAA and elevated 2H-PG. In addition, these three markers were associated with a higher risk of T2D in the DPS.

In women, the same markers that were associated with central obesity (rs4828037 and rs5966709) were associated with elevated serum concentrations of CCL3 and CCL5. The sequence variation in the region of haploblock 2 was linked to concentrations of CCL5 and MIF and two of these markers were associated with risk of AMD. Therefore, one possible scheme is that the elevated serum levels of inflammatory factors, secondary to central obesity, facilitate the pathophysiological changes related to development of AMD (Figure 9b). In addition, due to its angiogenesis-inhibiting properties, *TNMD* might also contribute to the altered neovascularization.

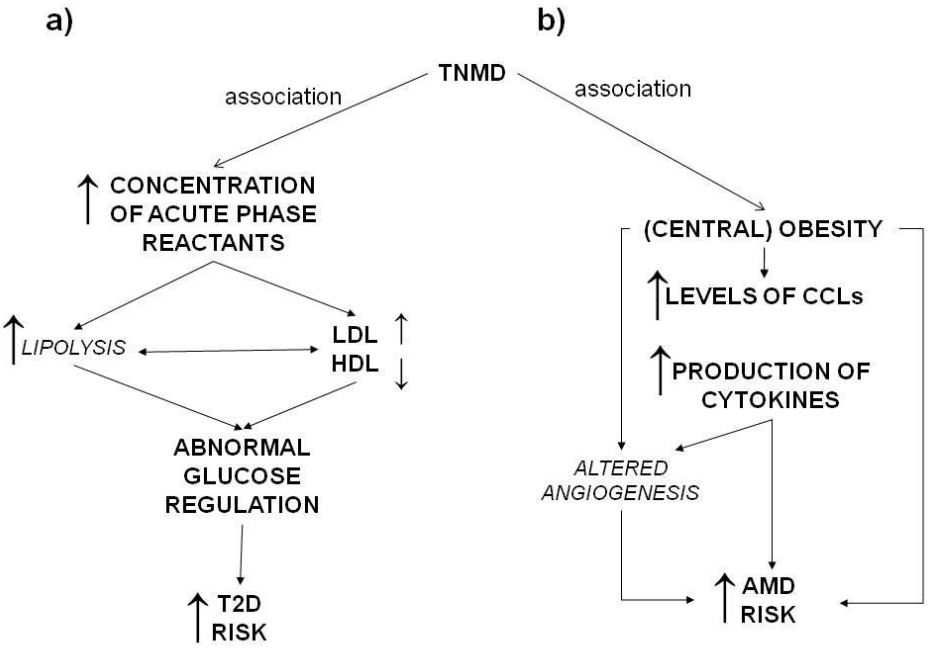


Figure 9. Hypothesis for the putative mechanism for the observed associations of *TNMD* markers in a) men and b) women. The events that were not measured in any of the study populations are indicated with *non-bolded cursive font***.** a) Acute phase reactants CRP and SAA can affect cholesterol metabolism and SAA also promotes lipolysis. Altered lipoprotein metabolism and increased lipolysis disturb glucose metabolism and thereby increase the risk of type 2 diabetes (T2D). b) Obesity, either general or central, is associated with accumulation of macrophages in adipose tissue. These cells secrete proinflammatory cytokines and other compounds, such as chemokine (C-C motif) ligands (CCLs) that further promote the production of proinflammatory factors. Obesity and inflammation are related to vascular changes. Inflammation and angiogenesis are essential in the pathogenesis of AMD, but obesity is also one of the known risk factors.

7.1 Future implications

Since we performed only genetic association studies, it is difficult to suggest how tenomodulin could participate in adiposity, glucose and lipid metabolism or inflammation and therefore *Studies I-IV* generate new research hypotheses rather than answering specific questions. The current published *in vivo* and *in vitro* studies with *TNMD* do not provide explicit clues on the potential mechanisms. However, *TNMD* shares interesting similarities with *TSP-1*. Both genes are expressed in adipose tissue and the mRNA-levels correlate with obesity-related traits (286,325). The expression of both genes is regulated *via* the extracellular-signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK)-signaling pathway (434-437) and high expression of both

genes is associated with inflammation (438-440). The mRNA expression of these two genes is also induced in the same phase during adipocyte differentiation (280,281). Several adipokines, such as TSP-1 (286) and vaspin (382) have been identified by investigating differences in mRNA expression in adipose tissue of obese and lean subjects, and therefore it would be interesting to investigate if also TNMD could be an adipokine. Our preliminary research has confirmed that TNMD is expressed in adipocytes and blood vessels (Figure 8).

The BRICHOS-domain with its connection to ER stress and the C-terminal angiogenesis-inhibiting domain provide interesting hypotheses for functional studies. Since the TNMD-null mice were studied in the context other than obesity or T2D and the mice did not harbour any drastic phenotypes in normal settings (345), the authors did not conduct more detailed analyses. It would be interesting to investigate how the *TNMD*-null mice would respond to a high-fat diet and to study the effect of *TNMD* overexpression in a suitable model system on the phenotypes of interest. In addition, the use of cell culture model systems might shed light on these putative connections.

8 SUMMARY

These studies were carried out to examine the association between the variation in the *TNMD* gene and obesity- and inflammation-related phenotypes. The conclusions from the studies can be summarized as follows:

Study I: Three markers of the *TNMD* gene were associated with risk of T2D in men and two other markers with central obesity in women during a follow-up of overweight individuals with IGT. The association with T2D was not replicated in a cross-sectional population-based sample. These discrepancies might be due to differences between study populations or that different genes operate in distinct phases of T2D development.

Study II: The markers that were associated with the risk of T2D in Study *I*, were connected to the serum levels of CRP and SAA in overweight men with IGT. Furthermore, two of these markers were linked to the serum concentrations of sICAM. The association was to some extent predictable, since the same genotypes which were linked to a higher incidence of T2D had higher serum levels of these inflammatory markers. In addition, the association between *TNMD* sequence variation and serum CCL5 concentrations was observed in men. In women, the genotypes that were associated with central obesity were related to higher serum CCL3 levels. Furthermore, an association with CCL5 and MIF was detected in women. These results suggest that inflammation might be the link between TNMD, impaired glucose regulation and obesity.

Study III: The same marker that was associated with T2D incidence and the proinflammatory state was also associated with serum total and LDL-cholesterol in two separate samples of Finnish men. These consistent, replicated findings further strengthen the link between TNMD, metabolic syndrome and inflammation.

Study IV: Two markers that were associated with serum levels of MIF were related to the higher prevalence of exudative AMD in women. This novel finding provides another link to an obesity-related condition, which also has strong relationship to angiogenesis and inflammation.

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ORIGINAL PUBLICATIONS

I-IV

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