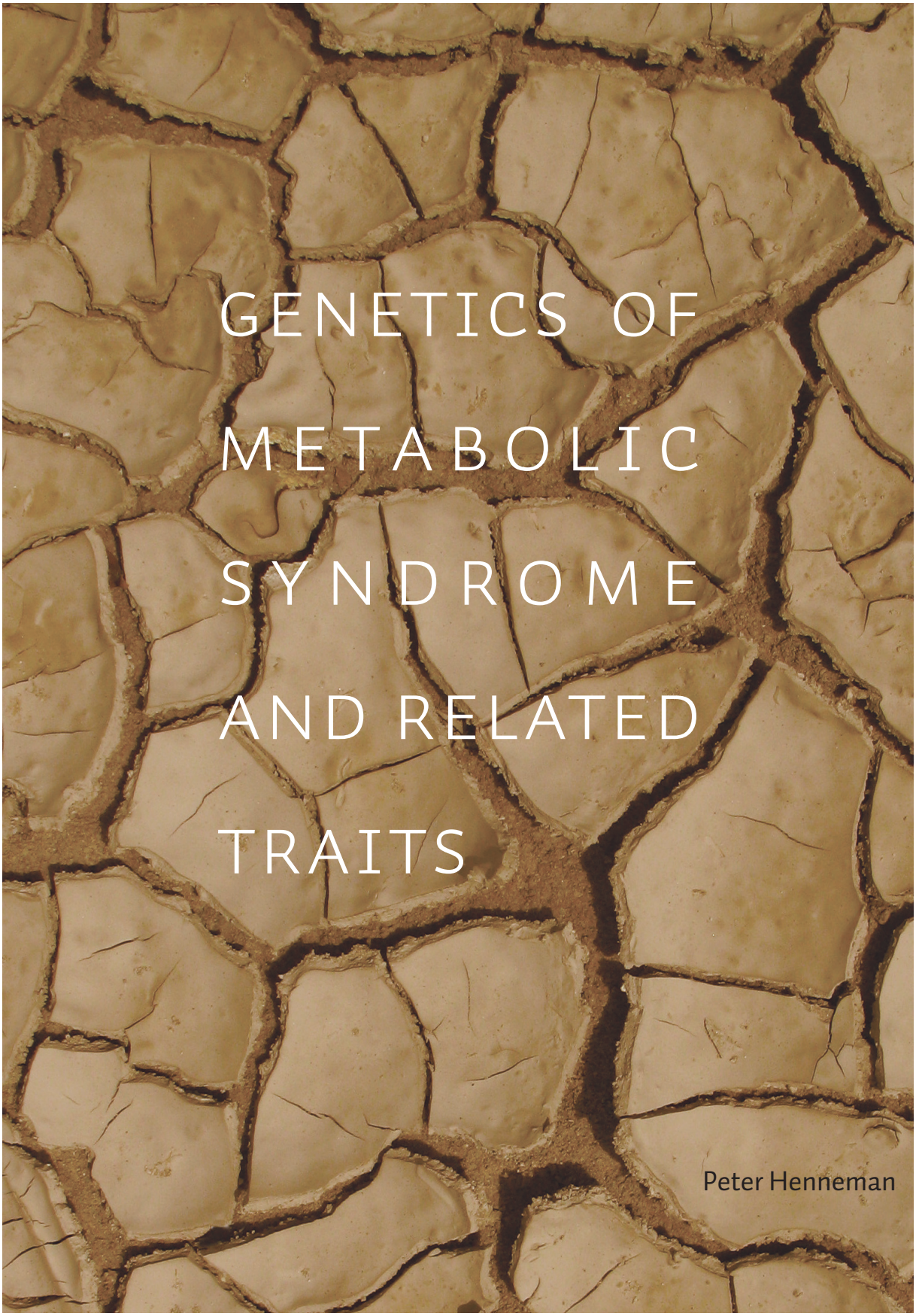
GENETICS OF
METABOLIC
SYNDROME
AND RELATED
TRAITS
Peter Henneman

Genetics of Metabolic Syndrome and related traits

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Chapter 1

General Introduction

METABOLIC SYNDROME

Metabolic syndrome (MetS) refers to a cluster of risk factors for type 2 diabetes (T2D), cardiovascular disease (CVD) and stroke (Figure 1) that are strongly associated with the Western life style[1,2,3,4]. This life style is a result of the overwhelming and readily available supply of high-energy food that can be consumed at relatively low cost in westernized societies and is characterized by excess food intake and limited physical exercise. The term syndrome implies a complex etiology and this is confirmed by the variety of definitions for MetS that have been formulated over the years (Table 1).

The concept of MetS has been recognized for at least 80 years and during this period the syndrome has been (re)defined several times. MetS was introduced for the first time in the 1920s by the Swedish clinician Eskil Kylin[5]. Kylin discovered that several individual risk factors for CVD, like hypertension, obesity, hyperglycemia and dyslipidemia, tend to cluster. Such individual risk factors are still considered part of MetS and involve an increased risk for T2D, CVD and stroke. However, other components such as urinary albumin content and different measures for obesity like body mass index (BMI) or waist to hip ratio (WHR) can be considered determinants for the diagnosis of MetS. Systemic inflammation is widely regarded as associated with MetS, but is not part of any MetS definition. Table 1 presents four different MetS definitions which were formulated over the last decade. Alternative definitions of MetS have been developed, such as the definition of the American Association of Clinical Endocrinologists (AACE), and these are more focused on diabetes and insulin resistance[6,7,8]. Applying the different criteria to a single data set will lead to different patients being classified as MetS

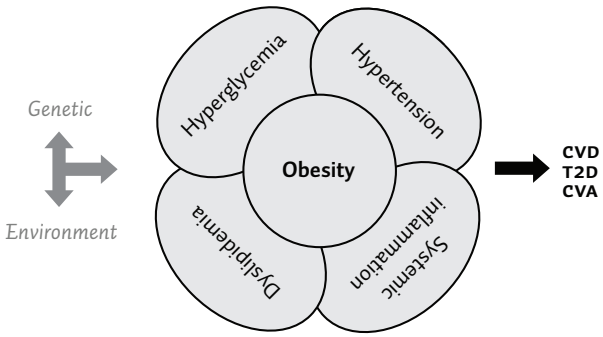

Fig. 1: Schematic representation of biologically relevant components contributing to the metabolic syndrome (MetS): Obesity, Hyperglycemia, Hypertension, Dyslipidemia and Systemic inflammation. The latter component is virtually absent in MetS definitions. MetS refers to a clustering of risk factors for cardio vascular disease (CVD), type II diabetes mellitus (T2D) and stroke.

The International Diabetes Federation MetS definition (2006) includes central obesity as an essential component for the manifestation of the syndrome. The obligatory presence of obesity in this definition is driven by the observed strong association between excess energy intake over expenditure and MetS. As such, obesity is a clear indication that excess energy intake has taken place for some period of time in an individual.

ENERGY HOMEOSTASIS

In the fasting state, the body relies on the production of glucose and lipids by the liver for the supply of energy to peripheral organs. The brain requires glucose while skeletal and heart muscle can also utilize fatty acids (FA) as substrates for oxidation. Glucose is secreted directly into the blood and FA's are packaged into VLDL particles after secretion by adipose tissue or liver in the form of triglycerides (TG).

Upon ingestion of a meal composed of lipids and carbohydrates, the body will respond by producing a variety of hormones and neuronal signals which cause the system to switch the system from a catabolic to an anabolic state. Carbohydrates are converted to glucose in the gut and are directly

Table 1 Four metabolic syndrome definitions

	WHO (1999)	EGSIR (1999)	ATPIII (2001)	IDF (2006)[a]
Components (N) [b]	2 + OGTT	2 + IR	3	2 + WC
_ WC (cm)	-	≥ 94	>102	≥ 94
_ WC (cm)	-	≥ 80	> 88	≥ 80
BMI (kg/m2)	> 30 [c]		-	-
_ WHR	> 0.9 c		-	-
_ WHR	> 0.85 c		-	-
FPG (mmol/L)	-	≥ 6.0	≥ 6.1	≥ 5.6 [d,f]
AE (_g/min)	≥ 20	-	-	-
OGTT	< glucose 25%	-	-	-
IR	-	> insulin 25% [c]	-	-
SBP (mm Hg)	> 140	≥ 140	≥ 135 e	≥ 130 e
DBP (mm Hg)	> 90	≥ 90	≥ 85 e	≥ 85 e
_ HDL-C (mmol/L)	< 0.9 [g]	< 1.0	< 1.0	< 1.03 [g]
_ HDL-C (mmol/L)	< 1.0 [g]	< 1.0	< 1.3	< 1.29 [g]
TG (mmol/L)	≥ 1.7 [g]	≥ 2.0	≥ 1.7	≥ 1.7 [g]

WHO; World Health Organization, EGSIR; European Group for the study of Insulin Resistance, IDF; International diabetes federation, ATPIII; National Education Control Panel Adult Treatment Panel III. a Europids. b Minimal composition of components required for diagnosis MetS. WC; waist circumference, BMI; body mass Index, WHR; waist to hip ratio; FPG; fasting plasma glucose, AE; albumin excretion, OGTT; oral glucose tolerance test, IR; insulin resistant, SBP; systolic blood pressure, DBP; diastolic blood pressure, HDL-C; HDL-cholesterol, TG; total plasma triglycerides., c BMI or WHR, d top 25% of fasting insulin values from non-diabetic population, e pharmacological treated hypertensive patients included, f pharmacological treated type 2 diabetes patients included, g pharmacological treated dyslipidemic patients included.

absorbed in the blood. Lipids are converted to chylomicrons and enter the blood via the lymph. The production of insulin by the pancreas is considered as the most metabolically important hormone signal. Insulin secretion is prompted by the relatively rapid increase in plasma glucose. Insulin represses the secretion of glucose by the liver and increases the uptake of glucose by adipose and muscle tissue. Insulin also represses the secretion of VLDL by the liver.

From an evolutionary perspective, the system is exquisitely suited to ensure survival of prolonged periods of chronic food deprivation, but seems much less suited to deal with chronic over consumption. A chronic excess intake of energy leads to obesity and is associated with low grade inflammation, disturbances in both glucose and lipid metabolism and high blood pressure. These aspects of MetS are discussed in detail below. In addition to quantity, the quality of the food plays a role in the development of pathology. For example, the Mediterranean diet has been associated with less pathology presumably due to the increased dietary levels of unsaturated FA inherent to a high intake of olive oil and fish[9,10]. The mechanism of excess of food intake leading to MetS is under debate. Although most of the processes that are involved are not disputed to play a role in MetS, their relative contribution and the sequence of events leading to MetS are the source of the debate. These processes and their overlaps are shown in Figure 1.

PATHOPHYSIOLOGY OF THE METABOLIC SYNDROME

OBESITY

A misbalance between energy intake and expenditure is thought to be the cause of the current increase in prevalence of MetS[11,12]. Although obesity may not be the first pathological metabolic consequence of excess food intake, its presence does prove that, for a prolonged period of time, there has been higher

energy intake than is required for expenditure has taken place. Expansion of adipose tissue requires an increased influx of FA, but also extensive tissue proliferation and remodeling including precursor cell differentiation, extracellular matrix breakdown and neovascularization. Excess adipose tissue is also associated with increased effluxes of FA and altered function of the adipose tissue itself.

In the definitions of MetS in Table 1, obesity is defined by a threshold BMI or WC. The major difference between these two obesity measures is the focus on body composition (BMI) versus central obesity (WC) and the corresponding risk for MetS. BMI is the most widely used general measure for obesity and is defined by the ratio of weight and squared height (kg/m^2). Because BMI involves the total sum of bone, fat and muscle mass, it does not make a distinction between specific fat depots. WC specifically measures central obesity. Although WC is more region specific than BMI, WC measures the total sum of visceral and subcutaneous fat depots and does not distinguish between these two functionally different fat depots. Alternatively, body composition can be expressed as waist to hip ratio (WHR) and is defined by the ratio between specific fat depots in hip and waist. This measure is generally used to distinguish between the benign pear-shaped overweight individuals and the more pathogenic apple-shaped overweight individuals. As an isolated measure, WHR does not actually represent the level of obesity.

ADIPOSE TISSUE

It is generally accepted that adipose tissue functions as an endocrine organ and can respond to neuronal and hormonal input by secreting biologically active substances in addition to FA, namely adipocytokines or adipokines. Chronic disturbances in the endocrine function of adipose tissue clearly play a role in the pathology associated with MetS. In this respect, the different adipose tissue depots, such as visceral and subcutaneous fat, are not each other's equivalent in terms of the production and secretion of adipokines[13]. Visceral fat directly drains on the portal vein, and is thus likely to have a much more direct signaling and metabolic relation with the liver in comparison to subcutaneous fat. The adipokine family can be divided in two types of signaling molecules, namely those with a metabolic/immunological function, which include interleukins 1β, 6, 8, 10 or 18, tumor necrosis factor alpha (TNF-α) and transcription growth factor beta (TGF-β), and those with an endocrine function, which include leptin, retinol binding protein-4 (RBP-4), adiponectin and resistin. All adipokines are thought to affect many different tissues throughout the body, including liver, gall bladder, skeletal muscle, brain and pancreas.

The metabolic/immunological adipokines are, in general, pro-inflammatory and positively associated with obesity and trigger a response which results in infiltration of immune cells, such as macrophages. Macrophages are involved in the clearance of all forms of debris, including dead cells, but are themselves also a source of pro-inflammatory cytokines. Once macrophages are abundant in adipose tissue, they maintain the inflammatory state of the adipose tissue in a vicious circle, which results in chronic systemic low-grade inflammation. This inflammatory state is far less severe than pathogen-induced inflammation[14,15].

The adipokines with endocrine function do not only affect the liver, skeletal muscle and pancreas, but also the central nervous system (CNS)[16]. The adipokine leptin affects the arcuate nucleus in the brain, which is involved in the regulation of appetite and energy expenditure. Increased plasma leptin functions as a signal of increased adipose tissue mass. As such leptin is, potentially, an ideal weight loss hormone. However, obesity is associated with leptin resistance limiting the use in

weight reduction[17,18]. The adipokines resistin and adiponectin are negatively associated with adipose tissue mass and insulin sensitivity, whereas RBP-4 is positively associated with adipose tissue mass and insulin sensitivity[19,20,21,22,23,24]. The precise role of the adipokines resistin and adiponectin in the development of obesity and insulin resistance remains to be fully characterized. The role of RBP-4 in the manifestation of T2D seems to involve a secondary mechanism[25]. Still, in animal intervention studies it has been shown that modulation of adipokine levels can determine the development of insulin resistance[24,16]. Although pharmacological interventions in patients that improve insulin sensitivity clearly ameliorate plasma adipokine levels, interventions that are directly aimed at adipokines have yet to be performed[26].

HYPERGLYCEMIA

Hyperglycemia is defined by elevated levels of fasting plasma glucose. The threshold values of these levels vary between the different MetS definitions (Table 1). Hyperglycemia is thought to be characterized by insulin resistance (IR). IR is defined as an impaired response of a specific process or organ to insulin. In a normal physiological state, insulin suppresses glucose production by the liver and increases glucose uptake by peripheral organs such as muscle and fat. As a consequence of IR, plasma insulin levels increase to achieve normal plasma glucose levels[27].

Many physiological and pathological processes have been identified that can modulate the response of cells to insulin and thus affect insulin sensitivity. These processes include nutritional status, circadian rhythms, inflammation, ER stress and intracellular lipid levels[28,29,30,31]. A current important challenge lies in the understanding of the integration of these (patho) physiological processes in the development of insulin resistance.

Hyperglycemia is generally defined as T2D when fasting plasma glucose levels consistently rise up to 7 mmol/L. T2D is a specific risk factor for retinopathy, neuropathy and nephropathy[32]. The progression of insulin resistance to diabetes is thought to result from the chronic nature of the triggers that induce insulin resistance. It seems more than likely that as time progresses, some of these triggers exacerbate as a consequence of aging-induced changes, i.e. hormonal status and physical activity. At some point, the insulin resistance may fail to be compensated by increased insulin secretion, resulting in failure of the glucose homeostasis and thus in hyperglycemia. Since high levels of glucose are cytotoxic, the hyperglycemia will contribute further to the overall deterioration of the system. Eventually, this vicious cycle may progress to pancreatic beta-cell deterioration, at which point the diabetic state has become irreversible.

It should be noted that the threshold levels for fasting hyperglycemia in the various definitions of MetS (5.6-6.1 mM/L, Table 1), are all below the accepted level that is considered to be minimal for treatment (>7.0 mM/L).

DYSLIPIDEMIA

The dyslipidemia associated with MetS is defined by hypertriglyceridemia and low HDL-cholesterol levels. Both have been identified as risk factors for cardiovascular disease[33] and especially hypertriglyceridemia is a target for drugs aimed at CVD prevention. These drugs include fibrates, which act mainly via the nuclear receptor PPAR-alpha. Similar to hyperglycemia, the level of hypertriglyceridemia required for MetS diagnosis is well below the level required for pharmacological intervention.

The lipids TG and cholesterol are transported via lipoproteins in the blood. After a meal, food derived TG and cholesterol are packaged into chylomicrons in the intestine and secreted via the lymph into the circulation. The main fate of the TG in chylomicrons is lipolysis in peripheral muscle and storage in fat. In the fasting state, TG and cholesterol are transported from the liver to the periphery via very-low-density-lipoprotein particles (VLDL). Once chylomicrons and VLDL are depleted of TG, their remnants are cleared by the liver. A fraction of VLDL remnants progresses to be metabolized in cholesterol-rich LDL particles. LDL particles are specific transporters of cholesterol to the periphery and LDL cholesterol is a well defined risk factor for CVD. A separate lipoprotein, HDL, is responsible for cholesterol transport from the periphery to the liver, which is referred to as reverse cholesterol transport. High levels of HDL cholesterol are associated with low levels of CVD[34,35].

From epidemiological studies it is well known that plasma TG and HDL-cholesterol are highly correlated inversely. The enzyme Cholesterol-Ester Transfer Protein (CETP) balances the levels of TG and HDL-cholesterol and is thus responsible for the mutual exchange of TG and cholesterol ester between apoB-containing lipoproteins (chylomicrons, VLDL and LDL) and HDL. It has been suggested that CETP activity explains some of the high TG levels and low HDL levels, observed in persons with MetS[36].

It has recently been hypothesized that hepatic insulin resistance of glucose and lipid metabolism may be differentially affected in persons with MetS. In individuals with MetS, insulin-mediated suppression of hepatic glucose output may be decreased, but insulin-mediated stimulation of *de novo* lipogenesis may be increased. This will result in increased hepatic lipid accumulation, increased VLDL production and thus ensuing hypertriglyceridemia[37,38].

HYPERTENSION

Hypertension is characterized by chronically elevated systolic (SBP) and/or diastolic (DBP) blood pressure. The diagnosis hypertension can be subdivided in two types: primary hypertension, which involves an unknown cause or origin and secondary hypertension, which involves a known cause or origin. Threshold values for hypertension within the different MetS definitions differ slightly (Table 1). For diastolic blood pressure this threshold range between 85-90 mm Hg and for systolic blood pressure the threshold ranges between 135-140 mm Hg. The increased blood pressure found in persons with MetS is in general of unknown cause (primary hypertension). Similar to the previously discussed MetS traits, the threshold for hypertension in the definition of MetS is well below the level that requires pharmacological treatment.

The exact cause for the increased blood pressure associated with MetS is not known but there is evidence suggesting that genetic predisposition plays an important role. However, it has also been suggested that MetS components hyperglycemia, dyslipidemia and low grade systemic inflammation affect the functioning of the vascular endothelium. A dysfunctional endothelium will not properly respond to physiological stimuli that increase NO production, an important signaling molecule involved in vascular contraction-relaxation and subsequent hypertension[39]. Such mechanism may occur independently of a joint genetic etiology.

Alternatively, mild hypertension may be involved in the etiology of MetS in that hypertension can contribute to the presence of MetS as an independent risk factor. Especially when hypertension is the consequence of endothelial dysfunction not directly associated with MetS (i.e. genetic factors or smoking), this endothelial dysfunction may, in turn, affect blood supply to organs and thus affect

tissue function. Insulin sensitivity of the blood supply to adipose tissue, muscle and liver is an important regulator of glucose and fat fluxes.

INFLAMMATION

Low grade systemic inflammation and/or hepatic inflammation are generally not included in MetS definitions. However, in addition to the inflammation associated with adipose tissue as discussed above, hepatic inflammation is also strongly associated with MetS[14,15]. Markers such as C-reactive protein, several hepatic enzymes like alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) and cytokines like IL-6 can serve as determinants (biomarkers) of the inflammatory state[40]. Hepatic inflammation associated with MetS but not due to excessive alcohol intake is termed non-alcoholic steatohepatitis (NASH). NASH is thought to be a consequence of hepatic lipid accumulation. NASH is also characterized by hepatic insulin resistance. As discussed above, the differential insulin resistance of hepatic glucose output and hepatic *de novo* lipogenesis may explain the development of NASH in individuals with MetS. This explanation is attractive in its simplicity, but remains to be thoroughly investigated and confirmed. For example, the specific triggers that lead from lipid accumulation to inflammation remain to be fully characterized. Moreover, the role of immune cells, such as macrophages, in relation to the inflammatory state of the hepatocyte remains to be characterized.

As discussed above, the low grade systemic inflammation has been hypothesized to present a continuous insult to the endothelium, leading to endothelial cell dysfunction and abnormal blood pressure regulation. The loss of cell function can also extend to the endothelial barrier. When the endothelial barrier function is compromised, LDL and other potentially harmful substances may enter the sub-endothelial space more easily and trigger an inflammatory response. The chronic accumulation of LDL, subsequent oxidation of LDL and uptake by invading macrophages leads to foam cell formation, which is considered the first step in the development of an atherosclerotic plaque. As such, chronic low grade inflammation, hypertension and dyslipidemia all represent chronic triggers for the development of atherosclerosis[34,41,42].

EPIDEMIOLOGY OF THE METABOLIC SYNDROME AND ITS COMPONENTS

PREVALENCE OF METS

The prevalence of MetS has increased dramatically over the last decades in societies with a Western lifestyle. In the US, studies have shown that the prevalence of MetS in adults and, in particular, in adolescents and female adults was growing constantly over the years 1984 to 2000. In this time frame, the prevalence of MetS in adults ranged from approximately 25% to 30%, and in it adolescents ranged from approximately 4% to 9%, with a higher prevalence in males than in females[43,44,45]. In Europe, studies in the general Caucasian population of the prevalence of the metabolic syndrome are scarcer and quite diverse due to different study methods. For example, the prevalence of MetS among healthy French families ranged from approximately 7% to 9%[46], whereas the prevalence in an urban and rural German population ranged from 20% to 40%[47]. Although consistent and comparable information about the increase of the prevalence of the metabolic syndrome in European is scarce, there is no doubt that the prevalence of metabolic components such as obesity and hyperglycemia

is increasing in Europe[48,49,50]. The macro-economic consequences but also individual morbidity and mortality associated with an increase in the prevalence of MetS, are substantial[51,52].

PREVALENCE OF OVERWEIGHT AND OBESITY

In westernized countries the prevalence of overweight and obesity has increased dramatically over the last few decades[53,54,55,56]. A particularly worrying development is the rise in the manifestation of overweight and obesity in young children and adolescents[57]. In 2002, the prevalence of overweight (BMI ≥ 25 kg/m²) or obesity (BMI ≥ 30 kg/m²) in young US children, adolescents and adults, exceeded the astonishing level of 60%. In a time span of approximately 25 years, a 25% increase of overweight was seen in children from age 6 to 11 [58]. In the period 1997-2001, the prevalence of European obese children between 13 and 17 years old ranged, in general, from 10 to 15%. However, in Greek children the prevalence of obesity was much higher, at 22 to 30%[59,57].

The prevalence of overweight and obesity in the Dutch population is carefully monitored by the "Centaal Bureau voor Statistiek" (CBS; http://www.cbs.nl) and by the "Rijksinstituut voor Volksgezondheid en Milieu" (RIVM; http://www.rivm.nl). These studies are cross sectional and prospective and include males and females, children and adolescents. Figure 2 illustrates the cross sectional increase of the prevalence of overweight in the Dutch population between 1987 and 2007. The mean prevalence of overweight in Dutch adults (>20 years old) increased from 33% in 1983 to 45% in 2007.

The prevalence of overweight (BMI>25 kg/m²) and/or obesity (BMI>30 kg/m²) in Dutch men and women (age> 20 years old) rose with 10% in the timeframe of 1981 to 2007. In general, the prevalence in men is approximately 10% higher than in women. The prevalence of obesity alone in this timeframe rose from 4% to 10% in women and from 6% to 12% in men. In Dutch adolescents, a similar increase of the prevalence of overweight is seen. Especially in children / adolescents (age > 8 years old) the prevalence of overweight increased by approximately 10% between the years 1997 and 2004 in both genders.

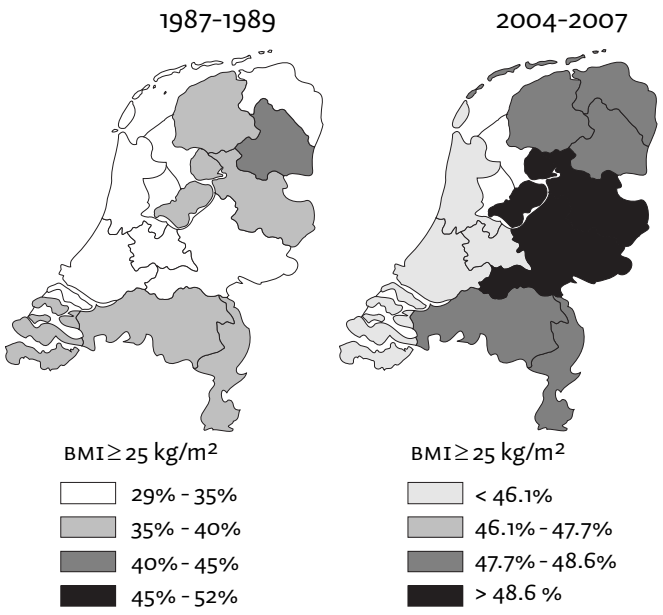

Fig. 2: Prevalence of overweight in Dutch population in the periods between 1987-1989 and 2004-2007. Prevalence adjusted for gender and age. source: CBS

PREVALENCE OF HYPERGLYCEMIA

The prevalence of hyperglycemia as entity in general populations is mainly monitored by the prevalence of diabetes mellitus (fasting plasma glucose > 7mM/L). However, within MetS an impaired glucose metabolism is diagnosed using a variety of measurements (see Table 1). There are large differences in the definition of impaired glucose metabolism between the four MetS definitions. The WHO uses the oral glucose tolerance test (OGTT), whereas the EGSIR includes fasting plasma glucose or the top 25% of fasting insulin values from the (non-diabetic) population. Both the NCEP ATPIII and IDF use fasting plasma glucose levels, though with different thresholds. The choice of such a parameter is a matter of availability (within clinics), cost-efficiency and personal preference. The outcome of epidemiological studies of the presence of impaired glucose metabolism and MetS are affected by these methodological differences.

The increase of the prevalence of hyperglycemia or T2D is as dramatic as the increase of the prevalence of obesity[58,60]. In 2007 the incidence of diagnosed T2D in the general US population was 5.9%. It should be noted that about 2% of the population was estimated to be suffering from undiagnosed diabetes (source: National Diabetes Information Clearinghouse: NCDIC; http://diabtes.niddk.nih.gov/DM/PUBS/statistics).

The prevalence of hyperglycemia (fasting plasma glucose > 6.1, T2D included) was monitored in two cross-sectional Dutch cohorts, the MORGEN and PREVEND studies[61]. The prevalence of hyperglycemia ranged between 5%-20% in men and 3%-9% in women. The age of the individuals in these studies ranged from 28 to 59 years old. The overall incidence of T2D in 2007, as monitored by the RIVM (http://www.rivm.nl), was approximately 4.5%. The incidence of T2D in the Dutch population has increased in the last decade. Between 1990 and 2007, the incidence of T2D increased by approximately 50% in men and 40% in women. However, it should be noted that in addition to the increase of obesity, the increase in average age of the Dutch population also contributes to this increase of the prevalence of T2D.

Although T2D is typically a late onset disease, the increasing incidence of T2D is also seen in young children and adolescents[57]. The prevalence of T2D or other rare forms of diabetes among US children ranged between 1-2%. In the last decade however, several reports have indicated an increase of incidence of up to 50% of newly identified non immune-mediated diabetes in US young children[62]. In Europe the increase in the prevalence of T2D in children is limited[63]. Recently a prevalence of T2D or impaired glucose tolerance of 2.5% was reported in German children with a low socioeconomic status[64].

PREVALENCE OF DYSLIPIDEMIA

Two of the 5 traits defining the metabolic syndrome are dyslipidemias, namely high TG and low HDL-cholesterol. These traits are not independent, since high TG and low HDL are strongly correlated. This correlation may be caused by the activity of the enzyme Cholesteryl Ester Transfer Protein (CETP), as discussed above[65,36]. The prevalence of HTG (> 1.7 mM/L) in the MORGEN and PREVEND studies, ranged between 13% in women and 24% to 29% in men (age ranging from 28 to 59 years old)[61]. The prevalence of low HDL-cholesterol (men <1.0 and women <1.3 mM/L) ranged between 28% and 36% in the MORGEN and PREVEND studies[61].

Both high TG and low HDL are classical risk factors for CVD and stroke[66]. However, the other classical lipid risk factor for CVD and stroke, LDL-cholesterol, is not part of the definition of the

metabolic syndrome. This is due to the independent association of LDL cholesterol with CVD/stroke risk. High levels of plasma LDL-cholesterol do not consistently cluster with other components of MetS, such as obesity and insulin sensitivity. In some cases, a specific genetic cause (Familial Hyper cholesterolemia; FH) lies at the basis of this particular impairment[67].

PREVALENCE OF HYPERTENSION

Similar to T2D, hypertension is a late onset and common disease in the general population. Hypertension in the general adult US population shows a prevalence of approximately 25 up to 36% (SBP ≥ 140 mm Hg and DBP ≥ 90mm Hg or use of medication, reported from 1988 to 1998)[68,44]. This prevalence of hypertension in general USA adults increased up to 41% in the period between 1999-2000[44]. In general, other countries show lower percentages of hypertension[68,69]. The prevalence of hypertension (according to NCEP ATPIII, see Table1) in the MORGEN and PREVEND studies, ranged between 42% and 44% in men and from 21% to 26% in women (age ranging from 28 to 59 years old)[61].

Analogous to hyperglycemia, dyslipidemia and hyperglycemia, many hypertensive patients are not aware that they are suffering from elevated blood pressure. 45% of US adults in the period between 1988 and 1993 were not aware of their elevated blood pressure. Hypertension develops gradually and eventually does result in overt problems in the patient. Since anti-hypertension medication is generally prescribed life long and may have unwanted side-effects, a large proportion of patients does not adhere to therapy; 29% of US adults suffering from hypertension in the period between 1988 and 1993 did not adhere properly to therapy[68,70].

APPROACHES IN GENETIC EPIDEMIOLOGY

INTRODUCTION TO GENETIC EPIDEMIOLOGY

Genetic diseases can be divided in disorders with a monogenic inheritance pattern and disorder with a complex inheritance pattern. Monogenic disorders show autosomal or X-linked dominant or recessive inheritance patterns. Complex disorders are characterized by inheritance patterns where only some of the mutation carriers are affected. This is referred to as reduced penetrance. In complex disorders, environmental variables or additional genetic factors contribute to the manifestation of the disease. For the genetic part, this means that multiple genes and interactions may contribute to the disease according to a threshold model.

An example of a monogenetic x-linked recessive disorder is Duchenne muscle dystrophy (DMD). DMD is characterized by a progressive dystrophy of skeletal muscles and eventual respiratory or heart failure. The genetic basis of this neuralmuscular disorder lies, in general, in a disrupted reading frame in the dystrophin gene (Xp21) caused by nucleotide insertions or deletions of variable length. Such disruption of the reading frame results in a truncated (dysfunctional) or absent dystophin protein[71]. An example of a complex disorder is the disease hyperlipidemia (HLP) type III, which is characterized by elevated plasma levels of VLDL triglyceride and cholesterol. In the last 3 decades, researchers have found that patients suffering from HLP type III were predominantly homozygote carriers of the apoE2 protein variant. Since homozygosity of this variant is mainly present in healthy controls, this metabolic disorder is characterized by a reduced penetrance. Functional analyses indicated that apoE2 has a defect in binding to the hepatic LDL receptor and is thus poorly cleared

from the circulation[72,73,74]. In addition, a number of rare variants of apoE have been identified that contribute to the expression of type III HLP or HTG[75,76]. Family analyses of these variants revealed clear co-segregation of disease and variant and these variants were completely absent from healthy controls. This provided convincing evidence for causation of disease, which was confirmed by *in vivo* analyses in transgenic mouse models[77,78]. Thus, monogenic disorders are completely or predominantly caused by variations in one single gene. In contrast, complex disorders result from joint effects of multiple genetic -and environmental causes with each factor having only a minor contribution to the expression of the disease[79].

In general, two methods are available for the identification of loci involving monogenetic or complex diseases and these are illustrated in Figure 3. Linkage analysis is a family based method and was the first robust method in genetic epidemiology. The second method is association analysis which is based on cases and controls or quantitative trait analysis. Both methods are described below with regard to their design and statistical power in the following sections.

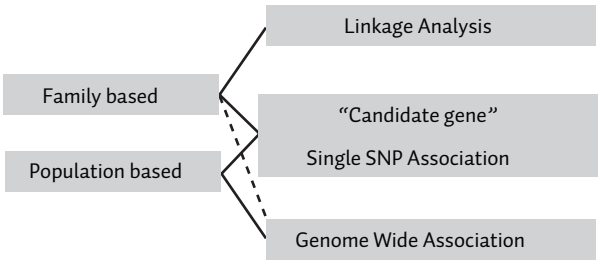

Fig. 3: Two main types of cohorts with regards ro feasible typs of analyses in genetic research

LINKAGE ANALYSIS

Early techniques which made large scale genotyping possible enabled a novel strategy for the identification of disease loci, namely large scale linkage analysis. This type of analysis is based on the characterization of a large number of short tandem repeats (STRs) or micro-satellites distributed over the genome. At present, large genotyping platforms involving SNPs are also used in linkage analysis, as described in the section below.

Linkage analysis focuses on chromosomal regions that are transmitted to diseased offspring more often than expected. Linkage analysis is based on the fact that particular loci do not show independent inheritance patterns. This means that between such loci the probability of recombination approaches o within a family of closely related subjects. This phenomenon of linked loci is also called linkage[80]. Linkage analysis is thus a family-based approach where the segregation of the disease within the families can be linked to a specific chromosome region (locus). Linkage analysis was and still is a robust method to identify novel disease loci. After determining the chromosomal location of the causal gene, these loci often contain multiple interesting genes with regard to the disease of interest. The most interesting genes overlap or involve a certain pathway which is impaired in the disease. These are called "candidate genes". The method for validation or replication of the involvement of such candidate genes in the disease is described in the section "validation and replication". In addition, new pathways not implicated earlier in the disease may also be discovered. Linkage analysis can be performed with a relatively small number of samples and genotypes. Nevertheless, information about the pedigree structure is essential.

GENOME WIDE ASSOCIATION

Family based genome wide linkage analysis is especially powerful for the detection of association of rare genetic variants with rare diseases, since large chromosomal regions are linked to the disease. Traditionally, genetic association has been used for fine mapping of the linked region. Moreover, the last decade (genome wide) genetic association was also used. The basic underlying assumption is the "common disease – common variant" (CD-CV) hypothesis. This hypothesis involves the idea that a prevalent disease in the general population (common) is caused by many common genetic variants. Thus, according to the CDCV hypothesis, proposed in the last decade of the 20th century, common prevalent diseases like hypertension, CVD or T2D, might be caused by (multiple) common variants in genes throughout the general population[81]. Discovery of novel loci using linkage analysis is not suitable in CDCV because different genes may be involved in the same family. Thus, for the search for common variants, causing common disease, preferably large cohorts with extensive genotype data are used. Within such large cohorts, cases and controls can be selected for binary association or alternatively, quantitative trait analyses can be performed. The statistical power (see section *statistical power*) of the two study designs, linkage analysis and genome wide association, are illustrated in Figure 4.

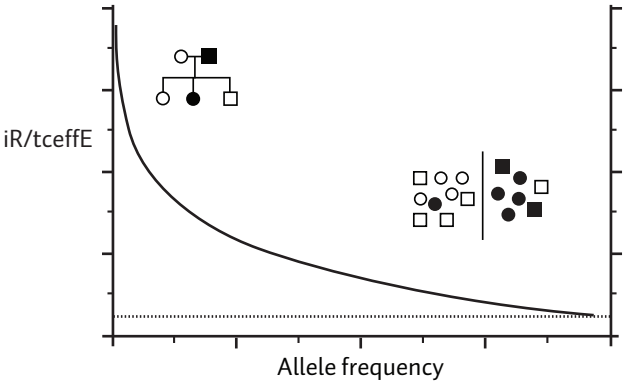

Fig. 4: Schematic overview of the most powerful approach for discovering genes based on minor allele frequency.

To find novel loci according the CDCV hypothesis, extensive genotyping is necessary and the available techniques have evolved rapidly in recent years. Extensive genotyping techniques are based on micro-array technology. Two major companies, Affymetrix and Illumina, have developed micro-arrays, among which SNP-arrays. At present, SNP micro-arrays involving 6K to 1000K SNPs are available. The SNPs present on the Illumina SNP arrays are based on their tag property. This implies that these SNPs were selected because each SNP covers a relatively large region in linkage disequilibrium (LD). Such tag property of the SNPs is of less importance in the Affymetrix design. By contrast, Affymetrix SNP arrays also contain known "coding" mutations.

Since it is likely that common variants are associated with small effects, large cohorts are needed to achieve sufficient statistical power. Therefore, in Genome Wide Association studies (GWAS), the aim is to accumulate the highest achievable number of genotypes of as many subjects as possible. When GWAS results in novel loci, the "candidate gene" approach is generally chosen to search for and validate the causal variant[82].

VALIDATION AND REPLICATION

A classical method in genetic research of disease is the "candidate gene" approach. Candidate genes can be selected after indication obtained through several different methods, namely by means of: (1) linkage analysis, (2) GWAs or by (3) selecting a gene / protein according to its biochemical characteristic in the disease process or pathway. In general, fine mapping of the gene of interest is performed using sequencing analysis. Replication of a candidate gene is, mostly, performed using association analysis. This association analysis can be performed using SNPs and is based on the fact that most genetic variants causally related to the trait are expected to be more or less prevalent in patients than in controls, depending on whether they increase or decrease the risk of disease. However, also the SNPs close to the causal ones are expected to be increased or decreased in patients, when SNPs are close to each other. This phenomenon is called linkage disequilibrium (LD).

Thus causal mutations can be identified using SNP analysis or sequencing analysis. Mutations that cause overt changes in protein function (i.e. reading frame shifts leading to stop codons) provide strong proof for being the cause of a genetic disease. Less overt changes that nevertheless lead to protein dysfunction (i.e. missense mutations) can be identified by comparing the prevalence of the variants in patients versus controls. Putative dysfunction of the proteins encoding these genes can subsequently be characterized *in vitro* in material (i.e. blood cells) derived from patients versus healthy controls. Finally, a SNP that is located in a gene desert may very well influence the expression of a gene in the region (*cis* regulation) or elsewhere (*trans* regulation).

ALTERNATIVE STUDY COHORT

Most association studies are conducted in the general population. Alternatively, the design of a study can be based on a genetically isolated population. In short, this design is a mix between a family based cohort and a general cohort. It requires an extensive and more expensive collection procedure of study subjects, as in most cases a pedigree confirmation is required to rule out possible admixture with the general population. The statistical power of association analysis in such genetically isolated populations or founder populations is thought to be much stronger due to the fact that it is based on a limited gene pool[83,84,82]. This limited gene pool is a result of a limited number of founders in combination with a fast expansion of the population. Furthermore, a genetically isolated population is characterized by minimal immigration, due to social, geographical or religious reasons. Genetically isolated populations are liable to genetic drift. Genetic drift is defined by the phenomenon that rare genotypic variants disappear or, vice-versa, that rare variants become overrepresented with regard to the general (out bred) population. Common genetic variants are, however, generally not affected in genetically isolated populations and their frequencies are expected to be similar to those in the general population[83].

STATISTICAL POWER IN ASSOCIATION STUDIES

To perform linkage analysis or GWAs with sufficient statistical power, the study cohort must be of sufficient size and this requires significant effort and finances. Statistical power represents the measure of confidence to detect an (genetic) effect in a particular number of samples. However, the

problem with both linkage analysis and GWAs is the enormous number of tests which might result in false positive signals; type I errors. Statistical methods generally use a 95% confidence interval, which implies that five percent of the associations that are found are actually type I errors. When performing a single test, the 5% probability of finding a false positive result is acceptable. However, when performing half a million tests such as in GWA, the amount of false positive results will be large.

To address the multiple test correction issue in GWAs, methods like the method of Bonferroni are used to overcome the problem of accumulating type I errors[85,86]. In brief, the method of Bonferoni decreases the probability of a true finding by dividing the confidence (represented by the P-value) by the number of independent tests performed. This method is, however, a very stringent multiple test correction which might result in a high probability of type II errors; or false negatives. Therefore, other multiple test correction methods, like the method of Benjaminii – Hochberg, have been developed. This correction method also reduces the number of false positive associations, but also takes into account the possibility of false negative findings[85,86].

META-ANALYSIS OF ASSOCIATION STUDIES

To validate GWAs results and tackle the remaining probability of both false positive and false negative associations, meta-analyses are performed[87]. Meta-analysis is a statistical method to compare and strengthen similar observed candidate loci for disease associations in different studies/cohorts. Meta-analysis is capable of detecting in several different study cohorts consistent, yet small significant associations, but is also capable of excluding single significant false positive associations[88]. Meta-analysis is now generally accepted as a powerful tool in genetic epidemiology and the application of meta-analysis in the field of genetic epidemiology is widely used. Large international consortia have been formed over the last years, resulting in studies exceeding 30.000 samples. Meta-analysis on such large number of samples resulted for example in the discovery of several new loci associated with T2D and obesity and several quantitative traits such as plasma lipids[89,90,91,92].

Three common problems in meta-analysis are (1) the use of different types of cohorts, (2) inconsistency in phenotyping and (3) inconsistency in genotyping. The use of different types of cohorts in GWAs meta-analysis, at least with regard to ethnicity, should be avoided or at least carefully monitored since population specific genetic associations might unjustly be disregarded. Differences in (the accuracy of) phenotyping might also result in false negative findings. For example, in a meta-analysis of plasma glucose GWAs, consistent use of information about the fasting state of the samples and the use of glucose lowering medication should be included in each individual GWA. Genotypic inconsistencies between cohorts are for example caused by the use of different genotyping platforms. In this respect, the design of two major suppliers of genome wide genotyping platforms, Illumina and Affymetrix, are totally different.

To overcome the problem of missing SNPs, the statistical tool of imputation was developed[93,94]. Imputation is based on the fact that most genetic variants are more or less in LD with nearby genetic variants. Based on the genetic data of Hapmap, which involves about 2.500.000 genetic variants, differences in genetic variants between platforms can be filled in using estimation of the missing genetic variants[95]. This way, different platforms (cohorts) can be forced towards similarity in genetic variation and thus be used in meta-analysis.

OUTLINE THESIS

In this thesis several aspects of metabolic syndrome are addressed. The focus involves questions concerning the genetics of obesity, TG and cholesterol and hyperglycemia. Since we hypothesized that obesity is the most important trigger of metabolic impairment, the MetS definition in this thesis was chosen to include the obesity measure waist circumference as an essential component. In the study described in **chapter 2**, the heritability of the metabolic syndrome was addressed and compared to the heritability of its individual components. Since the individual components of MetS were shown to be more heritable than MetS itself, the studies described in **chapter 3 and 4** focused on the genetics of the individual MetS component plasma TG. For this purpose, a candidate gene approach was employed using HTG patients and healthy controls. The involvement of a series of candidate genes was confirmed. The study described in **chapter 5** followed a similar approach to that used in the studies described in **chapter 3 and 4**. Several candidate genes were studied in patients suffering from hyperlipoproteinemia (HLP) type III, which is characterized by elevated levels of total plasma cholesterol and plasma TG. HLP type III is characterized by APOE2 homozygosity. Contributing genetic factors in the (metabolically stressed) APOE2/2 environment were confirmed. Plasma adiponectin, an adipose tissue secreted hormone (adipokine), has been suggested to be a biomarker for MetS. In **chapter 6** we describe a study which particularly aimed to determine the effect of menopause on the discriminating accuracy of adiponectin to predict MetS. Especially low levels of plasma adiponectin in postmenopausal women were found to be a risk for MetS. However, the discriminating accuracy of adiponectin for the presence of MetS was exceeded by BMI in men and pre –and post menopausal women. Since plasma adiponectin levels are very well correlated with MetS components or related traits, the study described in **chapter 7** addressed the question whether these correlations are caused by a genetic overlap (genetic correlation). The genetic correlation was mono-laterally validated with regard to the adiponectin gene (*ADIPOQ*). **Chapter 8** describes a study towards finding novel loci associated with adiponectin or loci that are possibly involved in the genetic overlap between adiponectin and MetS components or related traits. This study followed a genome-wide association (GWA) approach. The results of this GWA were used in a joined analysis with two other cohorts in a meta-analysis. In addition, a selected proportion of SNPs was submitted for replication in several cohorts. **Chapter 9** provides a general discussion by reviewing all previous chapters in the thesis. Furthermore, chapter 9 includes suggestions and proposals for future analyses towards unraveling genetic and environmental factors involved in the expression and manifestation of metabolic risk factors.

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Chapter 2

Prevalence and heritability of the metabolic syndrome and its individual components in a Dutch isolate: The Erasmus Rucphen Family (ERF) study

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ABSTRACT

Background: Metabolic syndrome (MetS) is defined by a combination of abnormalities that are all individual risk factors for the development of type 2 diabetes and/or cardiovascular disease. The etiology of MetS includes both an environmental and genetic component. We studied prevalence and heritability of MetS and its individual components in a Dutch genetic isolate.

Methods: The Erasmus Rucphen Family study (ERF) consists of some 3000 genealogically documented individuals from a Dutch genetic isolate. Data on waist circumference (WC), blood pressure (BP), high density lipoprotein cholesterol (HDL-C), triglycerides (TG) and fasting plasma glucose values (FPG) are available. MetS was defined according to the International Diabetes Federation (IDF) (2003) and National Cholesterol Education program Adult Panel III (NCEP ATPIII) criteria. Variance component analysis was applied to extended family data to test for evidence of heritability.

Results: The prevalence of MetS in the ERF cohort ranged from 23-37% depending on MetS definition and gender considered. Low HDL-C and high WC are the main contributors to MetS. The heritability of MetS corrected for sibship effect was 10.6% ($P = 0.01$) according to IDF and 13.2% ($P = 0.07$) according to NCEP ATPIII criteria. In addition, the heritability of individual components of MetS were analyzed and found to range from 21.9 to 42.9%. The highest heritability was found for HDL-C (42.9%, $P < 0.0001$) and WC (37.8%, $P < 0.0001$). In addition, WC, SBP, HDL-C and TG, showed low to moderate genetic correlation (RhoG) between genders, whereas FPG and DBP showed absolute genetic correlation between genders.

Conclusion: Although the prevalence of MetS was high, the heritability of MetS in the ERF population was found to be moderate. The high heritability of the individual components of MetS indicates that the genetic dissection of MetS should be approached from its individual components.

INTRODUCTION

In populations that have adopted the Western life style, risk factors for type 2 diabetes (T2D) and cardiovascular disease (CVD) are strongly increased. These include high blood pressure (BP), low plasma high density lipoprotein cholesterol (HDL-C), elevated values of plasma triglyceride (TG), increased waist/hip ratio (WHR) or body mass index (BMI) and elevated levels of fasting plasma glucose (FPG). The combined presence of several of these risk factors has been termed the Metabolic Syndrome (MetS) or Syndrome X. Several definitions of MetS have been formulated over the years[1,2].

MetS as entity has proven to help clinicians in risk estimation for CVD and T2D. The question we raise in the present study is whether this entity is also of use in genetic research. Clustering of obesity and other cardiovascular risk factors might be explained by a common genetic origin, thus combining factors into a single outcome, as defined by MetS, may improve the power of gene discovery. However, recently the interest in defining gene specific endo-phenotypes in genetic research, in which syndromes are decomposed in traits with a homogeneous genetic origin, has increased. This approach does not favor gene discovery in MetS as entity, but favors studies of individual components. As the heritability of a trait is one of the main determinants of the power of gene finding, we compared this parameter in MetS and its individual components.

In the present study, we determined the prevalence of MetS and estimated the heritability of MetS and its individual components (by means of a general polygenic model, a polygenic household model

and polygenic gender model, respectively) in a Dutch genetically isolated population with extensive phenotypic and genealogical information. As in all isolated populations, our isolate is characterized by genetic drift and founder effect[3]. Genetic drift reduces the genetic variation. In recent simulation studies of genetic isolation, it was shown that rare variants may disappear completely or become common but it is unlikely that common alleles disappear[4]. Thus, genetically isolated populations will provide insight in both common and rare genetic variants that predispose to common complex diseases such as the metabolic syndrome[5,6].

SUBJECTS AND METHODS

In the present study, we used data of the Erasmus Rucphen Family study (ERF) which is embedded in a rural isolated population (Genetic Research in Isolated Populations; GRIP). This young, genetic isolate in the southwest of the Netherlands was founded in the mid-eighteenth century. Minimal immigration and/or marriages occurred between surrounding settlements due to social and religious reasons. This population experienced a fast expansion, at the moment this region counts roughly 20.000 inhabitants. The ERF population includes 3,000 individuals, who were not selected based on health information, but rather comprises of living descendants of 22 couples with at least 6 children baptized in the community church around 1850-1900 (approximately a total of 150 founders). Details about the genealogy of the population are described elsewhere[6,4,3]. The study protocol was approved by the medical ethics board of the Erasmus MC Rotterdam, the Netherlands. All investigations were carried out in accordance with the Declaration of Helsinki.

Plasma concentrations of triglyceride (TG), high density lipoprotein – cholesterol (HDL-C), fasting plasma glucose (FPG), diastolic and systolic blood pressure (DBP and SBP), waist circumference (WC), anti-diabetic (insulin, oral hypoglycemic) treatment, antihypertensive treatment and lipid lowering agents were determined as described previously[5,6,7].

We applied two different MetS definitions (table 1) in our study, namely the definition formulated by the National Cholesterol Education program Adult Panel III (NCEP ATPIII) and the definition formulated by the International Diabetes Federation (IDF).

Spss 11.0.1 (SPSS, Chicago, IL, USA) was used for selection, descriptive and regression analysis. For the prevalence of MetS according to the IDF definition, the Caucasian WC threshold values of >94 cm for males and >80 cm for females were used[8]. Correlations between components were based on transformed data. Pearson correlation coefficients were used to examine the association of MetS components and age, to confirm the presence of important cluster of traits within the MetS. Gender differences were evaluated by means of the Chi squared algorithm or Student's T-test. Heritability estimates were calculated using the SOLAR software version 2.05 (http://www.sfbr.org/solar/4.0.7.doc/00.contents.html). The individual components, WC, HDL-C, S/DBP, and TG showed a small deviation from a normal distribution within the ERF population, of which the software is extremely sensitive. Therefore heritability estimates of these components were based on the log normal transformed values. SOLAR's tdist function was used when traits remained to show some kurtosis. Where necessary, traits were multiplied with a factor (suggested by the software) for an optimal trait standard deviation.

The polygenic model was applied for all traits (MetS as binary trait, individual components as quantitative traits) which is assuming that an infinite number of genetic factors with a small additive effect contribute to the trait variance. In family based heritability estimations, the shared environment

within one household and shared genetic dominant effect between sibs within one household, may inflate the outcome of the heritability estimate. Therefore we corrected our heritability estimations for sibship effect (s). This shipship effect is an estimate of phenotypic similarity, induced by the progeny of the same parents. Thus, the sibship effect is a combination of effects induced by sharing early childhood environment and dominant genetic variation. Detailed description of calculation of heritability estimates was described by others[4,6]. To estimate the effect of gender specific heritability and the genetic correlation of a trait between genders (RhoG), bivariate polygenic analysis (trait + gender, covariates: age and inbreeding coefficient) was applied on all individual traits of the MetS[9,10].

RESULTS

Threshold values for MetS definitions according to the IDF and the NCEP ATPIII are given in table 1. Values for WC; HDL-C; TG; FPG; DBP and SBP were available for 2860 individuals. Table 2 shows the mean demographic characteristics of the study population. The mean age of the 1286 males was 49.4 years old (range 17.6 to 86.5) and the mean age of the 1574 females was 48.3 years old (range from 16.7 to 86.1).

Table 1: Metabolic syndrome definitions

	IDF (2003)[a]	ATPIII (2001)
male waist circumference (cm)	≥ 94	>102
female waist circumference (cm)	≥ 80	> 88
Fasting plasma glucose (mmol/L)	≥ 5.6 [b,c]	≥ 6.1
Systolic blood pressure (mm Hg)	≥ 130 [c]	≥ 135 [c]
Diastolic blood pressure (mm Hg)	≥ 85 [c]	≥ 85 [c]
male HDL-cholesterol (mmol/L)	< 1.03 [c]	< 1.0
female HDL-cholesterol (mmol/L)	< 1.29 [c]	< 1.3
Triglycerides (mmol/L)	≥ 1.7 [c]	≥ 1.7

a Europids

b included previously diagnosed type 2 diabetes patients

c included treatment patients.

The prevalence of MetS was 36.8% in males and 31.0% (P = 0.01) in females according to the IDF MetS definition and 26.7% in males and 22.8% (P = 0.02) in females according to the NECP ATPIII definition. As expected, the prevalence of MetS strongly correlated with age. We divided the population in six age groups: younger than 30 years old (N=294), 30-40 years old (N= 534), 40-50 years old (N=657), 50-60 years old (N=686), 60-70 years old (N=505) and 70 years old or older (N=184). Figure 1a illustrates the gender specific percentages within each age group of the prevalence of MetS according to the IDF definition. Figure 1b represents the same graph for the NCEP ATPIII definition. Using the IDF definition, in the group of 30-40 years old and using the NCEP ATPIII definition, in the groups of 30-40 and 40-50 years old the prevalence of MetS was significant higher in males compared to females.

According to the IDF definition of MetS, WC plus a minimum of any two other components are necessary for the expression of MetS. In contrast, the NCEP ATPIII definition does not require WC as an essential component for MetS expression. In this study, we investigated which combination of components contributed most to both the prevalence of the IDF MetS and NCEP ATPIII MetS. Table 3 represents all possible combinations of components in both genders, which can contribute to the expression of MetS. In these calculations, the blood pressure component was combined (BP =

Table 2: Demographic characteristics of the ERF population

Trait	Mean ± SE total	Mean ± SE males females	Mean ± SE	*P*-value [a]
N	2860	1286	1574	
Medication lipids	366(12.8)	193(15.0)	173(11.0)	< 0.01
Medication DM	137(4.8)	74(5.8)	63(4.0)	< 0.05
Medication hypertension	492(17.2)	241(18.7)	251(15.9)	< 0.05
Age (years)	48.8 ± 0.27	49.4 ± 0.40	48.3 ± 0.36	< 0.05
Waist circumference (cm)	87.7 ± 0.25	94.2 ± 0.33	82.3 ± 0.32	< 0.001
Fasting plasma glucose (mmol/L)	4.7 ± 0.21	4.84 ± 0.034	4.51 ± 0.026	< 0.001
Systolic blood pressure (mm Hg)	140.1 ± 0.38	143.6 ± 0.512	137.2 ± 0.538	< 0.001
Diastolic blood pressure (mm Hg)	80.4 ± 0.19	82.05 ± 0.276	79.0 ± 0.248	< 0.001
HDL-cholesterol (mmol/L)	1.3 ± 0.007	1.13 ± 0.009	1.40 ± 0.009	< 0.001
Triglycerides (mmol/L)	1.3 ± 0.146	1.49 ± 0.025	1.23 ± 0.016	< 0.001

a Difference between genders, *P* value by t-test. Value presented as N (%) or mean ± standard error.

SBP and/or DBP). For calculation within the IDF MetS, in total 11 combinations are possible with the components WC, HDL-C, TG, FPG and BP. Six of these combinations consisted of three components (for example WC + HDL-C + TG), four consisted of four components and one contribution included all components. For calculation within the NCEP ATPIII MetS in total 16 combinations are possible with the components WC, HDL-C, TG, FPG and BP. Ten of these combinations consisted of three components, five consisted of four components and one combination included all components. To determine the most prevalent contributions of combinations of the traits to MetS according to both definitions in the ERF population, we calculated Pearson correlations for all MetS components and age. These data are presented in table 4. Three main clusters of components contributing to MetS can be observed (bold in table 3). For males, these clusters are the same for the two definitions, [WC / HDL-C / BP] and [WC / HDL-C / TG / BP]. For females differences in composition of these clusters was observed between both definitions. The main female clusters according the IDF definition are [WC / HDL-C / BP] and [WC / HDL-C / TG / BP], but for the NCEP ATPIII definition [HDL-C / TG / BP] and [WC / HDL-C / TG / BP] contribute the most to the prevalence of MetS. Significant correlations between individual components (bilateral) were more prevalent in females than in males (table 4).

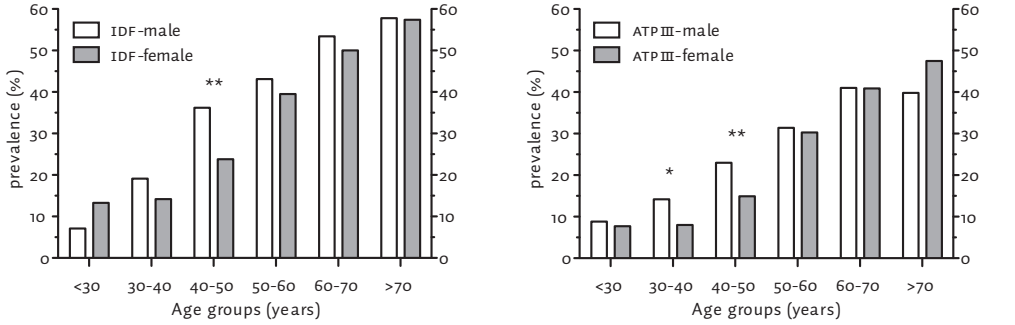

*Figure 1 Prevalence (%) of the metabolic syndrome (MetS) in age groups <30 years, 30–40, 40–50, 50–60, 60–70, and >70 years in the MetS subgroup of the Erasmus Rucphen Family population according to two different MetS definitions. Panel A: International Diabetes Federation (IDF). Panel B: US National Cholesterol Education Program Adult Panel III (NCEP ATPIII). *P<0.05; **P<0.001.*

WC in females correlates with all other components. HDL-C and TG are correlated in both genders. The BP components showed a somewhat lower correlation with WC and no correlation with HDL-C in females.

Table 3: Contribution (%) of possible combinations of individual components to MetS expression (both IDF MetS and NCEP ATPIII MetS).

Parameter combination	IDF MetS (%)		NCEP ATPIII MetS (%)	
	male [a]	female [b]	male [c]	female [d]
1 WC / HDL-C / TG	3.6	4.7	4.7	5.2
2 WC / HDL-C / FPG	0.6	0.2	0.6	0.3
3 WC / TG / FPG	0.2	0.0	0.0	0.0
4 WC / TG / BP	10.3	5.9	4.7	7.6
5 WC / HDL-C / BP	**27.3**	**40.8**	**31.5**	14.0
6 WC / FPG / BP	3.8	3.1	1.9	1.7
7 HDL-C / FPG / BP	-	-	2.2	3.8
8 HDL-C / TG / BP	-	-	11.7	**24.5**
9 HDL-C / TG / FPG	-	-	0.0	0.3
10 TG / FPG / BP	-	-	0.0	2.3
11 WC / TG / FPG / BP 4.7	1.0	0.8	2.6	
12 WC / HDL-C / FPG / BP	0.0	0.0	6.1	5.2
13 WC / HDL-C / TG / BP	**26.4**	**22.3**	**25.9**	**22.2**
14 WC / HDL-C / TG / FPG	11.8	10.9	0.0	0.9
15 HDL-C / TG / FPG / BP	-	-	2.8	3.2
16 WC / HDL-C / TG / FPG / BP	11.4	11.1	7.0	6.1

WC, waist circumference; HDLC, high density lipoprotein cholesterol; TG, triglycerides; FPG, fasting plasma glucose; BP, blood pressure; IDF, International Diabetes Federation; NECP ATPIII, National Cholesterol Education program Adult Panel III.
- , combination not represented by definition.

For both MetS definitions, we estimated the heritability in the ERF population (N=2488). Furthermore, we estimated the contribution of the sibship effect (early childhood environmental factors and genetic dominant effects) for both definitions. Table 5 illustrates the heritability estimates without consideration of the sibship effect (s) and table 6 illustrates the heritability estimates with consideration of the sibship effect. For both estimations, with and without sibship effect (s), the covariates we included were: gender, age and inbreeding coefficient. The heritability of the IDF-defined MetS, even when corrected for sibship effect, still reached significance (P = 0.01) whereas the heritability of the NCEP ATPIII-defined MetS was not but borderline significant (P = 0.07). In addition to the heritability of MetS definitions, we also estimated the heritability of the individual components of MetS in the ERF population (N=2488). SOLAR's analysis of covariance is not sensitive for altered trait values due to (limited) medication use (data not shown). Because all traits showed some skewness or extra kurtosis, these heritability estimates were based on natural log transformed data (table 5) and were necessary, t-distribution modeling was applied. In addition, we estimated the contribution of the sibship effect to the heritability of each component (table 6). This sibship effect inflated the heritability of all traits, with exception of HDL-C.

Table 4: Pearson correlation coefficients all components in ERF MetS (IDF) subjects

male n =473 [a]	Age	HDL-C	WC	TG	FPG	SBP		
		SBP	FPG	TG	WC	HDL-C	Age	**female n =488** [a]
HDL-C	0.09	0.40 **	0.06	-0.04	0.16 **	-0.01	-0.05	*DBP*
WC	0.03	-0.08	0.22**	0.04	0.12 **	0.06	0.50 **	*SBP*
TG	-0.16**	-0.11**	0.06	0.16**	0.29 **	-0.00	0.20 **	*FPG*
FPG	0.19**	0.03	0.13**	0.02	0.15 **	-0.12 **	0.10 *	*TG*
SBP	0.30**	0.15 **	0.07	-0.03	0.17 **	-0.16 **	0.04	*WC*
DBP	0.01	0.13 **	0.10*	0.16**	0.04	0.10 *	0.18 **	*HDL-C*

HDL-C, high density lipoprotein cholesterol; WC, waist circumference; TG, triglyceride; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; IDF, International Diabetes Federation. a Above diagonal (in italics) represents female correlation values.
*P<0.05, **P<0.01.

Table 7 represents gender specific heritability estimates and the genetic correlation of a trait between genders (RhoG), for all individual MetS traits. Inclusion of the sibship effect in this model was not appropriate (overmodelling). The lowest significant genetic correlation between genders (RhoG) was observed for WC and SBP (RhoG WC = 69.9 ± 14.0%, RhoG SBP = 61.5 ± 19.4%), whereas absolute genetic correlation between genders was observed for FPG and DBP (both RhoG = 100.0%). Both lipid traits, HDL-C and TG, showed moderate genetic correlation between genders (RhoG – HDL-C = 82.5 ± 12.6%, RhoG – TG = 85.5 ±17.5%)

Table 5: Heritability of IDF and NCEP ATPIII MetS definitions and its natural log transformed individual components in ERF population.

Parameter	**h2 (%)**[a]	**SEM**	***P*-value**[b]
n	**2488**		
IDF	14.3	4.8	$<10^{-06}$
ATPIII	19.2	5.6	$<10^{-06}$
ln WC	40.5	4.2	$<10^{-06}$
ln FPG	28.2	4.0	$<10^{-06}$
ln SBP	27.9	3.9	$<10^{-06}$
ln DBP	27.0	3.9	$<10^{-06}$
ln HDL-C	42.9	4.1	$<10^{-06}$
ln TG	30.8	3.9	$<10^{-06}$

IDF, International Diabetes Federation; NCEP ATPIII, National Cholesterol Education program
Adult Panel III; WC, waist circumference; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol TG, triglyceride. a Heritability based on polygenic model without consideration of the sibship effect (S), covariates: gender, age and inbreeding coefficient
b *P* values were derived using the likelihood ratio test.
*P<0.05, **P<0.01.

DISCUSSION

The prevalence of MetS in this Dutch genetic isolate according the IDF definition is 36.8% in males and 31.0% (*P* < 0.01) in females. According to the NECP ATPIII definition the prevalence of MetS was 26.7% in males and 22.8% (*P* < 0.02) in females. The two most important clusters of individual components, contributing to the manifestation of MetS (in both genders and both definitions) were [WC / HDL-C / BP] and [WC / HDL-C / TG / BP]. Another important cluster according the NECP ATPIII definition, in females was [HDL-C / TG / BP]. The heritability of MetS in our genetically isolated population was 10.6% ± 5.3% according to the IDF definition (p=0.07) and 13.2% ± 6.0% according to the NECP ATPIII definition (p<0.05). The heritability of the individual components ranged from 21.9% (DBP) to 42.9% (HDL-C). Low significant genetic correlations between genders were

Table 6: Heritability of IDF and ATP MetS definitions and its log normal transformed individual components in ERF population.

Parameter	h2 (%)[a]	SE	P-value[b]	s (%)[c]	SE	P-value[b]
n	2488					
IDF	10.6	5.3	< 0.05	9.05	6.2	0.07
ATPIII	13.2	6.0	0.07	15.89	7.4	< 0.05
ln WC	37.8	4.6	$<10^{-06}$	5.6	3.4	< 0.05
ln FPG	22.7	4.7	$<10^{-06}$	9.6	3.6	< 0.01
ln SBP	25.4	4.3	$<10^{-06}$	7.8	3.3	< 0.01
ln DBP	21.9	4.6	$<10^{-06}$	2.7	3.3	0.20
ln HDL-C	42.9	4.1	$<10^{-06}$	0.0	-	-
ln TG	28.3	4.4	$<10^{-06}$	4.1	3.3	0.10

IDF, International Diabetes Federation; NCEP ATPIII, National Cholesterol Education program Adult Panel III; WC, waist circumference; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol TG, triglyceride.a Heritability based on polygenic household model with consideration of the sibship effect (s), covariates: gender, age and inbreeding coefficient.
b *P* values were derived using the likelihood ratio test.
*P<0.05, **P<0.01.

observed for WC and SBP (RhoG: 69.9% and 61.5% respectively) Absolute genetic correlation between genders was observed for FPG and DBP (RhoG both 100.0). Moderate genetic correlation between genders, were observed for HDL-C and TG (RhoG: 82.2% and 85.5% respectively).

Although our genetically isolated population was not selected on disease phenotype, the population in general suffers from mild overweight (mean WC male = 94.2 ± 0.33 cm and mean WC female = 82.3 ± 0.32 cm) and mildly elevated blood pressures (mean SBP male = 143.6 ± 0.5 mm Hg, mean DBP male = 82.05 ± 0.3 mm Hg). It cannot be excluded that these phenotypic profiles are the consequence of enrichment of (rare) pathogenic alleles due to genetic drift associated with genetically isolated populations. Moreover, the ERF cohort is from a rural region, of which has been shown to be enriched in unfavorable metabolic profiles as compared to urban regions[11].

Relatively little information is available on the prevalence of MetS in Dutch populations. In a Dutch familial hypercholesterolemia (FH) patient population, a prevalence of 31% of MetS was found.

Table 7: Heritability of gender specific natural log transformed individual components in ERF population.

	h2 (%)[a]	SE	P[b]	h2 (%)[a]	SE	P[b]	RhoG[a]	SE	P[b]
	Female N =1397			Male N = 1091					
ln WC	53.7	6.6	$<10^{-06}$	37.6	8.2	$<10^{-05}$	69.9	14.0	0.03
ln FPG	34.4	6.5	$<10^{-06}$	25.4	6.1	$<10^{-04}$	100.0	-	-
ln SBP	42.9	6.6	$<10^{-10}$	20.3	7.5	< 0.01	61.5	19.4	0.05
ln DBP	42.6	6.7	$<10^{-09}$	14.7	4.6	< 0.01	100.0	-	-
ln HDL-C	53.0	7.1	$<10^{-13}$	42.0	7.7	$<10^{-07}$	82.2	12.6	0.16
ln TG	35.6	6.7	$<10^{-06}$	31.7	7.4	$<10^{-04}$	85.5	17.5	0.41

WC, waist circumference; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol TG, triglyceride.
a Heritability and RhoG (% shared genetics between genders) based on polymod model, RhoE (% shared environment between genders) forced to 0, covariates: age and inbreeding coefficient. b *P* values were derived using the X_2 test.

This study also reported, in a population suffering from CVD or other vascular disease, a prevalence of MetS ranging from 41-58%[12]. Data on two populations in the Southwest of Germany[11] showed a notably higher prevalence of MetS in rural as compared to urban populations, (urban 10%-15%, rural 20%-25%). Others[13] reported the prevalence of MetS in different ethnic groups. A Hispanic cohort (enriched for T2D) had a very high prevalence of MetS (73%). Overall, the prevalence of MetS in the Western world is estimated to vary between 10-35% (depending on age)[14,15,16]. Thus, the prevalence of MetS in the ERF population is relatively high. The higher prevalence of MetS according to the IDF definition as compared to the NCEP ATPIII definition is caused by the less stringent threshold values of the IDF definition. The significant difference in prevalence of MetS between genders is clearly a consequence of differences of prevalence in the first three age groups, < 30, 30-40 and 40-50 years old (figure 1). Women in the pre-menopausal period are apparently protected from MetS, as also reported by others[17].

In this study, we calculated the contributions to the total prevalence of MetS of all different combinations of individual components. The two most important contributors, for both genders, to the IDF MetS were [WC / HDL-C / BP] and [WC / HDL-C / TG / BP] (bold in table 3). The three most important contributions to the NCEP ATPIII MetS were [*male* WC / HDL-C / BP], [*female* HDL-C / TG / BP] and [*both genders* WC / HDL-C / TG / BP]. (bold in table 3). These findings were partly confirmed by Pearson correlation analysis of all traits, age included (table 4). In male IDF MetS subjects, a clear and significant correlation was found between HDL-C, TG and both BP components. In female IDF MetS subjects, HDL-C mainly correlated with WC and TG. The clustering of TG, HDL-C and WC is in line with previous findings[18,19,20]. The early metabolic syndrome definition was mainly determined by insulin resistance. Interestingly, FPG, is virtually absent in the most important clusters, contributing the most to MetS in our population. The result that FPG not only plays a minor role in MetS clusters, but also shows a relative moderate heritability estimate (22.7 %) in comparison with for example HDL-C, is in concert with findings of others. Apparently the environmental contribution to alterations in FPG seems to be prominent.

A heritability of 24% for MetS (NCEP ATPIII) in a Caribbean-Hispanic population has been reported by Lin *et al*[21]. Factor analysis in this study suggested two clusters of components: A lipid / obesity / plasma glucose cluster and a second blood pressure cluster. The first cluster showed the highest heritability (44%). Bayoumi *et al* reported a heritability of MetS (binary trait) in healthy Omani Arab families (N=1277) of 38%. Individual components as HDL-C and BMI showed the highest heritability in these families[22].

The heritability of MetS in our genetic isolated population was moderate (IDF = 10.6% $\pm$ 5.3%, NCEP ATPIII = 13.2% $\pm$ 6.0%) in comparison with data of others. However, our estimates of heritability of MetS were based on a complex pedigree structure, far more complex than a family pedigree. This complex pedigree structure allowed us to correct for the sibship effect, which should be a standard procedure to avoid substantial inflation of the heritability estimate due to early childhood environment (household-effect) and dominant genetic factors.

In the present study, the heritability estimations for the individual components of MetS were based on quantitative values. The heritability estimation found for individual components of MetS, ranged from 21.9% $\pm$ 4.6% for the DBP component to 42.9% $\pm$ 4.1% for the HDL-C component. Interestingly, we did not find evidence for a contribution of the sibship effect to this trait, which suggests a dominant role for strong genetic factors causing variation in plasma HDL-C values. The heritability of our MetS individual traits correspond well with findings of others in general populations (Omani Arab families)

and genetically isolated populations as the Hutterite population and Sardinian population. The Sardinian population is a large genetically isolated population which has already been useful in the identification of genes involved in several Mendelian disorders. Pilia *et al* found that, in a Sardinian cohort, the heritability of the MetS individual traits: fasted serum glucose, HDL-C, TG and WC was 31.8%, 47.1%, 29.6%, and 31.2% respectively[23]. The Hutterite population lives on nine communal farms (colonies) in South Dakota, USA and involves participants which are descendants of only 64 Hutterite ancestors who were born in the mid-eighteenth century. Abney *et al*[24] and Ober *et al*[25], both reported on the heritability in the Hutturite population. They found heritabilities of 37% for plasma TG, 45% for SBP and 21% for DBP.

Gender specific genetic architecture (heritability) of several individual MetS traits and several MetS related traits (such as plasma insulin, BMI and low density lipoprotein cholesterol, LDL-C) was reported by Weiss *et al*[9] and Pan *et al*[10] in the genetically isolated Hutterite population. The latter concluded that the individual MetS traits SBP and TG showed significant X chromosome effects, HDL-C showed a non-X chromosome gender effect and DBP showed no significant gender effects. The majority of our findings are in concert with these conclusions of Pan *et al*. Gender specific genetic architecture (genetic correlation or Rho G between genders) was found for the individual MetS traits WC, SBP, HDL-C, and TG whereas for FPG and DBP no evidence was found for differences in the genetic architecture between genders (RhoG = 100%).

Our findings suggest that genetic dissection of MetS as compound syndrome has a low power and should be approached by studying the genetics of the individual traits of MetS[21]. However, the observation that a limited set of clustered risk factors are the predominant contributors to MetS indicates that these clusters may have a common genetic origin. The power of genetic dissection using limited sets of risk factors is compromised by the limited number of subjects in the sub-selection. Alternatively, the interaction between clustered components within MetS can be studied by means of an epistasis model to study gene-gene interactions in the whole population[26].

Difference in the genetic architecture between genders is striking. Our findings and findings of others[9,10] indicate that these differences seem to be common in quantitative traits. Four of six of our MetS individual traits: WC, SBP, plasma HDL-C and plasma TG, show large to moderate differences in genetic architecture between genders. These findings indicate that analyses for novel loci should imply gender specific analysis

In conclusion, the present study on the prevalence and heritability of MetS and the individual components in a Dutch genetically isolated population provides a good basis for future genetic analyses to identify genetic risk factors for individual components contributing to MetS.

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Plasma apoAV levels are markedly elevated in severe hypertriglyceridemia and positively correlated with the APOA5 S19W polymorphism

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ABSTRACT

Objective: The recently discovered apoAV is hypothesized to affect triglyceride metabolism by stimulating the lipolysis of triglycerides in VLDL and chylomicrons. We set out to determine the association between increased serum TG levels, plasma apoAV levels, and polymorphism of the *APOA5* gene, with specific emphasis on the *APOA5* S19W variation. This mutation alters the endoplasmic reticulum signal peptide and is hypothesized to impair apoAV secretion into the circulation.

Methods and Results: Two haplotype-tagging *APOA5* polymorphisms, *APOA5* S19W and *APOA5* –1131T>C and plasma apoAV levels were determined in a previously characterized population of patients with severe hypertriglyceridemia (HTG). As compared to a random control population, the allele frequencies of the *APOA5* S19W and –1131T>C rare variants were significantly increased in HTG patients. Furthermore, the HTG population exhibited markedly elevated plasma apoAV levels that were positively correlated with serum TG levels. Plasma apoAV levels were positively correlated with occurrence of the *APOA5* S19W rare variant.

Conclusions: The increased allele frequencies of the *APOA5* S19W and –1131T>C rare variants in the HTG population are in agreement with previous reports. Our data show a positive correlation between apoAV and TG levels. Moreover the finding of a positive association between apoAV levels and the *APOA5* S19W rare variant is in disagreement with the hypothesis that this variant is poorly secreted.

INTRODUCTION

The recently identified apolipoprotein (apo) AV[1,2] has been shown to affect triglyceride (TG) levels in mouse models. Serum TG concentrations in mice were 4-fold increased upon deficiency of the endogenous *APOA5* gene and were decreased by 65% by overexpression of the human *APOA5* gene [1]. Adenovirus-mediated transfer of murine *APOA5* to mice resulted in a dose-dependent reduction of serum TG[3,4]. Thus, in these animal model studies, plasma apoAV levels are negatively correlated with serum TG levels.

In a large number of human populations, polymorphisms in the *APOA5* gene have been associated with variation in serum TG level (for reviews see[5-7]). Moreover, deficiency for apoAV has been associated with HTG[8-10], confirming the TG-lowering activity of apoAV. The *APOA5* gene is localized in a gene cluster containing *APOC3*, *APOA1* and *APOA4*, characterized by a high level of linkage disequilibrium. Single nucleotide polymorphisms (SNPs) in the *APOA5* gene fall into three common haplotypes: *APOA5**1, with common alleles at all sites; *APOA5**2, with rare alleles of -1131T>C, -3A>G, 751G>T, and 1891T>C; and *APOA5**3, distinguished by c.56C>G (S19W)[11,12].

ApoAV is a 39 kDa protein (343 amino acids) that may enhance LPL mediated TG hydrolysis, most likely in concert with heparin sulphate proteoglycans[3,13,14]. The *APOA5* S19W mutation is located in the endoplasmic reticulum signal peptide and is hypothesized to decrease the apoAV secretion rate [1]. Recent studies using a secreted alkaline phosphatase as reporter protein for signal peptide function are in agreement with this hypothesis[15].

In the current study, we have screened a severely hypertriglyceridemic population for the frequency of the most common *APOA5* haplotype tagging SNP's, -1131T>C for *APOA5**2 and S19W for *APOA5**3. In addition, we have determined the plasma apoAV levels and correlated these levels with serum TG levels and *APOA5* haplotype tagging SNPs. We find an increased frequency of the *APOA5* –1131C>T

and APOA5 S19W rare variants in the HTG population and markedly elevated plasma apoAV levels. Moreover, the apoAV levels are positively associated with serum TG levels. These data do not support the previously reported negative association between plasma apoAV and TG levels[16,17] and presumed secretion defect of the APOA5 S19W rare variant.

MATERIALS AND METHODS

PATIENT AND CONTROL POPULATION RECRUITMENT

Hypertriglyceridemic subjects were recruited from our lipid clinic meeting the following criteria: serum triglyceride >3.8 mmol/L, VLDL-cholesterol >1 mmol/l, LDL-cholesterol < 4.5 mmol/L and no apoE2/E2 phenotype[18,19]. The diagnosis was based on the means of two fasting blood samples obtained after a step one diet of the NCEP for at least 8 weeks. Patients with renal-, liver- or thyroid disease and/or alcohol consumption >40g/day were excluded. 29 (21%, N=139) HTG patients had type II diabetes. The random control panel include a total of 175 anonymous subjects. Data on age, body mass index and lipid levels of random control subjects were not available. The random control population consisted of 100 anonymous blood donors, blood supplied by Sanquin (Leiden, the Netherlands) and 75 anonymous subjects of a population-based study among 2018 randomly selected 35-year-old men[20] (Table 1). Informed consent was given by each participant and the study was approved by the Medical Ethics Committee of the LUMC. All blood samples were collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Lipids and lipoproteins were measured after ultracentrifugation using standard methods[21].

GENETIC ANALYSIS

Identification of the polymorphisms was performed using PCR followed by restriction enzyme analysis. The following SNPs were analyzed: APOA5 c.56 C>G (S19W) and APOA5 –1131T>C (SNP3)[12,22]. For amplification of the APOA5 S19W fragment (c.56 C>G) the following primers were used: 5'-CCA GAA GCC TTT CCG TGC CTG GGC GGC-3' (sense) and 5'-TGT AAA ACG GCC AGT - AAA AGG AAA A-CGG CCG-GTG CTC ACC TGG GCT GCT CTT C-3' (antisense). The penultimate base in the forward primer (sense) was changed to a C to create a Eco521 recognition site in the common allele. This recognition site was also introduced in the reverse primer (antisense), which served as a digestion control. To establish an optimal visualization, the 3' primer also was elongated with a 5' additional M13 tail. Fragments of 24, 156 and 27 bp represented the Ser-19 allele and the fragments 24 and 184 bp represented the Trp-19 allele.

The primers for amplification of the APOA5 -1131T>C SNP were as follows: 5'-CCC CAG GAA CTG GAG CGA AAT T-3' (sense) and 5'-TGT AAA ACG GCC AGT AAA AGG AAA AGG AAA AGG TTA AGA TTG ATT CAA GAT GCA TTT AGG AC-3' (anti-sense). The penultimate base in the forward primer (sense) was changed to a T to create a Tru1 recognition site in the common allele. This recognition site was also introduced in the reverse (antisense) primer, which served as a digestion control. To establish an optimal visualization, the 3' primer also was elongated with a 5' additional M13 tail. Fragments of 21, 168 and 36 bp represented the –1131T allele and the fragments 36 and 189 bp represented the –1131C allele.

ANALYSIS OF PLASMA APOAV LEVELS

Total plasma apoAV levels were measured by means of a sandwich ELISA assay [23]. A subset of the population, representing individuals from all tertiles with respect to serum TG and apoAV levels (as determined by ELISA) was analyzed by semi-quantitative Western blot analyses as previously described[2].

STATISTICAL ANALYSIS

For both polymorphisms and haplotypes, the Hardy-Weiberg equilibrium was calculated using the gene-counting method and differences were assessed by chi-squared analysis. *P*-values lower than 0.05 were considered as significant differences. Differences in sex were evaluated with Chi-squared analysis. Data in table 1 are expressed as mean ± SD. Data in figure 1,2 and 3 are expressed as mean ± S.E.M. Since total TG and apoAV levels showed non-Gaussian distributions, the mean differences of these parameters between groups were calculated by means of Mann-Whitney analysis. Association analysis between the TG concentration and apoAV concentration was performed by subdividing TG levels in quartiles (3.80-6.31, 6.53-9.42, 9.45-15.71 and 16.64-82.14 mmol/L resp.) All statistical analyses were performed with SPSS for windows 11.0.1 (SPSS, Chicago, IL, release November 2001).

RESULTS

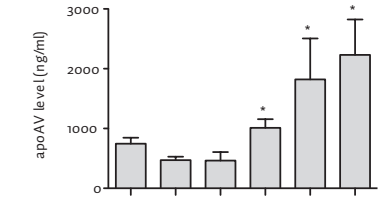
FREQUENCY OF APOA5 HAPLOTYPE-TAGGING SNP'S IN HTG AND CONTROL POPULATION

The occurrence of 2 common haplotype-tagging APOA5 SNPs, APOA5 -1131 T>C (haplotype APOA5*2) and APOA5 c.56C>G (S19W, haplotype APOA5*3) were determined in 141 patients with severe HTG and 175 control individuals (random control panel) (for population characteristics, see Table 1). Allele frequencies for both polymorphisms in the HTG and random control subjects were in Hardy-Weinberg equilibrium. Allele frequency of the rare APOA5 -1131 T>C SNP is 5.9% in the control population versus 23.5% in the HTG population ($P<0.05$). Similarly, allele frequency of the rare variant of the APOA5 S19W is 4.4 % in the control population versus 19.1 % in the HTG population ($P<0.05$). Thus, both the APOA5 –1131T>C and APOA5 S19W rare variants are significantly increased in the HTG population.

Table 1: Characteristics of patients with hypertriglyceridenia and controls

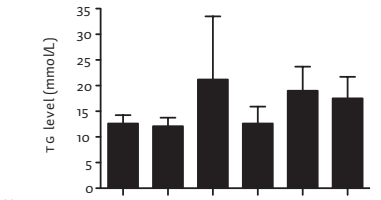
	HTG	N	RCP	N
Male	119 (84.4 %)	141	171 (97.7 %)	175
Female	22 (15.6 %)	141	4 (2.3 %)	175
Age	53.9 ± 10.7	141		
range	26-78			
BMI (kg/m2)	27.7 ± 3.4	141		
Total triglycerides (mmol/L)	14.3 ± 13.8	141		
VLDL-triglycerides (mmol/L)	12.0 ± 12.3	132		
apoAV (ng/ml)	965.2 ± 1391.8	129		
Total cholesterol (mmol/L)	8.8 ± 3.9	141		
HDL-C (mmol/L)	0.68 ± 0.20	137		
VLDL-C (mmol/L)	5.0 ± 4.1	132		
LDL-C (mmol/L)	2.8± 1.5	132		
HOMA index	14.0 ± 16.7	135		

RCP indicates random control panel; N indicates total number of subjects with available data about respective parameter; Age, body mass index, lipid levels, HOMA index and apoAV levels are presented as mean ± SD



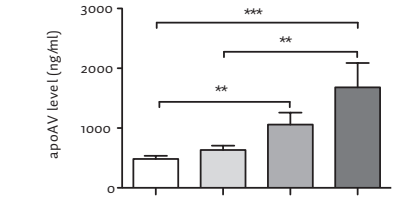
N	38	27	5	9	19	7
S19W	11	11	11	12	12	22
-1131 T>C	11	12	22	12	11	11
APOA5 haplotype	1+1	1+2	2+2	2+3	1+3	3+3

*Fig.1. Association between the APOA5 S19W and APOA5 –1131T>C polymorphisms and plasma apoAV level in HTG patients. ApoAV concentration is expressed as mean ±S.E.M. Data are shown for all individuals characterized for the parameters involved. Differences between non-carriers and carriers of the rare variants were evaluated with Mann-Whitney analysis. *P < 0.05 as compared with S19W 11/–1131 T>C 11; S19W 11/–1131 T>C 12; S19W 11/–1131 T>C 22. †Haplotype definitions as described by others [6,12,26,28].*



N	41	31	6	11	20	7
S19W	11	11	11	12	12	22
-1131 T>C	11	12	22	12	11	11
APOA5 haplotype[a]	1+1	1+2	2+2	2+3	1+3	3+3

Fig.2. Association between the APOA5 S19W and APOA5–1131T>C polymorphisms and serum TG level in HTG patients. Triglyceride concentration is expressed as mean ±S.E.M. Data are shown for all individuals characterized for the parameters involved. Differences between non-carriers and carriers of the rare variants were evaluated with Mann-Whitney analysis. ÜHaplotype definitions as described previously [6,12,26,28].



N	33	32	31	33
TG range (mmol/L)	3.80-6.31	6.53-9.42	9.45-15.71	>16.64

*Fig.3. Association between the serum TG concentration and plasma apoAV concentration in HTG patients. The HTG population was divided in quartiles based on TG concentration. ApoAV levels are expressed as mean ±S.E.M. Data are shown for all individuals characterized for the parameters involved. Differences between groups were evaluated with Mann-Whitney analysis. Significant differences are expressed as: *P < 0.05; **P < 0.01; ***P < 0.001.*

ASSOCIATION BETWEEN PLASMA APOAV, TG LEVELS AND APOA5 POLYMORPHISMS

Plasma apoAV levels in the HTG population were determined by sandwich ELISA. ApoAV levels in a selected set of individual sera were confirmed by an independent ELISA and also by semi-quantitative Western Blot analyses (data not shown). All replicate data were in near perfect concordance. Average plasma apoAV concentration in the HTG population was 0.97 ± 1.39 ug/ml, which is 4 to 5–fold increased as compared to reported apoAV levels in normal individuals[16,17,23].

Twenty HTG patients were taking antihypertensive medication. The apoAV levels were not significantly different between the treated and untreated groups of patients. Association studies were performed between APOA5 polymorphic variants and plasma apoAV and TG levels. Figure 1 shows that there is a significant positive correlation between presence of the APOA5 S19W rare variant and plasma APOA5 levels ($P<0.05$). No association was found between presence of the APOA5 –1131T>C rare variant and plasma APOA5 levels.

Neither the APOA5 S19W nor the APOA5 –1131T>C rare variants were correlated with serum TG levels (Fig. 2). The plasma apoAV levels were positively correlated with serum TG levels. The apoAV level in the lowest quartile of serum TG level (up to 6.31 mmol/L) is significantly different from the apoAV levels in all subsequent quartiles of serum TG level (Fig. 3).

DISCUSSION

Here, we show that the frequencies of the APOA5 S19W and -1131T>C variant allele carriers are about four times higher in patients with severe hypertriglyceridemia than in the control population, indicative for a causal association of the APOA1/C3/A4/A5 locus with this complex lipid disorder. Plasma apoAV levels are approximately 5-fold increased as compared to normal controls[16] and positively correlated with serum TG levels and the APOA5 S19W rare variant. The increased frequencies of the APOA5 S19W and -1131T>C rare variants in the HTG population are in line with previous reports, that have demonstrated a positive correlation between both these variants and TG levels in

various populations[24-27] (for reviews see [5-7]). Somewhat surprisingly, within the HTG population itself, the correlation between TG levels and presence of the APOA5 S19W or APOA5 -1131C>T rare variants was not significant (Fig. 2). However, since a trend seems present, this may be a power problem due to the size of the population. In addition, the lack of correlation may be caused by the selection criteria used for inclusion of patients in the HTG population. Half of the HTG population is characterized by TG levels of more than 9 mmol/L (upper two quartiles of the population (Fig. 3). It is more than likely that especially these quite extreme HTG patients will have mutations in additional genes causing the high TG levels that will be dominant over the effects associated with the APOA5 variants.

The increased average apoAV levels in the presence of HTG and the positive correlation between apoAV levels and TG levels indicate that, at least in these patients, the presumed LPL-stimulatory activity of apoAV does not normalize circulating TG levels. This observation contrasts with a study in Japanese indivuals that demonstrated a negative correlation between plasma apoAV and serum TG levels[16] and with two other independent studies which both found that apoAV deficiency is associated with HTG in humans[8,9]. Interestingly, individuals that are heterozygous for apoAV deficiency have a highly variable phenotype[8,9], indicating that additional factors may be necessary for the expression of apoAV-associated hyperlipidemia. The observation that the phenotypes of apoAV overexpression and apoAV-deficiency in mice are so clear cut, could indicate that these factors are skewed in a species-specific manner as to emphasize the role of apoAV. The identity and role of these putative modifiers remain to be established.

Several explanations could be invoked to explain the positive correlation between apoAV and TG levels and some of these may be affected by species-specific modifiers. *First*, the disturbance in TG metabolism in a large fraction of the HTG patients is dominant over the presumed apoAV TG-lowering effect. For example, if more potent inhibitors of lipolysis are up-regulated. It is possible that apoAV may actually even be up-regulated as a consequence of the HTG, but to no avail. Analysis of the production and turn-over rate of apoAV should clarify this issue. *Second*, the increased plasma levels of apoAV are a consequence of the altered lipoprotein structure present in HTG. If apoAV has for example a higher affinity for large-sized TG-rich particles, the observed increase in plasma apoAV levels is a symptom of HTG[23]. *Third*, apoAV could be up-regulated as an indirect consequence of HTG. It is conceivable that apoAV may have an as yet unknown function in liver homeostasis. For example, it is unknown what the mechanism is underlying the up-regulation of APOA5 expression after partial hepatectomy[2]. It should be noted that plasma apoAV levels did not correlate with SAA levels and thus presumably not with liver dysfunction per se (data not shown). Independent of the explanation underlying the increased apoAV levels in HTG patients, it is obvious that in the majority of individuals of this HTG population, the TG-lowering effect of apoAV is not sufficient to normalize TG levels.

The analyses of APOA5 variant allele frequencies and correlations with apoAV and TG level were performed under the assumption that the APOA5 S19W and APOA5 -1131T>C rare variants are allele-tagging variants. Although we have not performed a haplotype analysis in the HTG population, this assumption seems reasonable since the APOA5 haplotypes are in Hardy-Weinberg equilibrium in the HTG population. Moreover, the haplotype structure has been confirmed in numerous population studies[6,12,26,28]. Since we have not studied other polymorphic sites in APOA1/C3/A4/A5 locus, we cannot draw conclusions on the causality between the presence of the APOA5 S19W and APOA5 -1131T>C rare variants and HTG or apoAV level. However, it is of interest to note that the APOA5 S19W variant allele is characterized by presence of the most common (wild type) variants of other SNP's in the APOA1/C3/A4/A5 locus[12,26,28]. The APOA5 S19W variation occurs in the signal peptide for co-translational transport

into the endoplasmic reticulum. Computer modeling of this variation indicated a conformational change and *in vitro* analysis using a secreted alkaline phosphatase as reporter demonstrated that the variant signal peptide resulted in 50% less efficiently secretion as compared to the wild type form [15]. Therefore, the positive correlation between the presence of the APOA5 S19W rare allele and serum apoAV levels is unexpected.

This study was designed to test the hypothesis that apoAV levels are negatively correlated with HTG. The positive correlation between plasma apoAV levels and TG levels on the one hand and apoAV levels and the APOA5 S19W rare variant on the other hand provide evidence against (1) a dominant role for apoAV in reducing serum TG levels in HTG patients and, (2) a secretion defect associated with the APOA5 S19W variant. These observations illustrate that the postulated role of apoAV as dominant gene determining serum TG levels in humans may require adjustment.

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Chapter 4

Estrogen induced hypertriglyceridemia in an apolipoprotein AV deficient patient

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INTRODUCTION

Occasionally severe hypertriglyceridemia (HTG) may develop or exacerbate in females during pregnancy or use of exogenous estrogens[1-3]. Estrogens might increase production of triglyceride-rich very low density lipoproteins (VLDL) by the liver impair lipolysis of triglycerides through reduction of the concentration of lipoprotein lipase and hepatic lipase[4,5]. These effects might be induced as a result of a decrease of insulin sensitivity by estrogens[6,7].

Recently, a new apolipoprotein designated AV (apoAV) was discovered which is associated with VLDL-production in the liver and might stimulate lipoprotein lipase (LPL) in hydrolyzing triglycerides in fatty acids[8]. *APOA5* is closely linked to a well-studied apolipoprotein cluster located on chromosome 11q23, which involves the genes *APOA4*, *APOC3* and *APOA1*. ApoCIII plays an important role as inhibitor of lipoprotein lipase (LPL) in hydrolyzing triglycerides in fatty acids[8]. Several reports have shown that deficiency for apolipoprotein AV (apoAV) in humans is associated with HTG[9,10].

METHODS AND RESULTS

Here, we report a patient deficient for apoAV, yet having a variable TG phenotype. The study was approved by the medical ethical committee of our institution. A healthy 31-year old woman was referred to our Lipid clinic for HTG, detected by routine medical examination. She had no physical complaints, rarely consumed alcohol and used an oral anticonceptive, ethinylestradiol/desogestrel, for more than four years. No abnormalities were detected by physical examination. Her body mass index (BMI) was 23 kg/ m^2.

Laboratory examination showed HTG: plasma-TG 19.4 mmol/L, elevated VLDL-cholesterol and VLDL-TG and low HDL-cholesterol (data not shown). Her apoE phenotype was E3E2. The apoAV, determined by ELISA[11,12], was not detectable. She was advised to adhere to a diet low in fat (National Cholesterol Education Program 2). During the diet, her plasma TG did not normalize (6.0 – 13.3 mmol/L). However, after she stopped with the oral anticonceptive medication her plasma TG normalized 1.8 mmol/L. During her pregnancy, plasma TG increased to 54.0 mmol/L and normalized after delivery (Figure 1). Since then, plasma TG have remained normal (data not shown). The plasma apoCIII levels determined by ELISA[11,12] decreased from 37.5 mg/dl in the presence of HTG to 9.3 mg/dl in the normo-triglyceridemic state (Table 1).

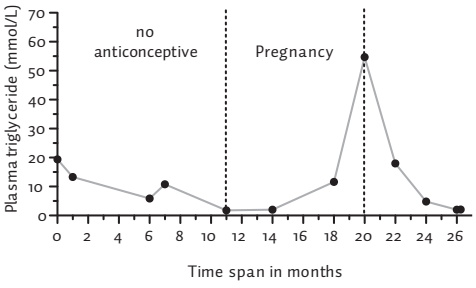

Fig.1. The variable plasma triglyceride phenotype of the apoAV deficient patient over a time span of 26 months; stop intake oral anticonceptive at 0 months, start pregnancy at 11 months, delivery at 20 months.

Sequence analysis revealed that the patient was homozygous for a novel mutation in the *APOA5* gene: c.161 + 5G (guanine) >C (cytosine). Computational splice site analysis[13] indicated that the c. 161 +5G>C variant severely decreased its binding capacity as donor splice site in intron 3 (SC35 weight matrix; fold change = -3.2), which suggests a splicing defect. Both parents of the apoAV-null patient were heterozygous for the same variant. Her parents did not show any alterations in plasma lipid levels nor plasma apoAV and apoCIII levels (Table 1), except for a mild combined hyperlipidemia (T-cholesterol

Table 1: Single nucleotide polymorphism (SNP) data of proband and parents

SNP	Mother	Father	Proband
APOA5 c.161 + 5G>C	1/2	1/2	2/2
APOA5 S19W	1/1	1/1	1/1
APOA5 SNP3	1/2	1/2	2/2
APOC3 Sst-1	1/2	1/2	2/2

1: common allele, 2: rare allele.

6.7 mmol/L and TG 3.9 mmol/L) of the obese (BMI 30.2 kg/m^2) mother of the proband.

The following additional SNPs in the APOA5-APOC3 locus were analyzed in proband and parents: APOA5 S19W (rs3135506), APOA5 SNP3 (rs662799) and APOC3 Sst-1(rs5128), using PCR followed by restriction enzyme analysis[14]. Interestingly, the c.161 + 5G>C variant is linked to the rare alleles of the APOA5 SNP3 and APOC3 Sst-1 variants and the patient is thus homozygous for both rare variants (Table 1). These data indicate that apoAV-deficiency alone was not sufficient for the induction of HTG in this patient. Exogenous estrogens and hyperestrogenemia of pregnancy may be the additional factor causing HTG in the presence of genetic susceptibility.

DISCUSSION

Whether the genetic susceptibility in the patient is defined by the APOA5 splice mutation (and associated apoAV-deficiency) or the presence of the linked variants APOA5 SNP3 and/or APOC3 Sst-1 cannot be concluded from these analyses. However, it is intriguing to note that many of the patients with null-mutations in APOA5 described to date, are carriers of TG-raising alleles in the APOA5-APOC3 gene locus (reviewed by Talmud[15]).

Thus, the patient described here confirms the notion that expression of HTG in apoAV-deficiency requires the presence of additional genetic and/or environmental factors.

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Chapter 5

The expression of type III hyperlipoproteinemia: involvement of lipolysis genes

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ABSTRACT

Type III hyperlipoproteinemia (HLP) is mainly found in homozygous apolipoprotein (APO) E2 (R158C) carriers. Genetic factors contributing to the expression of type III HLP were investigated in 113 hyper- and 52 normolipidemic E2/E2 subjects, by testing for polymorphisms in *APOC3*, *APOA5*, *HL* and *LPL* genes. In addition 188 normolipidemic Dutch controls (NDCP) and 141 hypertriglyceridemic (HTG) patients were genotyped as well.

No associations were found for four *HL* gene polymorphisms and two *LPL* gene polymorphisms and type III HLP. The frequency of the rare allele of *APOC3* 3238 G>C and *APOA5* -1131 T>C (in linkage disequilibrium) was significantly higher in type III HLP patients compared with normolipidemic E2/E2 subjects, 15.6% *vs.* 6.9% and 15.1% *vs.* 5.8% respectively ($P<0.05$). Furthermore, the frequencies of *APOA5* c.56 G>C polymorphism and *LPL* c.27 G>A mutation were higher in type III HLP patients, though not significant. Some 58% of the type III HLP patients carried either the *APOA5* -1131 T>C, c.56 G>C and/or *LPL* c. 27 G>A mutation as compared to 27% of the normolipidemic APOE2/2 subjects (odds ratio 3.7, 95% confidence = 1.8 – 7.5, $P<0.0001$). The HTG patients showed similar allele frequencies of the *APOA5*, *APOC3* and *LPL* polymorphisms, whereas the NDCP showed similar allele frequencies as the normolipidemic APOE2/2. Patients with the *APOC3* 3238G>C / *APOA5* -1131 T>C polymorphism showed a more severe hyperlipidemia than patients without this polymorphism.
Polymorphisms in lipolysis genes associate with the expression and severity of type III HLP in APOE2/2.

INTRODUCTION

Patients with type III hyperlipoproteinemia (HLP) are characterized by elevated levels of total cholesterol and triglycerides due to high plasma levels of chylomicron and very low density lipoproteins (VLDL) remnants enriched in cholesterol esters and APOE.[1,2]

APOE, a major constituent of chylomicron and VLDL remnants, serves as a ligand for the receptor-mediated uptake of these particles by the liver.[3] In type III HLP, *APOE* mutations lead to an impaired clearance of remnant lipoproteins by hepatic lipoprotein receptors. There are three common genetic variants of APOE: APOE2 (Arg158 →Cys), APOE3 (Cys112; Arg158) and APOE4 (Cys112 → Arg). These isoforms are encoded by three co-dominant alleles that are located at one single gene locus on chromosome 19. In comparison to the other two isoforms, APOE2 has less than 1% binding capacity for the hepatic low density lipoprotein receptor (LDLR).[4] Most type III HLP patients (> 90%) are homozygous carriers of APOE2 (Arg158 →Cys).[4] In Caucasian populations, APOE2 homozygosity occurs with a frequency of about 1%, whereas the frequency of type III HLP is about 1 to 7 per 5000.[5,6] A minority of the APOE2 homozygous subjects will develop type III HLP, indicating that type III HLP is a multifactorial disorder requiring additional genetic and environmental factors for its clinical manifestation.[4,7,8] It has been suggested that contributors to the expression of type III HLP include factors causing (1) an overproduction of lipoproteins, (2) an impaired lipolysis of lipoproteins or (3) an impaired hepatic uptake of remnants.[4,9] Insulin resistance is associated with high triglyceride levels caused by an increased VLDL production.[10,11] Earlier we found an association of high insulin levels with the expression of type III HLP.[6,12]

Several groups studied genetics factors that may contribute to the expression of type III HLP. [13-16] However, in the present study we have studied a larger type III HLP cohort. In addition, we used a

normolipidemic APOE2 homozygote cohort as control group. Mutations in genes involved in lipolytic conversion, such as *LPL* (lipiprotein lipase), *HL* (hepatic lipase) and *APOC3* have been associated with hyperlipidemia (for reviews see references).[17-20] In addition, Zhang *et al*.[13] observed an increased allele frequency for the *LPL* N291S mutation in type III HLP patients when compared with the general population. SNPs in the *APOA5* gene (11q23) were found to be strongly associated with plasma triglyceride levels.[21,22]

Data from family studies on type III HLP indicate that one or more genes are possible additional genetic factors predisposing to type III HLP.[4] However, from these studies it was not evident which additional genes were involved or the study population was very small. In the present study, a significant population of both normolipidemic and hyperlipidemic E2/2 subjects was collected to determine additional genetic risk factors contributing to the expression of type III HLP in APOE2 homozygotes. For comparison, these genetic risk factors were also typed in hypertriglyceridemic (HTG) patients [23,24] - they partly match their elevated VLDL/TG phenotype with type III HLP patients – and a normolipidemic Dutch control panel (NDCP).

METHODS

SUBJECTS

The study population consisted of 167 unrelated homozygous carriers of APOE2 (Arg158 →Cys). Type III HLP patients were defined as having total cholesterol and triglyceride levels ≥ 90th percentile, VLDL cholesterol/triglyceride ratio > 0.3 (mg.dL^{-1}/ mg/dL) and/or VLDL cholesterol/VLDL triglyceride ratio of > 0.8 (mmol/L/mmol/L) whereas normolipidemic E2/2 subjects had total cholesterol and triglyceride levels < 90th percentile according to the age- and sex-related percentile levels of the Prospective Cardiovascular Münster Study (PROCAM).[25] Ultracentrifuge data were not available from 24 HLP type III patients. Two HLP APOE2 homozygotes were excluded from the analysis because their plasma triglyceride levels were normal.

In total, 102 type III HLP patients and 9 normolipidemic E2/2 subjects were recruited from the outpatient Lipid Clinics of the University Medical Centers of Leiden, Nijmegen, Amsterdam and Rotterdam. Ten unrelated carriers (two type III HLP patients and eight normolipidemic E2/2 subjects) were detected during a population-based study among 2018 randomly selected 35-year-old men.[26] Forty-four E2/2 subjects (9 type III HLP patients and 35 normolipidemic E2/2 subjects) were collected from the Rotterdam Study, a prospective cohort study of 6870 healthy participants aged 55 years and older, investigating determinants of chronic diseases .[27]

In this study we included 113 type III HLP patients and 52 normolipidemic E2/2 subjects. Clinical data and blood samples from type III HLP patients were collected before lipid-lowering medication. One normolipidemic E2/2 subject and one type III HLP patient with a history of pancreatitis were not included in the study.

The selection of the 141 hypertriglyceridemic (HTG) patients was described earlier.[23] The 188 normolipidemic Dutch controls (NDCP) were selected on APOE genotype, and total plasma cholesterol, triglycerides and HDL levels, between the 25th and 75th percentile. Informed consent was given by each participant and the study was approved by the Ethics Committee of our hospital.

Vascular disease was defined as the presence of coronary artery disease (angina pectoris, 70% stenosis on coronary arteriography, myocardial infarction, coronary by-pass or percutaneous

transluminal coronary angioplasty) and/or cerebrovascular disease (stroke or transient ischaemic attack) and/or peripheral vascular disease. Hypertension was defined as systolic blood pressure of ≥ 160 mm Hg, or diastolic blood pressure of ≥ 90 mm Hg, or the use of medication for hypertension.[28] Less than 20% of the Type III HLP patients were treated with antihypertensive medication: 11% with a bètablocker and/or diuretic and 8% with other antihypertensive drugs. About 6% of the normolipidemic subjects were treated with antihypertensive medication. Four patients received anti-diabetic medication.

Hyperinsulinemia was defined as fasting insulin concentrations ≥ 100 pmol/L. The diagnostic criterion for diabetes mellitus was fasting blood glucose ≥ 7 mmol/L. Smoking was defined as the consumption of at least ten cigarettes per day. The non-smokers also included ex-smokers, who stopped smoking for at least one year. Alcohol consumers were defined as subjects with alcohol consumption of two or more grams per day.

APOE PHENOTYPING AND GENOTYPING

APOE phenotypes were determined by isoelectric focusing of delipidated serum samples and after cysteamine treatment followed by immunoblotting with a polyclonal anti-APOE antiserum as described.[29] The results were confirmed by *APOE* genotyping.[30]

LIPID AND LIPOPROTEIN ANALYSIS

With the exception of the sampling of 38 E2/2 subjects originating from the Rotterdam Study, all blood samples were collected after an overnight fast. Serum was obtained after centrifugation at 1500 *g* for 15 min at room temperature. Total serum cholesterol and triglyceride levels were measured enzymatically, using commercially available kits. Serum HDL-C concentration was measured after precipitation of VLDL and LDL with phosphotungstic acid and $MgCl_2$.[31] Three milliliters of serum was ultracentrifuged for 15 hours at 232000 *g* at 15°C in a TL-100 tabletop ultracentrifuge. The ultracentrifugate was divided in a density (d) < 1.006 and 1.006 < d < 1.25 g/mL fraction, designated as the VLDL and IDL+LDL-HDL fraction, respectively.

SERUM INSULIN AND GLUCOSE MEASUREMENTS

Insulin and glucose concentrations were only measured in fasting blood samples. The insulin concentration was determined by a radioimmunoassay (Ins-Ria-100, Medgenix). The antibody of this assay cross-reacts with proinsulin (40%) but not with C-peptide (< 0.001%).[32] Serum glucose was determined by the automated hexokinase method of Hitachi 747, Boehringer Mannheim-Hitachi.

DNA ANALYSES

Genomic DNA was isolated from leucocytes according to Miller *et al.*[33] The following mutations or polymorphisms were indentified with PCR followed by restriction enzyme analysis as previously described: The 3238 C>G (Sst-1; rs5128) [34] polymorphism in 3'-UTR of exon 4 of the *APOC3* gene, *LPL* c.27 G>A (D9N; rs1801177), *LPL* c.1342 C>G (S447X; rs328) [35], *LPL* c.874 A>G (N291S; rs268) [13], *HL* c.219 G>A (V73M; rs6078), *HL* –480 C>T (rs8192701) [36], *HL* c.1005 A>G (L334F; rs3829462) [37] and *HL* c.609 C>G

(T202T; rs6084) [38] in respectively the *LPL* and *HL* gene. Analysis of both the *APOA5* polymorphisms -1131 T>C (SNP3; rs662799) in the *APOA5* promoter region and the c.56 G>C (S19W, rs3135506) in exon 3 of the *APOA5* gene, were analyzed as described earlier.[23]

STATISTICAL ANALYSES

Differences between groups were tested with the X^2 test for dichotomous and categorical variables and the unpaired Student's *t*-test for continuous variables. As total cholesterol, total triglyceride and plasma insulin levels showed non-Gaussian distributions, these parameters were logarithmically transformed before analysis. Untransformed levels are shown in the tables.

For each polymorphism or mutation, the Hardy-Weinberg equilibrium was calculated using the gene-counting method and differences were assessed by X^2 test. The X^2 test or Fisher's exact test were applied to compare genotype and allele frequencies between type III HLP patients, normolipidemic E2/2 subjects, NDCP and HTG patients. A logistic regression model was used to examine the association between the presence of a mutation and the occurrence of type III HLP. The strength of the association was estimated as the odds ratio (OR) with 95% confidence intervals (95% CI). *P*-values lower than 0.05 were considered significant. Selection of all SNPs in this study was based on literature data, describing association of these SNPs with lipid phenotype. Therefore adjustment for multiple testing was not applied (replication). Statistical analyses were performed with SPSS statistical software (version 14.01; SPSS, Chicago, IL). Linkage disequilibrium (LD) estimations in the *APOC3*/A5 region were estimated using Haploview 3.32.[39]

RESULTS

BASELINE CHARACTERISTICS OF THE STUDY POPULATIONS

The mean age of type III HLP patients was lower and their BMI was higher compared with normolipidemic E2/2 subjects (Table 1). The prevalence of vascular disease was increased in type III HLP patients, whereas no differences in the occurrence of hypertension, diabetes mellitus and the number of smokers and alcohol consumers were found between the groups. Furthermore, type III HLP patients had significantly higher total triglyceride and insulin levels compared with normolipidemic E2/2 subjects, whereas HDL-C was decreased. Characteristics of the normolipidemic panel (NDCP) and hypertriglyceridemic (HTG) patients are summarised in Table 1.

GENOTYPE AND ALLELE FREQUENCIES

Table 2 shows the genotype distributions and allele frequencies of polymorphisms in the *APOC3*, *APOA5*, and the *LPL* genes in the type III HLP patients, normolipidemic E2/2 subjects, NDCP and the HTG patients. The genotype or allele frequencies of all *HL* polymorphisms and a subset of the analyzed *LPL* polymorphisms did not differ significantly between the study populations and are not shown in Table 2. These include: *HL* -480 C>T, *HL* c. 219 G>A, *HL* c.1005 A>G, *HL* c.609 C>G, *LPL* c.874 A>G and *LPL* c.1342 C>G. All polymorphisms, in all groups, were in Hardy-Weinberg equilibrium.

Distribution of the genotypes of the *APOC3* 3238 G>C, *APOA5* -1131 T>C and *LPL* c.27 G>A polymorphisms differed significantly between the normolipidemic E2/2 subjects and the type III HLP

Table 1 Clinical and biochemical characteristics of type III HLP patients, normolipidemic E2/2 subjects, normal Dutch controls and hypertriglyceridemic patients

	Type IIIHLP		Normo		NDCP		HTG	
		N		N		N		N
Male	76 (67%)	113	32 (62%)	52	90 (48%)	188	119 (84)	141
Female	37 (33%)	113	20 (39%)	52	97 (52%)	188	22 (16%)	141
Age mean (years)	50.4 **	113	64.0	52	41	187	53	141
Age range (years)	27-80	113	27-80	52	6-78	187	26-78	141
Body mass index (kg/m2)	26.9 ± 3.8*	113	25.3 ± 4.0	49	-	-	-	-
Vascular disease	46 (41%)*	113	7 (15%)	39	-	-	-	-
Hypertension	31 (27%)	113	14 (26%)	42	-	-	-	-
Diabetes mellitus	11 (10%)	113	5 (10%)	40	-	-	-	-
Smokers	45 (40%)	112	19 (39%)	43	-	-	-	-
Alcohol consumers	78 (75%)	105	29 (78%)	30	-	-	-	-
Total cholesterol (mmol/L)	11.0 ± 3.5[a,]**	113	5.7 ± 1.0	52	5.1 ± 0.72[a]	188	8.8 ± 3.89	141
Total triglycerides (mmol/L)	6.7 ± 4.7[a,]**	113	2.0 ± 0.7	47	1.1 ± 0.35[a]	188	14.3 ± 13.80[a]	137
HDL-C (mmol/L)	1.0 ± 0.3 **	113	1.4 ± 0.3	48	1.3 ± 0.23[a]	188	0.7 ± 0.20	137
Glucose (mmol/L)	5.4 ± 1.2	100	5.2 ± 0.8	14	-	-	-	-
Insulin (pmol/L)	157.2 ± 138.0**	74	62.5 ± 41.4	14	-	-	-	-

Type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects; NDCP: normal Dutch control panel; HTG: hypertriglyceridemic patients. N, total number of subjects with available data about the respective parameter. Body mass index and plasma parameters are presented as mean ± SD.

a Selection criterion.

* $P < 0.05$, significantly different from normolipidemic E2/2 subjects.

** $P < 0.001$, significantly different from normolipidemic E2/2 subjects.

patients (Table 2). Distribution of the genotypes of the *APOC3* 3238 G>C, *APOA5* -1131 T>C and *LPL* c. 27 G>A, but not the *APOA5* c.56 G>C polymorphisms, differed significantly between NDCP and type III HLP patients. We observed a significant difference in genotype frequency between HTG patients and type III HLP patients for the *APOA5* -1131 T>C polymorphisms but we did not observe such difference for the *APOC3* 3238 G>C, *APOA5* c.56 G>C and *LPL* c.27 G>A polymorphisms.

The frequency of the minor allele of the *APOC3* 3238 G>C polymorphism and the (in LD) *APOA5* -1131 T>C polymorphism were increased in type III HLP patients and the HTG patients (15.6% and 15.1% in the type III HLP, 21.7% and 23.5% in the HTG patients, *vs.* a mean NDCP / normolipidemic APOE2 homozygotes of ˜7.2 and ˜6.1%, respectively). The *APOA5* c.56 G>C polymorphism showed a significantly increased frequency of the minor allele in type III HLP patients (11.8%) and in HTG patients (18.7%) when compared with the mean minor allele frequency of the NDCP / normolipidemic APOE2 homozygotes (˜6.2%). A significant enrichment of the minor allele of the *LPL* c.27 G>A mutation was observed among type III HLP patients and HTG patients (respectively, 6.2% and 4.8%), compared to the other study populations (NDCP and normolipidemic APOE2 homozygotes respectively, 2.6%, and 1.0%). LD estimation within the tightly linked cluster of the *APOC3*/A5 region, confirmed earlier findings of others that the *APOA5* -1131 T>C and *APOC3* 3238 G>C polymorphisms are in strong LD ($D' = 0.8$), whereas the *APOA5* c.56 G>C and -1131 T>C polymorphisms are not.

ASSOCIATION OF APOC3 3238 G>C, APOA5 -1131 T>C, APOA5 C.56 G>C AND LPL C.27 G>A WITH TYPE III HLP

Association between polymorphisms and the expression of type III HLP was tested by logistic regression analysis using a dominant model (i.e. 1/1 *vs.* 1/2 + 2/2). Table 3 shows the association of the APOC3 3238 G>C polymorphism (OR = 2.7, 95% CI 1.1 – 6.7, *P* = 0.03), the APOA5 -1131 T>C polymorphisms (OR = 3.1 95% CI 1.2 – 8.0, *P* = 0.02), the APOA5 c.56 G>C polymorphism (OR = 2.4, 95% CI 0.9 – 6.7, *P* =

Table 2 Genotype and allele frequencies

	APOE2 homozygotes			
Genotype / Allele frequency[a]	Type III HLP, N (%)	NORMO, N (%)	NDCP, N (%)	HTG, N (%)
APOC3 3238 G>C				
GG	78 (69.6)	44 (86.3) *	123 (83.9) **	71 (61.7)
GC	33 (29.5)	7 (13.7)	23 (15.4)	38 (33.0)
CC	1 (0.9)	0 (0.0)	1 (0.7)	6 (5.2)
1	189 (84.4)	95 (93.1) *	273 (92.6) *	180 (78.3)
2	35 ((15.6)	7 (6.9)	25 (7.4)	50 (21.7)
95 % CI	1.11 – 1.20	1.02 – 1.12	1.05 – 1.12	1.16 – 1.27
APOA5 -1131 T>C				
TT	75 (70.8)	45 (88.2) *	127 (88.2) **	67 (58.3) *
TC	30 (28.3)	6 (11.8)	16 (11.1)	42 (36.5)
CC	1 (0.9)	0 (0.0)	1 (0.7)	6 (5.2)
1	180 (84.9)	96 (94.1) *	270 (93.7) **	176 (76.5) *
2	32 (15.1)	6 (5.9)	18 (6.3)	54 (23.5)
95 % CI	1.10 – 1.20	1.01 – 1.10	1.03 – 1.09	1.18 – 1.29
APOA5 c.56 G>C				
GG	84 (79.2)	46 (90.2)	114 (85.1)	85 (67.5)
GC	19 (17.9)	5 (9.8)	20 (14.9)	35 (27.8)
CC	3 (2.8)	0 (0.0)	0 (0.0)	6 (4.8)
1	187 (88.2)	97 (95.1)	248 (92.5)	205 (81.3)
2	25 (11.8)	5 (4.9)	20 (7.5)	47 (18.7)
95 % CI	1.01 – 1.16	1.01 – 1.10	1.04 – 1.1	1.14 – 1.24
LPL C.27 G>A				
GG	100 (88.5)	51 (98.1) *	144 (94.7) *	103 (90.4)
GA	12 (10.6)	1 (1.9)	8 (5.3)	11 (9.6)
AA	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)
1	212 (93.8)	103 (99.0) *	296 (97.4) *	217 (95.2)
2	14 (6.2)	1 (1.0)	8 (2.6)	11 (4.8)
95 % CI	1.03 – 1.09	0.99 – 1.03	1.01 – 1.04	1.02 – 1.08

95% CI: 95% confidence interval; type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects; NDCP: normal Dutch control panel; HTG: hypertriglyceridemic patients.

a 1 represent common allele frequency, 2 rare allele frequency.

* Significant different genotype / allele frequency (P< 0.05) with type III HLP.

** Significant different genotype / allele frequency (P< 0.01) with type III HLP.

Differences between genotype or allele frequencies are based on linear-by-linear association.

0.12) and the *LPL* c.27 G>A mutation (OR = 6.6, 95% CI 0.8 – 52.1, $P = 0.07$) with the occurrence of type III HLP.

The *APOA5* -1131 T>C and *APOC3* 3238 G>C polymorphisms (in LD) showed a similar significantly increased risk on the occurrence of type III HLP. A significant association between the *APOA5* c.56 G>C polymorphism and the *LPL* c.27 G>A mutation and expression of type III HLP was not seen. However, the association (Table 3, cumulative) of carrying a rare allele of either *APOA5* c.56 G>C, -1131 T>C, *APOC3* 3238 G>C polymorphisms or *LPL* c.27 G>A mutation (OR = 3.7, 95% CI 1.8 – 7.5, $P < 0.0001$) and the expression of type III HLP was significantly increased.

To investigate the effect of the polymorphisms on plasma lipid levels, type III HLP patients were divided in carriers and non-carriers of the polymorphisms. Type III HLP patients with the *APOC3* 3238 G>C polymorphism and (in LD) the *APOA5* -1131 T>C polymorphism showed increased levels of total cholesterol compared with their counterparts without the polymorphism (Figure 1). Total triglyceride levels were significantly increased in carriers of the *APOA5* -1131 T>C polymorphism compared with non-carriers, whereas the increased levels of total triglyceride levels in the *APOC3* 3238 G>C carriers did not reach statistical significance (Figure 2). To further determine the association of the different cholesterol-rich lipoprotein fractions with genetic variation, we also investigated the effect of the polymorphisms, *APOC3* 3238 G>C, *APOA5* -1131 T>C and c.56 G>C, and *LPL* c.27 G>A on plasma HDL and VLDL cholesterol (Figure 3). We observed no effect of the polymorphisms on plasma HDL cholesterol (data not shown) whereas carriers of rare variants of both APOC3 3238 G>C and *APOA5* -1131 T>C, showed a significant association with plasma VLDL cholesterol levels. Evaluation of the association of the *APOA5* c.56 G>C, *APOA5* -1131 T>C, *APOC3* 3238 G>C and the *LPL* c.27 G>A variants with plasma lipid levels in the NDCP did not reveal any significant associations. The effect of the polymorphisms on plasma lipid (TG) levels in the HTG patients has been described previously.[23,24]

Table 3 Association of genotypes with the expression of type III HLP

	Type III HLP, N (%)	Normo, N (%)	OR (95% CI)	*P*-value
APOC3 3238 G>C				
1/1	78 (69.6)	44 (86.3)	2.7 (1.1– 6.7)	0.03
1/2 + 2/2	34 (30.4)	7 (13.7)		
APOA5 -1131 T>C				
1/1	75 (70.8)	45 (88.2)	3.1 (1.2–8.0)	0.02
1/2 + 2/2	31 (29.2)	6 (11.8)		
APOA5 c.56 G>C				
1/1	84 (79.2)	46 (90.2)	2.4 (0.9–6.7)	0.12
1/2 + 2/2	22 (20.8)	5 (9.8)		
LPL c.27 G>A				
1/1	100 (88.5)	51 (98.1)	6.6 (0.8– 52.1)	0.07
1/2 + 2/2	13 (11.5)	1 (1.9)		
Cumulative ¥				
non-carrier	48 (42.5)	38 (73.1)	3.7 (1.8 – 7.5)	< 0.0001
Carrier	65(57.5)	14 (26.9)		

Type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects. OR, odds ratio; 95% CI: 95% confidence interval. 1/1: homozygous for the common allele; 1/2: heterozygous for the common and rare allele; 2/2: homozygous for the rare alelle. ¥ Cumulative: non-carrier represents non-carrier of the rare allele of either the APOA5 C.56 G>C , SNP3, APOC3 -1131 T>C or LPL c.27 G>A and carrier represents carrier of the rare allele of either the APOA5 C.56 G>C , SNP3, APOC3 -1131 T>C or LPL c.27 G>A.

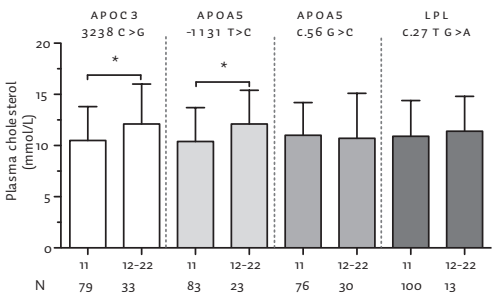

Fig.1. Effect of the APOC3 3238 G>C, the APOA5 -1131 T>C and APOA5 c.56 G>C, and the LPL c.27 G>A polymorphisms on plasma cholesterol levels in type III HLP patients. N represents number of type III HLP patients.
**P < 0.05.*

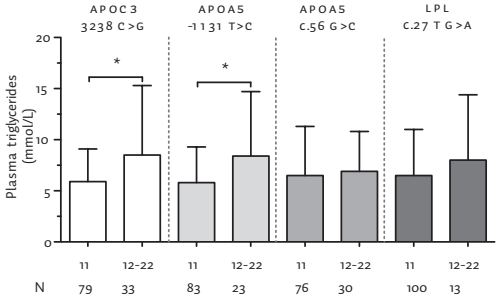

Fig.2. Effect of the APOC3 3238 G>C, the APOA5 -1131 T>C and APOA5 c.56 G>C, and the LPL c.27 G>A polymorphisms on plasma triglyceride levels in type III HLP patients. N represents number of type III HLP patients.
**P < 0.05.*

DISCUSSION

The clinical manifestation of type III HLP, in addition to defective hepatic clearance of remnant lipoproteins in E2/2 subjects, may be caused by impaired lipolysis.[4,9,40] Several groups reported about LPL and proteins involved in LPL activity, as apoCIII and apoAV in type III patients. [13-16] In accordance with this hypothesis we replicated in the largest type III cohort reported thus far, associations for polymorphisms in proteins involved in the lipolytic conversion of lipoproteins: the 3238 G>C polymorphism in the *APOC3* gene (in LD with APO5 -1131 T>C polymorphism) and the c.27 G>A mutation in the *LPL* gene. Our results show that the *APOC3* 3238 G>C/ *APOA5* -1131 T>C polymorphism exacerbates the hyperlipidemic phenotype of type III HLP patients, while the *LPL* c.27 G>A mutation has no additional effect on plasma lipid levels.

In an earlier study, we found an interaction between hyperinsulinemia and the *APOC3* 3238 G>C polymorphism, associated with severe hyperlipidemia in *APOE2* homozygotes.[12] By increasing the number of E2/2 subjects, data from the present study demonstrate that the *APOC3* 3238 G>C polymorphism *per se* is also an important contributor to type III HLP expression. The *APOC3* 3238 G>C polymorphism is strongly associated with elevated plasma triglyceride levels.[41] In accordance, our type III HLP patients carrying the *APOC3* 3238 G>C polymorphism showed a more severe hyperlipidemia, i.e. higher VLDL-cholesterol levels and a tendency of higher triglyceride levels than non-carriers. The molecular mechanism underlying the association with HTG is still unclear. The *APOC3* 3238 G>C polymorphism is located in the 3'-UTR of the *APOC3* gene and could be a causal variant→ by acting on mRNA stability which results in increasing apoCIII plasma levels. However, it is also possible that the *APOC3* 3238C>G polymorphism itself is not responsible for the triglyceride-raising effect. Other mutations within the APOA1-C3-A4 gene cluster, located near the *APOC3* 3238 G>C polymorphic site, could be candidates for being the causative mutations leading to HTG. Two polymorphisms located

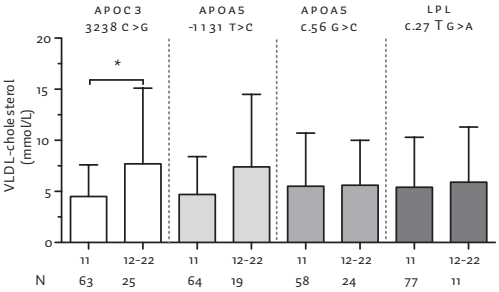

Fig.3. Effect of the APOC3 3238 G>C, the APOA5 -1131 T>C and APOA5 c.56 G>C, and the LPL c.27 G>A polymorphisms on VLDL-cholesterol levels in type III HLP patients. N represents number of type III HLP patients.
**P < 0.05.*

at positions –482 and –455 in the promoter region of the *APOC3* gene showed substantial linkage disequilibrium with the 3238 G>C polymorphism, as reported by others.[41-43] However, we found in a much smaller subgroup of our type III HLP patients, no evidence of an association between these polymorphisms and increased triglyceride levels [12], whereas Miller *et al.* suggested an association with these two *APOC3* polymorphisms and the metabolic syndrome. [40] Functional studies to elucidate the exact role on apoCIII protein levels of each of these three polymorphisms should be performed.

The *LPL* c.27 G>A mutation leads to an increased bridging of LDL and monocyte adhesion and is also associated with elevated levels of plasma triglycerides and reduced plasma HDL-cholesterol levels.[44] In our type III HLP patients, a tendency towards increased plasma cholesterol and triglyceride levels was observed in *LPL* c.27 G>A carriers, but this did not reach statistical significance, possibly due to the size of our groups. In Caucasians, the –93T G transition in the *LPL* gene promoter is in linkage disequilibrium with the *LPL* c.27 G>A mutation.[18,45] However, several studies have reported that the triglyceride-raising effect was solely attributable to the presence of the *LPL* c.27 G>A mutation.[45]

Data from genetic association studies on type III HLP are limited due to the low prevalence of APOE2 homozygosity in the population. To circumvent this problem, most studies have compared the allele frequency of mutations in type III HLP patients to that of the general population. Zhang *et al.*[13] found an increased allele frequency for the *LPL* N291S mutation in type III HLP patients compared with the Dutch population. However, this approach does not include the possibility that the mutation is also more prevalent in normolipidemic APOE2 homozygotes. Conversely, Evans *et al*[16] showed that the *LPL* mutations D9N, in particular N291S and S447X (c.27 G>A, c.874 A>G and c.1342 C>G resp.) were not associated with type III HLP.

Comparison of the Dutch population frequency for the *HL* -480 C>T mutation (19%) with that of our type III HLP patients (31%) revealed that the mutation was significantly more prevalent in patients ($P < 0.001$).[36] However, the fact that we did not find such a difference between patients and normolipidemic E2/2 subjects indicates that the *LPL* c.874 A>G and the *HL* -480 C>T mutations are not major contributors to the expression of type III HLP (data not shown). Moreover, also no association with type III HLP was found for *HL* c. 219 G>A, *HL* c.1005 A>G, *HL* c.609 C>G and *LPL* c.1342 C>G.

To further study the patient and population prevalences of the *APOA5*, *APOC3* polymorphisms and the *LPL* c.27 G>A mutation, two additional populations were screened: the NDCP and HTG patients. The prevalences of the *APOC3* 3238 G>C/*APOA5* -1131 T>C polymorphism, *APOA5* c.56 G>C and the *LPL* c.27 G>A mutation in the NDCP cohort are similar to those observed in the normolipidemic APOE2 homozygote individuals. This indicates that the normolipidemic APOE2 homozygotes were not a selected sub-population. In contrast, the prevalences of the *APOC3*, *APOAV* and *LPL* mutations in the HTG patients are similar to those of the type III patients, indicating that these polymorphisms are universal risk factors for hypertriglyceridemia.

Earlier studies have reported frequencies for the rare variant of the c.56 G>C polymorphism in type III patients.[14,15,46] In our type III patients, the rare variant of *APOA5* c.56 G>C polymorphism is far less frequent (11.8%) than reported thus far. In addition, 4.9% of the normolipidemic APOE2 homozygotes still was carrier of the rare allele of the *APOA5* c.56 G>C polymorphism. Moreover, we found no association between plasma triglyceride and cholesterol levels and the c.56 G>C polymorphism in hyper- and normolipidemic FDs. The fact that this association is not present and the frequency of the c.56 C-allele is somewhat increased in the type III HLP population suggests that the *APOA5* c.56 G>C polymorphism is a weak contributor to the clinical manifestation of type III HLP. The mechanism how this variant acts remains to be solved. It has been suggested that the *APOA5* c.56 G>C polymorphism

alters the signal peptide which results in a decreased secretion of apoAV protein. However, such correlation between APOA5 rare variant carriers and plasma apoAV, was not found in a cohort of HTG patients. [23]

As reported by others, our data show that the *APOC3* 3238 G>C and *APOA5* -1131 T>C polymorphisms show a high linkage disequilibrium. [41,47] Fifteen percent of type III HLP patients are carriers of the *APOA5* -1131 T>C polymorphism and 15.6% are carriers of the *APOC3* 3238 G>C polymorphism. In the present study we did not aim to elucidate a causal role of each polymorphism.

In total 58% of type III HLP patients *vs.* 27% of normolipidemic APOE2 homozygotes are carrier of the *APOC3* 3238 G>C/*APOA5* -1131 T>C polymorphism, *APOA5* c.56 G>C and/or the *LPL* c.27 G>A mutation, indicating that these mutations could partly explain the expression of type III HLP. Additional genetic and environmental risk factors remain to be identified. Earlier, we found that hyperinsulinemic APOE2 homozygotes have an increased risk for type III HLP.[6] It is likely that the suppression of VLDL-production by insulin in these hyperinsulinemic patients is reduced, resulting in chronically elevated VLDL-production.[10] Another possible mechanism is through an interaction between insulin and genes involved in the lipolysis of lipoproteins. Several studies support the hypothesis that insulin is involved in the regulation of the *APOC3* gene. The presence of the *APOC3* –455 and –482 polymorphisms may abolish the insulin responsiveness of the *APOC3* promoter.[48,49] Since the *APOC3* 3238 G>C polymorphism in E2/2 subjects is almost exclusively found in combination with the presence of the –455 and –482 promoter variants, it seems possible that in E2/2 subjects, who are carriers of the 3238 G>C polymorphism, the loss of insulin regulation results in overexpression of the *APOC3* gene and, as a consequence, in overt hyperlipidemia.[12,34] In addition, insulin is a major regulator of LPL activity. The combination of high insulin levels and decreased LPL activity, as found in carriers of LPL mutations, may further exacerbate the expression of HTG.[50]

In conclusion, our data indicate that the *APOC3* 3238 G>C, *APOA5* -1131 T>C and to a lesser extent the *LPL* c.27 G>A mutation associate with the elevated plasma TG phenotype of type III HLP patients, whereas *APOA5* c.56 G>C is perhaps a weak modifier.

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Chapter 6

Menopause impacts the relation of plasma adiponectin levels with the metabolic syndrome

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Objective: Plasma adiponectin is negatively correlated with metabolic syndrome (MetS) components obesity and insulin sensitivity. Here, we set out to evaluate the effect of menopause on the association of plasma adiponectin with MetS.
Design: Data on plasma adiponectin and MetS were available from 2256 individuals participating in the Erasmus Rucphen Family study. Odds ratios for MetS were calculated by logistic regression analysis using plasma adiponectin quartiles. The discriminative accuracy of plasma adiponectin for MetS was determined by calculating the area under the receiver-operator curve (AUC). Analyses were performed in women and men, pre- and postmenopausal women and younger and older men.
Results: Virtually all determinants of MetS differed significantly between groups. Low plasma adiponectin showed the highest risk for MetS in postmenopausal women (OR = 18.6, 95% CI = 7.9 – 44.0). We observed a high discriminative accuracy of age and plasma adiponectin for MetS in postmenopausal women (AUC = 0.76), but also in other subgroups (AUC from 0.67 – 0.87). However, in all groups, the discriminative accuracy of age and BMI for MetS was not exceeded by age and plasma adiponectin.
Conclusions: Low plasma levels of adiponectin are associated with increased prevalence of MetS, especially in postmenopausal women. Age and BMI have similar discriminatory accuracies for presence of MetS when compared to age and plasma adiponectin. Thus, we conclude that the association of plasma adiponectin with MetS is significantly affected by menopause but challenge the additional value of adiponectin for the discriminatory accuracy for presence of MetS.

INTRODUCTION

The prevalence of obesity and the metabolic syndrome (MetS) is increasing dramatically in populations with a western lifestyle[1-3]. Central obesity, dyslipidemia, impaired fasting plasma glucose (FPG) and hypertension are important components of MetS[4]. Given the increased morbidity and mortality associated with MetS, research has focused on associations of specific biomarkers with the (early) onset and progression of MetS[2,5,6].

Obesity plays a key role in the aetiology of MetS and related diseases. Adipose tissue functions as an endocrine organ which responds to changes in metabolic conditions by secreting biologically active substances also known as adipocytokines or adipokines[7,8,9]. Specifically, the visceral fat depot, which has rapid access to the systemic circulation via the portal vein, is thought to play an important role in adipokine secretion and MetS and related disorders[10]. Many groups have suggested that plasma adipokines are potential biomarkers for MetS, in particular plasma adiponectin[11,12].

Adiponectin is secreted form adipose tissue and is present in plasma in various multimeric forms. Plasma adiponectin levels have been shown to differ largely between genders[13]. In humans, plasma adiponectin levels are negatively correlated with obesity and type two diabetes (T2D)[14,15,16]. In mouse models, adiponectin has been shown to play a role in energy homeostasis by regulating insulin sensitivity of the liver[17]. Adiponectin is suggested to have an important role in vascular endothelium as modifier of monocyte adhesion by affecting anti-inflammatory properties. In addition, adiponectin has also been implicated in regulation of vasodilatation, via the endothelial NO synthase (eNOS) pathway[18].

Gender and age effects in the expression of MetS have been previously demonstrated[19]. Furthermore, it was shown that MetS is more prevalent in men than in premenopausal women[19,20]. After menopause, women experience a substantial increase in dyslipidemia and other MetS related

risk factors, leading to a risk profile comparable with that seen in men[21,22]. Thus, differences between pre- and postmenopausal women with regard to the prevalence of MetS and plasma adiponectin have been reported independent from each other. It has been suggested that plasma adiponectin is a promising biomarker for MetS[11,12]. However, within the general population the effect of menopause on the association of plasma adiponectin with MetS is not clear. Furthermore, it is not clear to what extent menopause affects the association between plasma adiponectin and MetS.

The present study aims to elucidate the effect of menopause on the association of plasma adiponectin with MetS. In addition, we investigate the discriminatory accuracy of plasma adiponectin for presence of MetS as a measure for a MetS biomarker. Analyses were performed overall and in subgroups: pre- and postmenopausal women and younger and older men (stratified on the mean age at halt of menstruation of women).

METHODS

STUDY POPULATION

We used data of the Erasmus Rucphen Family (ERF) study. This population was embedded in an isolated population (Genetic Research in Isolated Populations, GRIP). The ERF population includes individuals who were not selected based on health information, but comprises living descendants of 22 couples, who had at least 6 children baptized in the community church between 1850 and 1900. Details about the genealogy of the population are described elsewhere[23,24,25]. The study protocol was approved by the medical ethics board of the Erasmus MC Rotterdam, the Netherlands. A written informed consent was obtained from each participant and all investigations were carried out in accordance with the Declaration of Helsinki.

DATA COLLECTION

Blood of participants was obtained in the fasted state. Total plasma insulin was analyzed with the INS-Irma kit of DIAsource ImmunoAssays (formerly Bio-Source; Nivelles, Belgium; cat #: KIP1254), total plasma adiponectin with the Human adiponectin RIA kit (cat#: HADP-61HK) of Linco Research (St. Charles, MO, USA) and total plasma CRP with the US C-reactive protein ELISA (cat.# DSL-10-42100) of Diagnostic Systems Laboratories (Webster, TX, USA), Inc. All measurements were performed conform the manufactures protocol. The distribution of plasma CRP level was skewed, therefore upper plasma CRP levels exceeding three times the standard deviation of the mean were removed from the analyses. Menopause was defined by halt of menstruation whether it be naturally, due to medication or due to surgical intervention. Insulin sensitivity was based on the homeostasis model assessment insulin resistance (HOMA-IR; glucose* insulin divided by 22.5). Assessment of the following study variables have been given earlier by others[26,23,27,19]: Medication use; glucose lowering, lipid lowering or anti-hypertension treatment, body mass index (BMI), MetS (International Diabetes Federation – 2006, europids); waist circumference (WC, men ≥ 94 cm, women ≥ 80), fasting plasma glucose (FPG, ≥ 5.6 mmol/L, previously diagnosed/treated type 2 diabetes patients included), HDL-cholesterol (HDL-C, men < 1.03 mmol/L, women < 1.29 mmol/L, treated patients included), total plasma triglycerides (TG, ≥ 1.7 mmol/L, treated patients included), systolic blood pressure (SBP, ≥ 130 mm Hg, treated patients included), diastolic blood pressure (DBP, ≥ 85 mm Hg, treated patients included).

STATISTICAL ANALYSES

Women were stratified in two groups according to menopause. The mean age of premenopausal -and postmenopausal women was 37.8 and 60.2 years respectively and the mean age at halt of menstruation was 46.8 years. In order to be able to exclude an age effect in our analyses, we divided the men in two groups, younger -and older men, using the mean age at halt of menstruation of women. Younger men were defined by an age limit of < 46.8 years, older men by an age limit of > 46.8 years. Continuous variables did show some deviation from a normal distribution. Analysis of mean differences between groups was tested using analysis of variance (ANOVA) statistics using log normal transformed continuous variables or chi-squared statistics (categorical variables). All further analyses were based on the log normal transformed trait values with exception of the correlation analysis, which was performed using Spearman's correlation method. Odds ratios (OR) were calculated using binary logistic regression analyses, using quartiles of plasma adiponectin level, age and BMI as independent variables. Plasma adiponectin cut off values for these quartiles were 18.3 mmol/L, 11.1 mmol/L, 7.9 mmol/L and 4.7 mmol/L for group 1 to 4 respectively. The group with highest plasma adiponectin levels (18.3 mmol/L) was taken as reference group. The discriminative accuracy for MetS was based on predicted probabilities (binary logistic regression) according four different models; model 1: age; model 2: age and BMI; model 3: age and plasma adiponectin; model 4: age, BMI and plasma adiponectin. Predicted probabilities were calculated in pre –and postmenopausal women, total women, younger –and older men and total men. The binary logistic regression in total women and total men included next to independent variables also interaction terms of the stratification component (menopause or younger and older men) with each independent variable within the respective model. For example, in the total female group the first model (1) included age and age*menopause, the second model (2) age, BMI, age*menopause and BMI*menopause, the third model (3) age, adiponectin, age*menopause and adiponectin * menopause and the final model (4) included age, BMI, adiponectin, age*menopause, BMI*menopause and adiponectin*menopause. The discriminating accuracy is the extent to which test results can discriminate between individuals who will develop the disease and those who will not[28]. The area under the receiver operating characteristic curve (AUC) is commonly used to quantify this discriminative accuracy of a predicting model[29]. The AUC is the probability that the test correctly identifies the diseased individual from a pair of whom one is affected and one is unaffected, and ranges from 0.5 (total lack discrimination) to 1.0 (perfect discrimination)[28]. Analysis of the data was performed using SPSS 14.01 software. Statistical tests were not independent, which was a equirement for multiple test correction (e.g. Bonferoni). However, as we performed a considerable amount of statistical tests, only P-values below 0.01 were assumed significant.

RESULTS

Characteristics of the study population are described in Table 1. Data on plasma adiponectin, insulin and CRP levels were obtained for 2256 individuals. The population included 695 premenopausal and 581 postmenopausal women and 418 younger and 549 older men (stratified on mean age of onset menopause in women). The prevalence of MetS was 17.0% in premenopausal women, 43.2% in postmenopausal women, 22.2% in younger men and 48.5% in older men. All measurements, with exception of age and HDL-cholesterol, differed significantly ($P < 0.05$) between women and men (data

Table 1: General characteristics of the study population

	Women		Men	
	Premenopausal	Postmenopausal	Younger	Older
	(n=695)	(n=581)	(n=418)	(n=549)
Age (yr)	37.8 ± 9.6	60.2 ± 8.3 **	36.0± 7.8	59.5 ± 8.0 ***
Metabolic syndrome and its individual components				
Metabolic syndrome % (n)	17.0 (118)	43.2 (251) ***	22.2 (93)	48.5 (266) ***
Waist circumference (cm)	78.4 ± 11.3	86.1 ± 11.5 ***	90.5 ± 10.8	96.9 ± 11.2 ***
Glucose (mmol/L)	4.2 ± 0.8	4.7 ± 0.8 ***	4.5 ± 0.8	5.0 ± 1.1 ***
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.1 ± 0.3	1.2 ± 0.3
Triglycerides (mmol/L)	1.1 ± 0.6	1.4 ± 0.7 ***	1.4 ± 0.9	1.6 ± 0.9 *
Systolic blood pressure (mm Hg)	126.7 ± 15.2	147.4 ± 21.8 ***	136.9 ± 12.6	148.2 ± 20.0 ***
Diastolic blood pressure (mm Hg)	76.5 ± 9.6	80.9 ± 9.4 ***	78.8 ± 9.8	84.0 ± 9.2 ***
Metabolic syndrome related traits				
Body mass index (kg/m2)	25.5 ± 5.0	27.8 ± 4.7 ***	26.9 ± 4.1	27 .6 ± 4.0 ***
Insulin (_U/ml)	12.2 ± 5.5	13.6 ± 7.4 ***	13.9 ± 9.8	13.9 ± 8.1
HOMA-IR	2.3 ± 1.3	2.9 ± 1.9 ***	2.9 ± 2.5	3.2 ± 2.3
Adiponectin (mgl/L)	11.9 ± 5.3	12.9 ± 6.2 *	7.7 ± 3.8	8.2 ± 4.4 *
C-reactive protein (mg/L)	5.7 ± 4.6	6.3 ± 4.7 **	4.0 ± 3.6	5.9 ± 4.4 ***
Medication				
Glucose lowering % (n)	0.9 (6)	3.4 (20) ***	0.0 (0)	4.9 (27) ***
Lipid lowering % (n)	3.2 (22)	18.9 (110) ***	3.6 (15)	23.5 (129) ***
Blood pressure lowering % (n)	8.1 (56)	31.2 (181) ***	5.3 (22)	32.6 (179) ***

HDL: high density lipoprotein; HOMA-IR: Homeostatic model assessment-Insulin resistance. Values presented as mean ± SD for continuous variables and percent (number) for categorical variables. Plasma was taken in fasted state. Differences were calculated within gender group only, * P < 0.05, ** P < 0.01, *** P < 0.001. Significance of continuous variables based on ANOVA, for non-continuous variables based on Chi2.

not shown) and between pre- and postmenopausal women and between younger and older men.

Spearman's correlation coefficients between plasma adiponectin and MetS, MetS individual traits and MetS related traits are presented in Table 2. Correlation coefficients of plasma adiponectin level with MetS were -0.23, -0.37 in respectively pre- and postmenopausal women and -0.19, -0.22 in respectively younger and older men. We observed significant correlations of plasma adiponectin with each of the individual components of MetS with exception of diastolic blood pressure in all four groups. Correlation coefficients of plasma adiponectin level with MetS related traits: BMI, fasting plasma insulin level, HOMA-IR and plasma CRP level ranged from -0.11 to -0.34 ($P < 0.01$). Correlation coefficients differed in general more than 0.10 between pre- and postmenopausal women, whereas no difference was observed between younger and older men.

The presence of MetS was higher in subgroups with lower plasma adiponectin levels, indicated by the higher OR presented in Figure 1. The highest OR for the presence of MetS was observed for postmenopausal women in the lower plasma adiponectin quartile (OR = 18.6, 95% CI=7.9–44.0) which differed significantly from that seen in the premenopausal women (OR = 4.5, 95% CI=1.7–7.4). We did not observe such extreme differences in OR's between the two male groups.

To assess the value of adiponectin as biomarker for MetS, we analyzed the AUC as a measure of discriminative accuracy according to several models (Table 3). The AUC of age for MetS (model 1)

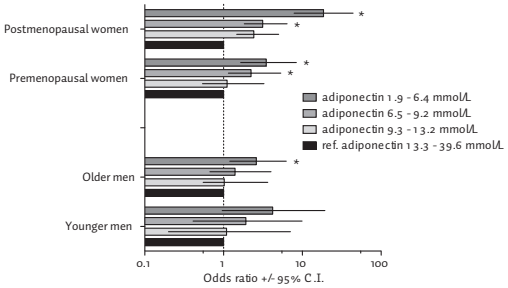

Fig.1. Odds ratio of adiponectin quartiles for presence of the MetS. Bars represent OR ± *95% confidence interval in pre- and postmenopausal women and younger men (<46.8 years old) and older men (>46.8 years old). * P < 0.01, Odds ratio based on logistic (binary) regression adjusted for age and body mass index.*

ranged from 0.55 (95% CI= 0.50 – 0.60) in older men to 0.71 (95% CI= 0.68 – 0.74) in total women. When the discriminatory accuracy for presence of MetS was based on age and BMI (model 2) AUC's ranged from 0.80 (95% CI= 0.76 – 0.83) in postmenopausal women to 0.92 (95% CI= 0.90 – 0.95) in younger men. In the third model, which included age and adiponectin (model 3), AUC's ranged from 0.67 (95% CI= 0.64 – 0.71) in older men to 0.87 (95% CI= 0.85 – 0.89) in the total female group. Finally, when age, adiponectin and BMI all (model 4) were included, we observed the largest AUC's, ranging from 0.84 (95% CI= 0.81 – 0.87) in postmenopausal women to 0.92 (95% CI= 0.90 – 0.95) in younger men.

Table 2: Spearman's correlation coefficients of plasma adiponectin levels with MetS, MetS individual components and MetS related traits.

	Women		**Men**	
	Premenopausal	**Postmenopausal**	**Younger**	**Older**
	(n=695)	(n=581)	(n=418)	(n=549)
Age	-0.03	0.07	-0.14 **	0.12 **
Metabolic syndrome and its individual components				
Metabolic syndrome	-0.23 ***	-0.37 ***	-0.19 ***	-0.22 ***
Waist circumference	-0.30 ***	-0.38 ***	-0.18 ***	-0.24 ***
Glucose	-0.09 *	-0.22 ***	-0.13 **	-0.07
HDL-cholesterol	0.33 ***	0.42 ***	0.29 ***	0.31 ***
Triglycerides	-0.19 ***	-0.34 ***	-0.18 ***	-0.17 ***
Systolic blood pressure	-0.03	0.00	-0.14 **	-0.08
Diastolic blood pressure	-0.01	0.01	-0.09	-0.06
Metabolic syndrome related traits				
Body mass index	-0.26 ***	-0.30 ***	-0.17 ***	-0.21 ***
Insulin	-0.27***	-0.33 ***	-0.18 **	-0.19 ***
HOMA-IR	-0.26 ***	-0.34 ***	-0.19 **	-0.18 ***
C-reactive protein	-0.11 **	-0.25 ***	-0.08	-0.14 **

HDL: high density lipoprotein; HOMA-IR: Homeostatic model assessment-Insulin resistance. Values presented as Spearman's correlation coefficient, * P < 0.05, ** P < 0.01, *** P < 0.001.

DISCUSSION

Low plasma adiponectin is associated with obesity and insulin resistance[15,30] and plasma adiponectin has been proposed as a biomarker for MetS[16,31]. Large differences in mean plasma adiponectin and correlation coefficients with Mets and MetS associated traits have been reported between genders[13,32,33]. Our observations confirmed a significant difference in plasma adiponectin level and

Table 3: Discrimanative accuracy (AUC) of age, BMI and plasma adiponectin in the prediction of MetS.

	Model 1	Model 2	Model 3	Model 4
	age	age / BMI	age / adiponectin	age / BMI / adiponectin
Premenopausal women	0.61 (0.56 – 0.67)	0.87 (0.84 – 0.90)	0.72 (0.68 – 0.78)	0.88 (0.85 – 0.91)
Postmenopausal women	0.60 (0.55 – 0.65)	0.80 (0.76 – 0.83)	0.76 (0.72 – 0.80)	0.84 (0.81 – 0.87)
Total women	0.71 (0.68 – 0.74)	0.85 (0.83 – 0.88)	0.87 (0.85 – 0.89)	0.87 (0.85 – 0.89)
Younger men	0.66 (0.60 – 0.72)	0.92 (0.90 – 0.95)	0.71 (0.66 – 0.77)	0.92 (0.90 – 0.95)
Older men	0.55 (0.50 – 0.60)	0.84 (0.80 – 0.87)	0.65 (0.61 – 0.70)	0.85 (0.82 – 0.88)
Total men	0.67(0.64 – 0.71)	0.88 (0.86 – 0.90)	0.73 (0.69 – 0.76)	0.89 (0.87 – 0.91)

BMI: body mass index. Values presented as AUC (95% confidence interval). Detailed description of models see methods.

virtually all aspects of MetS between genders and within gender subgroups (Table 1). As large mean differences are also observed between the male groups (stratified on mean age of onset menopause in women), age seems significant contributor to variation in plasma adiponectin.

We further questioned whether menopausal status, independent from age, affects plasma adiponectin levels and its association with MetS and associated traits. The spearman's ρ correlation coefficients for plasma adiponectin levels with MetS, MetS individual components and associated traits were different in women before and after menopause (Table 2). Furthermore, we observed higher correlation coefficients in postmenopausal women and we observed that correlation coefficients varied to a much lesser extent between both male groups. These findings indicate that menopausal status, independent of age, had a large effect on the correlation of plasma adiponectin with MetS, MetS individual components and MetS associated traits (with exception of diastolic blood pressure).

The correlation of plasma adiponectin with MetS was further investigated by determining the OR for MetS in pre- and postmenopausal women and younger and older men. It is well known that age and body composition strongly influence the manifestation of MetS and that obesity is strongly associated with plasma adiponectin levels. Therefore, we included in addition to age, BMI as covariates in the logistic regression. Although an effect was seen in premenopausal women and both male groups, our analysis showed that the OR of MetS in the lowest adiponectin quartile was the highest in postmenopausal women (OR = 18.6 95% CI = 7.9 – 44.0, figure 1). Because in men such high OR was not observed, these data indicate that the risk for MetS was especially high for postmenopausal women, independently of age.

Our data showed a moderate discriminative accuracy for the presence of MetS, expressed as AUC, of age alone (Table 3, model 1). As obesity is an essential component of MetS, our data further confirmed the high discriminative accuracy for the presence of MetS of age and BMI (Table 3, model 2). As the AUC of age alone was lower compared to the AUC of age and plasma adiponectin (Table 3, models 1 and 3), plasma adiponectin contributed significantly to the discriminative accuracy for the presence of MetS. Including BMI in the model, this discriminative accuracy was even better (Table 3, model 4) and consistent between the genders and gender groups (AUC $\geq$ 0.84). Thus, the contribution of BMI in model 4 was substantial in the discriminative accuracy for the presence of MetS. Our findings further showed that age and BMI are more accurate in detecting the presence of MetS than age and adiponectin (Table 3, models 2 and 3). As measuring BMI is far more cost effective than measuring plasma adiponectin, the clinical utility of plasma adiponectin for the diagnosis MetS is questionable. Nevertheless, the logistic regression analyses indicated a high relative risk for MetS in particular in postmenopausal women with low plasma adiponectin. Since OR's are frequently used

in genetic studies, our data indicate that ignoring menopause may result in underestimation of effect size in genetic association analysis of plasma adiponectin.

The MetS can be diagnosed according to several different definitions[34]. In addition to the IDF MetS definition, a widely used MetS definition has been formulated by the National Cholesterol Education Pogram Adult Treatment Panel III (NCEP ATP III). The IDF and the NCEP ATPIII definitions differ slightly in MetS component threshold values[19,34]. Moreover, in contrast to the NCEP ATPIII, the obesity measure waist circumference is essential in the IDF definition. The Pearson correlation coefficient between IDF and NCEP ATPIII is 0.68 in our study cohort. To investigate whether the differences in MetS definitions affected the associations of plasma adiponectin with MetS or the discriminative accuracies for the presence the MetS, we recalculated all analyses using the NCEP ATPIII MetS definition. These analyses showed similar results obtained with regard to the IDF MetS definition. For example, postmenopausal women in the lower plasma adiponectin quartile showed for the IDF an OR of 18.6 (95% CI=7.9–44.0) and the NCEP ATPIII showed an OR of 16.8, (95% CI=7.8–36.4). In addition, the AUC analyses showed highly similar results (data not shown). These analyses demonstrated that our calculation of OR and the discriminative accuracies for the presence the MetS associated with plasma adiponectin were not determined by the
IDF MetS definition.

In conclusion, the present study confirmed gender differences in the association of plasma adiponectin with MetS and demonstrated that this association was significantly affected by menopause. We showed that adiponectin *per se* was strongly associated with MetS, in particular in postmenopausal women, but then its discriminative accuracy was overruled by the obesity measure BMI. Nevertheless, the strong effect of menopause on the association of plasma adiponectin with MetS and MetS components indicates that inclusion of menopause in genetic association studies of plasma adiponectin will increase statistical power.

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Chapter 7

The genetic architecture of plasma adiponectin overlaps with the genetics of metabolic syndrome related traits

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ABSTRACT

Objective: Adiponectin, a hormone secreted by adipose tissue is of particular interest to metabolic syndrome (MetS), since it is inversely correlated with obesity and insulin sensitivity. However, it is unknown to what extent the genetics of plasma adiponectin and the genetics of obesity and insulin sensitivity are interrelated. We aimed to evaluate the heritability of plasma adiponectin and its genetic correlation with the MetS and MetS related traits and the association between these traits and ten *ADIPOQ* SNPs.

Research Design and Methods: We made use of a family-based population, the Erasmus Rucphen family (ERF) study (1258 women and 967 men). Heritability analysis was performed using a polygenic model. Genetic correlations were estimated using bivariate heritability analyses. Genetic association analysis was performed using a mixed model.

Results: Plasma adiponectin showed a heritability of 55.1%. Genetic correlation between plasma adiponectin HDL-C and plasma insulin ranged from 15% to 24% but were not significant for fasting glucose, TG, blood pressure, HOMA-IR and CRP. Significant association with plasma adiponectin was found for *ADIPOQ* variants rs17300539 and rs182052. Nominally significant association was found with plasma insulin and HOMA-IR and *ADIPOQ* variant rs17300539 after adjusting for plasma adiponectin.

Conclusions: The significant genetic correlation between plasma adiponectin and HDL-C, and plasma insulin should be taken into account in the interpretation of genome wide association studies. Association of *ADIPOQ* SNPs with plasma adiponectin was replicated and we showed association between one *ADIPOQ* SNP and plasma insulin and HOMA-IR.

The dramatic increase in the prevalence of the metabolic syndrome (MetS) in countries with a western lifestyle is precipitated by environmental variables. However, the individual susceptibility to the obesogenic environment is largely determined by genetic susceptibility[1]. Central obesity, dyslipidemia, impaired glucose metabolism and hypertension are the key elements determining the expression of the MetS[2] which is associated with an increased risk for type 2 diabetes (T2D) and cardiovascular disease (CVD)[2].

Adipose tissue is an active endocrine tissue which can respond to changes in metabolic conditions by secreting biologically active substances (adipokines). The adipokine family can be divided in two overlapping sets of signaling molecules, namely those with metabolic / immunologic function, which include interleukins 1ß, 6, 8, 10 or 18, tumor necrosis factor alpha and tumor growth factor beta, and those with endocrine function, which include leptin, retinol binding protein 4, adiponectin and resistin[3]. Human adiponectin is a protein of 247 amino acids (30-kDa), encoded by a gene (*ADIPOQ*) located on chromosome 3q27[4]. Adiponectin is secreted and present in plasma in various multimeric forms of which the biological significance remains to be determined. Yamauchi *et al* showed that binding of adiponectin to adiponectin receptors (ADIPOR1 and ADIPOR2) in mice results in increased AMPK activity and PPAR-α activity. In humans, both receptors are mainly expressed in skeletal muscle and adiponectin could thus play a role in energy metabolism[5].

Limited data on the overall heritability of plasma adiponectin is available. Furthermore, it is not known whether the genetics of plasma adiponectin overlap with the genetics of body weight and insulin sensitivity/diabetes or other individual components of the MetS. Several studies showed convincing association of genetic variants near and in the promoter region of the *ADIPOQ* gene with plasma adiponectin and T2D or T2D related traits[9,5,10].

In the present study, we set out to evaluate the heritability of plasma adiponectin and its genetic

correlation with the MetS and the MetS related traits BMI, insulin, HOMA-IR and plasma CRP.

RESEARCH DESIGN AND METHODS

STUDY POPULATION

In the present study, we used data of the Erasmus Rucphen Family (ERF) study, which is embedded into a rural genetically isolated population (Genetic Research in Isolated Populations; GRIP). This young, genetic isolate from the southwest of the Netherlands was initiated by <400 founders in the middle of 18th century. Minimal immigration occurred between the surrounding settlements due to social and religious reasons. The population experienced a fast expansion and at the moment this region counts roughly 20.000 inhabitants. The ERF population is a cross-sectional cohort and includes 3,000 individuals, who were not selected based on health information, but rather comprise living descendants of 22 couples who had at least 6 children baptized in the community church around 1850-1900. Details about the genealogy of the population have been described elsewhere[11,12]. In the current study, we included 2256 individuals of the ERF population from whom all study parameters were known. We did not exclude participants based on health status. The study protocol was approved by the medical ethics board of the Erasmus MC Rotterdam, the Netherlands. All investigations were carried out in accordance with the Declaration of Helsinki.

DATA COLLECTION

Blood from participants was obtained in a fasted state. Total plasma insulin measurements were analyzed with the INS-Irma kit of Biosource (cat.#: KIP1254), total plasma adiponectin with the Human adiponectin RIA kit (cat#: HADP-61HK) of Linco Research and total plasma CRP with the US C-reactive protein ELISA (cat.# DSL-10-42100) of Diagnostic Systems Laboratories, Inc. All measurements were performed conform the manufactures protocol. Insulin sensitivity was based on the homeostasis model assessment insulin resistance (HOMA-IR; glucose * insulin divided by 22.5). Plasma CRP showed kurtosis, therefore, upper plasma CRP levels exceeding three times the standard deviation of the mean were removed from further analyses. Data on plasma adiponectin, insulin, HOMA-IR and CRP was available for 2256 individuals. MetS was assessed according to the criteria of the International Diabetes Federation (IDF- 2006, Europids), which requires a minimum waist circumference (WC,) and two of the following abnormalities: high fasting plasma glucose (glucose), low HDL-cholesterol (HDL-C), high total plasma triglycerides (TG), and high systolic and/or diastolic blood pressure (SBP/DBP), described in detail in supplemental table 1. Prevalence and heritability of MetS and related traits in ERF have been reported previously[1,13].

STATISTICAL ANALYSES

Analysis of mean differences between groups was tested using analysis of variance statistics (ANOVA, continuous variables) or chi squared statistics (Chi^2, categorical variables). Correlations of plasma adiponectin with other traits were estimated in men and women separately adjusting for age and BMI. Heritability estimations were obtained using SOLAR software (version 2.05, http://solar.sfbrgenetics.org[11,1]. Heritability and genetic association analyses were performed using normal-log

Table 1: Characteristics of the ERF population

	Women (n=1258)	Men (n=967)
Age (yr)	48.0 ± 14.3	49.4 ± 14.1 *
range (yr) / min-max	68.0 / 18-86	68.5 / 18-86
Metabolic syndrome and its individual components		
Metabolic Syndrome n(%)	369 (29.3)	359 (37.1) **
Waist circumference (cm)	81.9 ± 12.0	94.2 ± 11.5 **
Glucose (mmol/L)	4.4 ± 0.9	4.8 ± 1.0 **
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.1 ± 0.3 **
Triglycerides (mmol/L)	1.2 ± 0.7	1.5 ± 0.9 **
SBP (mm Hg)	136.1 ± 21.2	143.4 ± 18.0 **
DBP (mm Hg)	78.6 ± 9.8	81.7 ± 9.8 **
Metabolic syndrome related traits		
Body mass index (kg/m2)	26.6 ± 5.0	27.3 ± 4.1 **
Insulin (_U/ml)	12.8 ± 5.8	13.9 ± 8.8 *
HOMA-IR	2.6 ± 1.6	3.0 ± 2.4 **
Adiponectin (mg/L)	12.4 ± 5.8	8.1 ± 4.1 **
CRP (mg/L)	11.1 ± 22.2	10.0 ± 28.5 **
Medication		
Glucose lowering med. n (%)	26 (2.1)	27 (2.8)
Lipid lowering med. n (%)	132 (10.5)	144 (14.9) *
Anti-hypertension med. n (%)	237 (18.8)	201 (20.8)

SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; CRP: C-reactive protein; med.: medication. Values presented are mean ± SD for continuous traits and number (percent) for categorical traits.
* Significant different from women (P-value < 0.05), ** (P-value < 0.01), continuous traits based on ANOVA, non- categorical traits based on Chi2 test.

transformed trait values and SOLAR's "tdist" function. The polygenic model (covariates: gender, age) was applied. Heritability estimations included a second variance component, the sibship effect (S), which is an estimate of phenotypic similarity, due to effects of a shared (early) environment and genetically dominant effects[1]. The effect of relevant medication use was assumed to be covered by the MetS definition (Models B). Inbreeding coefficients in heritability estimations were not significant and therefore excluded from further analyses. To determine the genetic correlations of plasma adiponectin with other traits we used bivariate heritability analysis. We also estimated gender-specific heritability using bivariate analysis of traits stratified by gender. Because the latter analysis is confined to one quantitative trait, the environmental correlation component is forced to be zero. Bivariate analysis was applied to plasma adiponectin with the MetS components and MetS related components using gender, age and BMI as covariates (Model A) or using gender, age and MetS as covariates (Model B). The bivariate heritability analysis yields estimates of the total overlapping genetic and environmental component (correlation) of these traits.

The following *ADIPOQ* variants were selected after literature review (9) and confirmed for tag property using Haploview ((r^2 > 0.8; MAF >10%) and CEU HAPMAP data: rs864265, rs822387, rs17300539 (-11391G/A), rs266729, rs182052, rs822396, rs2241766 (+45T/G), rs1501299 (+276G/T), rs3774262 and rs6773957. Selected variants were genotyped using Sequenom iPLEX (MALDITOF, Sequenom Inc. San Diego, USA). Genotypes were screened for Mendelian errors using the pedigree structure (14). All ten *ADIPOQ* variants achieved a call rate of >95% and all were in HWE (*P*>0.05). For *ADIPOQ* genotype-association we assumed an additive model. Analysis of plasma adiponectin included covariates gender, age and BMI (Model A) or covariates gender, age and MetS (Model B). To investigate whether *ADIPOQ* SNPs were independently associated with plasma adiponectin, we applied a backward linear regression model containing all SNPs which were associated with plasma adiponectin at FDR < 0.05. The following model was used: rs822387, rs17300539, rs182052, rs1501299, rs6773957, gender, age and BMI as independent variables and plasma adiponectin as dependent variable. The independence of the associated SNPs was confirmed in HAPLOVIEW and these SNPs were used in association analysis with the MetS related traits.

Association analyses of ADIPOQ variants with MetS related traits was performed using two models with different covariates: (1) gender, age, BMI, (2) gender, age, BMI and plasma adiponectin. Adjustment for family structure of the association model was based on a pedigree matrix obtained using Illumina 6K linkage chip data. Analysis was performed using model residuals in score test accounting for pedigree structure as implemented in GenABEL software[15] function "mmscore"[16]. The Bayesian information criterion (BIC) for plasma adiponectin was implemented on associated ADIPOQ SNPs, gender, age and BMI using R software. All other analyses were performed using SPSS 14.01 (September 2005) software.

Table 2: Genetic and environmental correlation of plasma adiponectin with MetS, MetS components and MetS related traits

Model A	**_G**	**SE**	***P***	**_E**	**SE**	***P***
Metabolic syndrome and its individual components						
Metabolic Syndrome	-42.7	11.3	<10-04	-19.4	9.4	0.04
Waist circumference	-20.4	7.3	<10-03	-18.8	5.8	<10-04
Glucose	-2.6	8.7	0.76	-12.0	5.6	0.03
HDL-cholesterol	24.7	7.2	<10-04	35.6	5.5	<10-9
Triglycerides	-12.2	8.9	0.17	-26.0	5.2	<10-6
SBP	-10.3	9.1	0.26	-7.1	5.3	0.18
DBP	-9.5	9.2	0.31	6.0	5.4	0.27
Metabolic syndrome related traits						
Body mass index ¥	-32.4	7.2	<10-05	-20.7	5.9	<10-04
Insulin	-20.0	9.9	0.04	-18.8	5.1	<10-04
HOMA-IR	-18.6	9.9	0.06	-19.6	5.1	<10-04
CRP	-11.6	12.3	0.34	-13.4	5.4	0.01
Model B	**_G**	**SE**	***P***	**_E**	**SE**	***P***
Metabolic syndrome individual components						
Waist circumference	-15.0	8.9	0.092	-22.6	5.6	< 10-04
Glucose	0.5	8.9	0.955	-11.3	5.5	0.040
HDL-cholesterol	15.2	7.7	0.048	37.2	5.4	< 10-11
Triglycerides	-11.9	9.8	0.225	-26.0	5.1	< 10-06
SBP	-6.3	9.3	0.498	8.5	5.3	0.100
DBP	-7.1	9.6	0.459	4.0	5.3	0.450
Metabolic syndrome related traits						
Body mass index ¥	-12.9	7.9	0.103	-14.6	6.1	0.017
Insulin	-19.1	10	0.056	-19.1	5.1	< 10-03
HOMA-IR	-16.4	10	0.101	-20.4	5.1	< 10-04
CRP	-12.3	11.7	0.293	-14.4	5.5	0.009

Model A: included covariates – gender, age and body mass index; Model B: covariates – gender, age and metabolic syndrome. _G: genetic correlation (%); _E: environmental correlation (%). SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; CRP: C-reactive protein. *P*: *P*-values were derived using Chi2 test, covariates gender, age and body mass index ¥ covariate gender and age only.

RESULTS

Characteristics of the ERF cohort are presented in table 1. The prevalence of the MetS, according to the IDF definition, was 29.3% in women and 37.1% in men. Virtually all mean values differed significantly ($P<0.01$) between genders.

To determine the association of plasma adiponectin with the MetS and related traits, we calculated the partial correlation coefficients of plasma adiponectin with each studied trait (supplemental table 2) adjusted for age and BMI. Plasma adiponectin showed a high correlation with the MetS (ρ= -0.20 and ρ= -0.13 in, respectively, women and men) and with the lipid components HDL-C and TG (all $\rho \geq 0.15$, $P< 0.01$). The correlation of plasma adiponectin with BMI (adjustment only for age), fasting plasma insulin, HOMA-IR and plasma CRP showed correlations coefficients ranging from 0.10 to 0.29 (all $P< 0.05$). In men, we observed in general for all components lower correlation coefficients, with the exception

Table 3: Association of variants in and around ADIPOQ with plasma adiponectin

Model A			Alleles	Frequency	Effect		
SNP	Position	n	M/m	(m)	(m)	SE	P
Prs864265	Pr	1918	G/T	0.19	-0.012	-0.023	0.601
rs822387	Pr	1917	C/T	0.95	0.146	0.040	**$3.0\ 10^{-4}$**
rs17300539	Pr	1919	A/G	0.95	0.155	0.040	**$9.3\ 10^{-5}$**
rs266729	Pr	1934	G/C	0.74	-0.025	-0.021	0.230
rs182052	Ex-1	1916	A/G	0.68	-0.070	-0.020	**$3.0\ 10^{-4}$**
rs822396	Ex-1	1925	A/G	0.22	0.043	0.021	0.049
rs2241766	In-1	1939	G/T	0.93	0.037	0.036	0.303
rs1501299	Ex-2	1921	T/G	0.73	0.057	0.021	**0.006**
rs3774262	Ex-2	1937	A/G	0.93	0.035	0.036	0.330
rs6773957	3'-UTR	1914	A/G	0.67	0.061	0.020	**0.002**
Model B			**Alleles**	**Frequency**	**Effect**		
SNP	**Position**	**n**	**M/m**	**(m)**	**(m)**	**SE**	**P**
rs864265	Pr	1904	G/T	0.19	-0.006	-0.023	0.788
rs822387	Pr	1904	C/T	0.95	0.143	0.040	**$3.6\ 10^{-4}$**
rs17300539	Pr	1905	A/G	0.95	0.166	0.040	**$2.7\ 10^{-5}$**
rs266729	Pr	1920	G/C	0.74	-0.017	-0.020	0.400
rs182052	Ex-1	1902	A/G	0.68	-0.066	-0.019	**$6.7\ 10^{-4}$**
rs822396	Ex-1	1911	A/G	0.22	0.043	0.022	**0.047**
rs2241766	In-1	1924	G/T	0.93	0.046	0.036	0.202
rs1501299	Ex-2	1907	T/G	0.73	0.055	0.021	**0.007**
rs3774262	Ex-2	1923	A/G	0.93	0.046	0.036	0.200
rs6773957	3'-UTR	1900	A/G	0.67	0.063	0.020	**0.001**

Model A: included covariates – gender, age and body mass index; Model B: covariates – gender, age and metabolic syndrome. m; effect allele. Pr; promoter, Ex; exon, In; intron, UTR; untranslated region. P: P-values based on Chi2 test. P-values printed in bold were selected for analysis in a backward linear regression model and were evaluated on linkage disequilibrium using Haploview (HAPMAP CEU).

of diastolic blood pressure.

We next estimated the heritability of plasma adiponectin and MetS related traits. Heritability estimates of the MetS and its individual components and BMI in ERF have been described earlier[1,13]. All heritability estimates were highly significant and none of the MetS related traits showed a significant sibship effect estimate (S). The heritability estimate of plasma insulin was 21.4 %, SE=5.2%, $P=<10^{-05}$ (S=6.1%, SE=4.2%, P=0.07), of HOMA-IR 22.0 %, SE=5.3%, $P=<10^{-05}$ (S=5.8%, SE=4.3%, P=0.08) and of plasma CRP level 21.0 %, SE=4.9%, $P=<10^{-05}$ (S=nihil). The highest heritability estimate was found for plasma adiponectin 55.1%, SE= 4.7, $P< 10^{-6}$ (S=nihil).

Next, we performed bivariate analysis of gender-stratified age-adjusted traits. We observed no significant difference in the heritability between the genders and found a high correlation (ρ =100%) between the heritability of plasma adiponectin in men (h^2 =59.6%, SE = 9.4) and women (h^2 =52.9%, SE = 7.7). Details on gender specific heritability of plasma insulin, HOMA-IR and plasma CRP are presented detail in supplemental table 3. Gender-stratified bivariate heritability estimates of the MetS and its individual components and BMI have been described earlier[1,13].

Bivariate heritability analyses were performed using trait by trait analyses on plasma adiponectin combined with the MetS and the MetS related traits. To investigate whether the interrelation between

the traits affected their genetic correlations, we used two statistical models: Model A included BMI as covariate and Model B included MetS as covariate in addition to age and gender in the trait by trait analyses. The outcomes of the bivariate heritability analysis of plasma adiponectin and the MetS and the MetS related traits are presented in table 2.

According to Model A, MetS, WC and HDL-C demonstrated a significant ($P<10^{-3}$) shared genetic component with plasma adiponectin (-42.7%, -32.4% and +24.7% respectively). TG, FPG and both SBP and DBP showed a low and insignificant shared genetic component with plasma adiponectin. BMI and plasma insulin showed a significant ($P< 0.04$) shared genetic component with plasma adiponectin of respectively -32.4%, -20.0%, while our finding for HOMA-IR was borderline significant (ρ G=-18.6%, P=0.06). The genetic correlation of plasma adiponectin and plasma CRP was found to be not significant. The environmental correlations of plasma adiponectin with plasma glucose (-12.0%), HDL-C (+35.6%), TG (-26.0%) and plasma CRP (-13.4%) exceeded their shared genetic component value. In contrast, the genetic correlation of plasma adiponectin and MetS was twice as high as their environmental correlation (ρ G = -42.7% and ρ E = -19.4%). According to Model B (covariates gender, age and MetS), HDL-C shares a significant (15.2%, $P< 0.05$) genetic component with plasma adiponectin. Plasma insulin shared a borderline significant (19.1%, $P = 0.056$) genetic component with plasma adiponectin. All observed environmental correlations obtained according to Model B exceeded their genetic correlations (Table 2).

To investigate to what extend the high heritability of plasma adiponectin is due to variants located in the *ADIPOQ* gene, we performed genetic association analysis. Table 3 presents association of ten *ADIPOQ* SNPs with plasma adiponectin, adjusted for gender, age and BMI (Model A) and adjusted for gender, age and MetS (Model B) . According to both Model A and B, nominally significant associations with plasma adiponectin were found for two promoter SNPs (rs822387 and rs17300539), one SNP located in exon 1 (rs182052), one SNP locate in exon 2 (rs1501299) and one SNP (rs6773957) located in the 3' untranslated region of the *ADIPOQ* gene. Four of these SNPs (except rs822396 and rs1501299) remained significant after conservative Bonferroni correction ($P<0.005$). The effect size of the promoter SNPs was substantially higher than the other significantly associated SNPs.

To determine the best model explaining plasma adiponectin we used the Bayesian information criterion (BIC)[17]. The parameters used in the BIC analysis for plasma adiponectin were gender, age, BMI and the *ADIPOQ* SNPs: rs822387, rs17300539, rs182052, rs1501299 and rs6773957 (N=1914). BIC values are presented in supplemental table 4. The lowest BIC value was found for the model including four *ADIPOQ* SNPs (rs822387, rs17300539, rs182052, and rs6773957).

Backward linear regression analysis of the five associated SNPs indicated that rs17300539 and rs182052 were independently associated with plasma adiponectin ($P<0.001$ and $P<0.004$, respectively, in the joint model). Evaluation of r^2 between the SNPs using Haploview, confirmed the independency of the two *ADIPOQ* SNPs ($r^2 = 0.2$, plot not shown). Next, we analyzed whether the two significant SNPs, *ADIPOQ* rs17300539 and rs182052, were also associated with the MetS and MetS related traits using two models. The first model included gender, age and BMI as covariates. The second model included gender, age BMI and plasma adiponectin as covariates. The second model was used to investigate whether MetS related traits were associated independently of plasma adiponectin. No significant associations were found for rs182052. *ADIPOQ* rs17300539 showed an significant association with plasma insulin (N= 1919, ß=0.072, SE=0.031, P= 0.022) and with HOMA-IR (N=1892, ß=0.084, SE=0.036, P= 0.021), according to model 2.

CONCLUSIONS

Here, we report a high heritability of plasma adiponectin (55.1%) and a similar genetic architecture between men and women. We also demonstrated that the genetic component of the HDL-C and plasma insulin overlap significantly with that of plasma adiponectin. The *ADIPOQ* rs17300539 and rs182052 were both found to contribute independently to the heritability of plasma adiponectin. Of these two SNPs, rs17300539 was also associated with plasma insulin and HOMA-IR illustrating their genetic overlap with adiponectin.

Our estimate of the heritability of plasma adiponectin is similar to heritability reported by Patel *et al*. (62%)[18]. Furthermore, our heritability estimates of the MetS related traits (plasma insulin, HOMA-IR and plasma CRP) are in agreement with earlier reports[19,20,21]. In our previous study[1] on the heritability of MetS and its individual components, heritability varied from 10.6% (MetS) to 42.9% (HDL-C). The heritability of plasma adiponectin is higher than the heritability of MetS or of its individual components, making it an attractive trait for genome wide association studies.

We did not find evidence for a gender-specific genetic component for plasma adiponectin[1,13]. Patel *et al*[18] studied in a large longitudinal family based cohort, the genetic correlation between plasma adiponectin and obesity traits and found that the genetic correlation (ρ G) of plasma adiponectin with BMI and WC was for both approximately -40%. This matches well with our estimate of the genetic correlation between adiponectin and BMI of -32.4% using gender, age and BMI as covariates (Model A). Furthermore, we found, using Model A, a high and significant shared genetic component between plasma adiponectin and the MetS (-42.7%). Moreover, this genetic correlation was twice as large as the environmental correlation. Furthermore, according to Model A, WC (-20.4%), HDL-C (+24.7%) and plasma insulin (-20.0%) shared a significant genetic component with plasma adiponectin. Since many of the study parameters are strongly associated with each other, we also studied the genetic correlations adjusting for MetS (Model B). Applying this conservative model, the genetic correlations found according to Model A were consistent, again revealing the genetic correlations between plasma adiponectin and HDL-C and plasma insulin. Our findings imply that genetic studies of plasma adiponectin might also lead to the identification of genes associated with HDL-C and plasma insulin.

The *ADIPOQ* gene has been found to be consistently and significantly associated with plasma adiponectin in genetic association studies or GWA[22,9]. To investigate the role of the *ADIPOQ* gene in the genetic overlap between plasma adiponectin and MetS traits, we performed association analysis using 10 *ADIPOQ* SNPs. Hivert *et al*[9] showed significant association of the *ADIPOQ* SNP rs17300539 with plasma adiponectin. Our study of 10 *ADOPOQ* SNPs showed that plasma adiponectin was significantly and consistently associated with six *ADIPOQ* variants using adjustment for gender, age and BMI (Model A) or adjustment for gender, age and MetS (Model B). Moreover, our study of 10 *ADOPOQ* SNPs showed that plasma adiponectin was significantly and independently associated with both *ADIPOQ* rs17300539 and rs182052 variants. *ADIPOQ* rs17300539 is located in the promoter region of the *ADIPOQ* gene whereas *ADIPOQ* rs182052 is located in exon 1 of the *ADIPOQ* gene. Whether these two variants are actually causal remains to be determined. Since our heritability and association analyses on plasma adiponectin and associated traits are in concert with findings of other studies in general cohorts or other genetically isolated populations, it seems unlikely that our findings are specific for the genetically isolated ERF cohort.

Plasma adiponectin is strongly associated with MetS, obesity, and in particular with plasma insulin and insulin sensitivity. There is evidence indicating that insulin directly affects plasma

adiponectin[23,24,25]. Thus it is likely that the genetics of plasma adiponectin and insulin overlap. Hivert *et al* reported that rs173766743 was associated with the incidence of T2D[9]. Our analyses showed that rs17300539 was also associated with plasma insulin and HOMA, independently of plasma adiponectin. We did not observed any effect on this association using MetS instead of BMI as covariate (data not shown). Since HOMA-IR is a measure for insulin resistance which reflects a pre-diabetic state, we analyzed the r^2 between *ADIPOQ* rs17300539 and rs173766743 in Haploview using HAPMAP CEU data. This analysis did not show any evidence of linkage disequilibrium between these variants (r^2=0). An explanation for this apparent discrepancy may be the adjustment for plasma adiponectin levels in our association analyses. Whether the association of rs173766743 with T2D is independent of plasma adiponectin was not reported. Since the association of rs17300539 with plasma insulin and HOMA-IR is independent of plasma adiponectin in our analyses, this implies a direct effect of this SNP on plasma insulin and insulin sensitivity. One explanation could be that *ADIPOQ* rs17300539 is associated with a functional variation of the adiponectin protein affecting insulin sensitivity independent of plasma levels of the protein. However, this association would have to be replicated in independent analyses before further investigation.

In conclusion, the present study confirms and extends the correlation of plasma adiponectin with HDL-C and plasma insulin. The high heritability of plasma adiponectin is promising for GWAS. The genetics of plasma adiponectin is similar between genders. The genetics of plasma adiponectin showed a significant and consistent overlap with the genetics of HDL-C and plasma insulin, which implies that GWAS of plasma adiponectin might also result in the detection of genetic variation associated with HDL-C , plasma insulin.

Genetic association analyses indicated that *ADIPOQ* variation is strongly associated with plasma adiponectin and indicated that *ADIPOQ* rs17300539 is associated with plasma insulin and HOMA-IR independently of plasma adiponectin. These genetic association data are thus in line with the observed genetic overlap between plasma adiponectin and plasma insulin.

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SUPPLEMENTARY MATERIAL

Supplemental table 1: Metabolic syndrome definition according to the International Diabetes Federation (IDF, 2006)[a]

_ Waist circumference (cm)	≥ 94
_ Waist circumference (cm)	≥ 80
Fasting plasma glucose (mmol/L)	≥ 5.6[b,c]
Systolic bloodpressure (mm Hg)	≥ 130[c]
Diastolic blood pressure (mm Hg)	≥ 85[c]
_ HDL-Cholesterol (mmol/L)	< 1.03[c]
_ HDL-Cholesterol (mmol/L)	< 1.29[c]
Triglycerides (mmol/L)	≥ 1.7[c]

a Europids
b included previously diagnosed type 2 diabetes patients
c included treatment patients.

Supplemental table 2: Partial correlation coefficients of plasma adiponectin with the MetS, its individual components and MetS related traits.

	Women (n=1258)	Men (n=967)
Metabolic syndrome and its individual components		
Metabolic Syndrome	-0.20 **	-0.13 **
Waist circumference	-0.23 **	-0.12 *
Glucose	-0.10 **	-0.04
HDL-cholesterol	0.35 **	0.27 **
Triglycerides	-0.21 **	-0.15 **
SBP	-0.03	0.01
DBP	0.03	-0.04
Metabolic syndrome related traits		
Body mass index ¥	-0.29 **	-0.20 **
Insulin	-0.17 **	-0.10 **
HOMA-IR	-0.18 **	-0.10 **
CRP	-0.12 **	-0.08 *

SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; CRP: C-reactive protein. Partial correlation coefficient: * P-value < 0.05; ** P-value < 0.01, covariates age and body mass index, ¥ covariate age only.

Supplemental table 3: Gender specific heritability of the MetS related traits

	Women			Men					
	h^2 (%)	SE	P	h^2 (%)	SE	P	_G	SE	P
Adiponectin	52.9	6.5	<10-15	59.6	7.5	<10-14	100.0	-	-
Insulin	28.2	7.4	<10-03	21.6	7.3	<10-03	100.0	-	-
HOMA-IR	28.1	7.7	<10-03	24.5	9.4	<10-03	100.0	-	-
CRP	32.2	8.1	<10-04	17.1	9.8	0.08	91.7	28.6	0.77

_G: genetic correlation (%); _E : environmental correlation (%). HOMA: Homeostatic Model Assessment-Insulin Resistance; CRP: C-reactive protein. P: P-values were derived using the Chi2 test.

Supplemental table 4: Bayesian information criterion for 5 ADIPOQ SNPs with plasma adiponectin

nr	rs822387	rs17300539	rs182052	rs1501299	rs6773957	BIC/1000
1	0	0	0	0	0	2.567
2	1	0	0	0	0	2.491
3	0	1	0	0	0	2.498
4	0	0	1	0	0	2.505
5	0	0	0	1	0	2.510
6	0	0	0	0	1	2.498
7	1	1	0	0	0	2.463
8	0	0	0	1	1	2.452
9	1	0	1	0	0	2.460
10	1	0	0	1	0	2.446
11	1	0	0	0	1	2.453
12	0	1	1	0	0	2.463
13	0	1	0	1	0	2.454
14	0	1	0	0	1	2.493
15	0	0	1	1	0	2.482
16	0	0	1	0	1	2.501
17	1	1	1	0	0	2.424
18	1	0	1	1	0	2.433
19	1	1	0	1	0	2.422
20	1	1	0	0	1	2.449
21	0	1	1	1	0	2.439
22	0	1	1	0	1	2.485
23	0	0	1	1	1	2.457
24	1	0	0	1	1	2.450
25	0	1	0	1	1	2.435
26	1	0	1	0	1	2.448
27	1	1	1	1	0	2.420
28	**1**	**1**	**1**	**0**	**1**	**2.410**
29	0	1	1	1	1	2.441
30	1	1	0	1	1	2.426
31	1	0	1	1	1	2.439
32	1	1	1	1	1	2.413

Thirty two Bayesian information criterion (BIC) models based on the log-likelihood estimates, calculated using the parameters gender, age, BMI and SNP(s). N=1914. BIC presented as BIC/1000.

Chapter 8

Clear detection of ADIPOQ locus as the major gene for plasma adiponectin: results of genome-wide association analyses including 4659 European individuals

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ABSTRACT

Objective. Plasma adiponectin is strongly associated with various components of metabolic syndrome, type 2 diabetes and cardiovascular outcomes. Concentrations are highly heritable and differ between men and women. We therefore aimed to investigate the genetics of plasma adiponectin in men and women.
Methods. We combined genome-wide association scans of three population-based studies including 4659 persons. For the replication stage in 13795 subjects, we selected the 20 top signals of the combined analysis, as well as the 10 top signals with p-values less than $1.0*10^{-4}$ for each the men- and the women-specific analyses. We further selected 73 SNPs that were consistently associated with metabolic syndrome parameters in previous genome-wide association studies to check for their association with plasma adiponectin.
Results. The *ADIPOQ* locus showed genome-wide significant p-values in the combined ($p=4.3*10^{-24}$) as well as in both women- and men-specific analyses ($p=8.7*10^{-17}$ and $p=2.5*10^{-11}$, respectively). None of the other 39 top signal SNPs showed evidence for association in the replication analysis. None of 73 SNPs from metabolic syndrome loci exhibited association with plasma adiponectin ($p>0.01$).
Conclusions. We demonstrated the *ADIPOQ* gene as the only major gene for plasma adiponectin, which explains 8.7% of the phenotypic variance. We further found that neither this gene nor any of the metabolic syndrome loci explained the sex differences observed for plasma adiponectin. Larger studies are needed to identify more moderate genetic determinants of plasma adiponectin.

INTRODUCTION

Plasma adiponectin is a quantitative parameter, which has a strong role in modulating insulin sensitivity and glucose homeostasis. It has been found to be decreased in humans with type 2 diabetes and cardiovascular disease (CVD)[1,2] and decreased plasma adiponectin was found to be associated with deteriorated levels of virtually all parameters of the metabolic syndrome[3-5]. Experiments in mice transgenic or deficient for the adiponectin gene have underscored the functional role of adiponectin on various components of the metabolic syndrome and diabetes mellitus[3,6,7].

Concerning CVD outcomes the observations on adiponectin are heterogeneous as recently reviewed extensively[8]: experimental data demonstrate that adiponectin stimulates the production of nitric oxide, positively affects inflammatory mechanisms, has anti-apoptotic properties and is involved in vascular remodeling. Clinical data are diverse depending mainly on the disease stage when investigated. Low levels seem to be associated with worse outcomes when measured in healthy conditions. However, there is accumulating data that in diseased states such as chronic heart failure or existing CVD high rather than low levels predict CVD and non-CVD mortality. Knowing the genes which affect plasma adiponectin might be helpful to disentangle adiponectin as cause or consequence of disease states using a Mendelian randomization approach[9].

Plasma adiponectin shows pronounced differences between men and women with about 1.5 times higher concentrations in women[10]. An explanation for these differences is lacking as plasma adiponectin is only moderately influenced by nutritional behavior, physical activity or other environmental components[5,8,11]. However, there is clear evidence for a high heritability of about 50%[4,12-14] which one study even suggested to be sex-dependent[14]. In line with lower plasma adiponectin in men, higher prevalences of type 2 diabetes and impaired fasting glucose were also reported in men[15].

Recent genome-wide association (GWA) scans have highlighted the potential of genetic factors with differential sex effects on concentrations of uric acid[16-18] and lipids[19], waist circumference[20] or schizophrenia[21]. Many of these phenotypes show pronounced sex-specific differences in plasma concentrations or prevalence. A sex-differential SNP association with a quantitative phenotype can even mask a real association if data are analyzed without stratification. One example is a SNP near the *LYPLAL1* gene which recently showed a strong association with waist-hip-ratio in women but not in men and would have been missed in the sex-combined analysis[20]. To our knowledge, sex-specific differences for genetic effects on plasma adiponectin have not been investigated so far.

In the study at hand, we aimed to identify not only novel genes modulating plasma adiponectin but also whether genetic effects are differential between men and women. We combined this meta-analysis with a candidate gene approach considering all genes which have recently been associated with singular components of the metabolic syndrome in GWA studies.

METHODS

STUDY COHORTS AND GENOTYPING

Our gene discovery included 4659 subjects (women=2562, men=2097) derived from three population-based studies, the Erasmus Rucphen Family Study (ERF, n=1820)[22], the follow-up of the third survey from the "Kooperative Gesundheitsforschung in der Region Augsburg" Study (KORA-F3, n=1644)[23], and the MICROS Study (n=1195)[24]. The replication contained 13795 subjects (women=7673, men=6122

from the study cohorts CoLaus (n=5381), Framingham (n=2228), GEMS (n=1780), ALSPAC (n=1415), TWINS UK (n=1399), InChianti (n=1027) and BLSA (n=565).

All studies had genotypes available from genome-wide SNPs imputed based on the HAPMAP CEU r22 reference sample after quality control. Measurement of adiponectin was made by ELISAs (from Mercodia, BioVendor and R&D Systems) or RIA (Linco). Details on study cohorts including the phenotyping for adiponectin measurements, genotyping methods, statistical analysis, and descriptive statistics are provided in the Supplementary Material and Supplementary Table S1.

STUDY DESIGN AND STATISTICAL ANALYSIS

The study design is summarized in Figure 1. GWA analyses (stage 1): GWA analyses were conducted using a standardized protocol in each of the three stage 1 studies. For each of the 2,585,854 SNPs, linear regression using an additive genetic model was performed for log-transformed adiponectin values adjusting for age, sex, and BMI and accounting for the uncertainty in the inferred genotype from the imputation by utilizing the estimated genotype probabilities (implemented in MACH2QTL and GenABEL/ProABEL, respectively). All analyses were repeated for men and women separately. Relatedness between study participants was accounted for where appropriate (ERF, MICROS). Genomic control was applied when appropriate with study-specific lambda factors being 1.05, 1.05, and 0.99 for ERF, KORA, and MICROS, respectively. The beta-estimates of the three cohorts were combined using a fixed effect model. Also, a scaling-invariant p-value pooling meta-analysis using a weighted Z-score method was applied. For each SNP, we tested for significant differences between pooled men-specific beta-estimates across the three GWA studies as well as women-specific beta-estimates (see Supplementary Material for details).

GWA SNP selection: We selected three types of interesting regions to identify potentially novel signals for plasma adiponectin: (1) from the sex-combined sample (20 loci), (2) from the analysis in women (10 loci) and (3) in men (10 loci). Loci were considered as interesting and one SNP per locus was selected, if the combined p-values were less than 1*10-4 and if study-specific MAF was greater than 5% and imputation quality r^2 greater than 0.2.

Replication analysis (stage 2): For the selected 40 SNPs, we attempted replication based on 7 studies with the same study-specific SNP analysis as for stage 1 studies. A stage 2 only and a joint analysis of stage 1 and 2 (n=18454) was performed using the scaling-invariant weighted Z-score method.

Further statistical issues: In stage 1, we had 92% power to detect a variant that explains 1% of the variance of plasma adiponectin with genome-wide significance (alpha=5*10-8). In the stage 1 and stage 2 combined analysis, we had 99% power to yield genome-wide significant evidence for the 40 selected SNPs if they explained 1% of more of the variance in plasma adiponectin.

Candidate gene approach: From the literature, we identified loci associated with metabolic syndrome parameters in large GWA studies to obtain a list of candidate gene SNPs for adiponectin levels. We examined the association of these SNPs with plasma adiponectin from our stage 1 sex-combined and sex-stratified meta-analyses. For this candidate gene approach, we had 92% power to detect a SNP association that explains 0.5% of the variance accounting for the 73 SNPs tested (alpha=0.0007).

Percentage of variance explained: The general population design of KORA enabled computation of the proportion of the adiponectin variance explained by all analyzable ADIPOQ SNPs (i.e. SNPs

available in all three GWA studies in the 50kb region of the ADIPOQ locus with MAF>5%), by an independent SNP set of these (i.e. selecting the SNP with the lowest p-value in the meta-analysis for each bin of SNPs with pairwise r^2>0.2; r^2 information was taken from HAPMAP), or by the top SNP alone. Computations were performed by linear regression on the standardized residuals (log of adiponectin concentrations adjusted for age, BMI and – if appropriate – for sex) and computing the R^2 measure of the model adjusting for the SNP(s) using PROC REG by SAS.

Heritability: The family-based design of MICROS allowed us to compute heritability of plasma adiponectin using a polygenic model for standardized residuals of plasma adiponectin (adjusted for age and BMI - and sex if applicable). Heritability was also computed with additional adjustment of the top ADIPOQ SNP, with the independent SNP set as described above (see above). Computations were performed using the R library GenABEL[25].

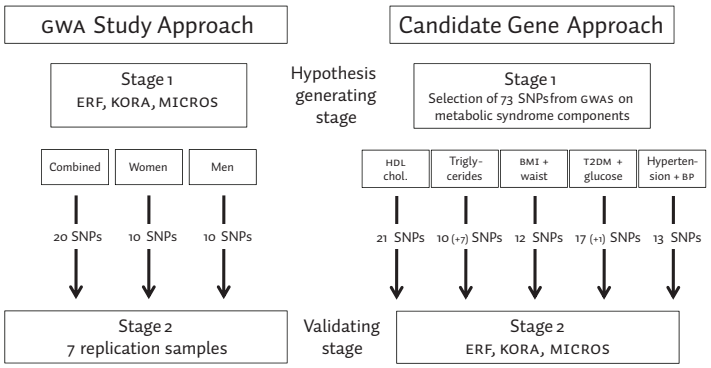


Fig.1. Study design illustrating the genome-wide association (GWA) study approach and the candidate gene approach.

Bioinformatic analysis: Bioinformatic analysis for potential functional SNPs was done in two stages, using bioinformatic tools outlined in the GenEpi Toolbox[26] (Supplementary Material).

RESULTS

GWA ANALYSIS (STAGE 1)

Figure 2 shows the p-value, *ADIPOQ*-region and q-q-plots from the meta-analysis results of plasma adiponectin of the three GWA studies, ERF, KORA and MICROS cohorts. Results are presented for the sex-combined (n=4659) analysis as well as stratified for women (n=2562) and men (n=2097). The combined analysis yielded one genome-wide significant locus (Figure 2A), the *ADIPOQ* locus ($p=4.3*10^{-24}$), which was consistent in women ($p=8.7*10^{-17}$) and men ($p=2.5*10^{-11}$) (Figure 2B). The q-q plot did not show evidence for bias due to population stratification in any of the analyses (Figure 2C). The top *ADIPOQ* SNP rs17366568 (Table 1) exhibited low imputation quality in ERF and MICROS that was genotyped using the Illumina platform in contrast to high imputation quality in KORA genotyped using the Affymetrix platform. However, other SNPs in this region such as rs3774261 reached genome-wide significance in the combined analysis ($p=3.0*10^{-16}$) and had good imputation quality in all three stage 1 samples ($0.82<r^2<0.97$).

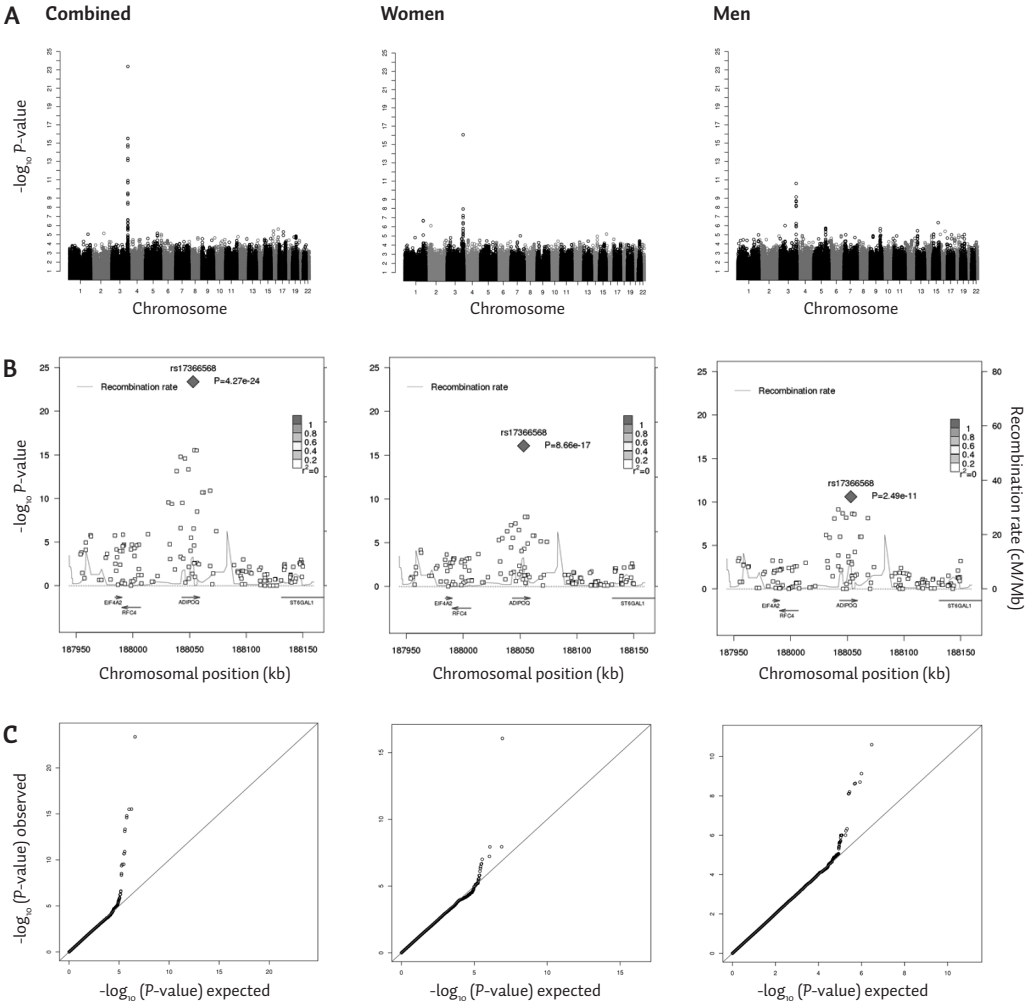


Fig.2. The analyses in panel A-C are provided for the combined sex analysis as well as the analysis stratified for women and men. A. Manhattan plots showing p-values of association of each SNPs in the meta-analysis with plasma adiponectin levels. SNPs are plotted on the X-axis to their position on each chromosome against association with plasma adiponectin on the Y-axis (shown as –log10 P-value). B. Regional Manhattan plots showing significance of association of all SNPs in the ADIPOQ region (3q27). SNPs are plotted on the X-axis to their position on chromosome 3 against association with plasma adiponectin on the Y-axis (shown as –log10 P-value). In each panel, the top-SNP rs17366568 is shown as red diamond. The SNPs surrounding this top-SNP are color-coded (see inset) to reflect their LD with the top-SNP using pair-wise r_ values from the KORA study. Estimated recombination rates from HAPMAP-CEU are plotted in blue to illustrate the local LD structure on a secondary Y-axis. Genes and their direction of transcription are provided below the plots using data from the UCSC genome browser. C. Quantile-quantile (QQ) plots of SNPs. Expected p-values are plotted on X-axis against the observed p-values plotted on the Y-axis.

REPLICATION ANALYSIS (STAGE 2)

Characteristics of the 40 SNPs taken forward for replication are provided in Supplementary Table S2. From the combined, women-, and men-specific GWA-analyses (n=13795, 7673, and 6122, respectively), only the *ADIPOQ* SNP remained significant in the combined analyses (Supplementary Table S3). P-values for rs17366568 were $1.09*10^{-41}$, $2.8*10^{-22}$ and $7.8*10^{-23}$ for the combined and the analysis stratified for women and men, respectively (Table 1).

SEX-SPECIFIC ANALYSES

In line with previous reports, plasma adiponectin in women was approximately 1.5 times higher than in men in each of the three stage 1 studies (Supplementary Table S1). Heritability computations in the family-based MICROS study showed slightly higher estimates of 65.1% for women and 54.0% for men (Table 2).

For each SNP, we evaluated whether the sex-specific beta-estimates combined across the three stage 1 studies were significantly different between men and women pointing towards a gender-SNP interaction. The q-q plot for the p-values of sex differences indicated some observed sex difference of genetic effects beyond that expected by chance (Supplementary Figure S1A), but not due to differences in the *ADIPOQ* region. For none of the SNPs in the GWA studies, the sex-specific beta-estimates were significantly different between men and women on a genome-wide level (Supplementary Figure S1B). For the *ADIPOQ* top SNP rs17366568 the p-value for sex difference was 0.62.

ASSOCIATION OF METABOLIC SYNDROME CANDIDATE GENE SNPS WITH ADIPONECTIN

From the literature, we identified loci associated with metabolic syndrome parameters in large GWA studies to obtain a list of candidate gene SNPs for adiponectin levels (Figure 1). These were partially overlapping for the various metabolic syndrome components and included 21 SNPs for HDL cholesterol, 17 for triglycerides (7 of them were also found for HDL cholesterol and were therefore only

Table 1: Genome-wide significant association of the rs17366568 (G>A) SNP in the ADIPOQ locus

				Combined			**Women**			**Men**	
Population	EAF*	Rsqr†	n	Beta‡	P	n	Beta‡	P	n	Beta‡	P
Stage 1											
ERF	0.91	0.37	1817	0.103	2.7E-07	1052	0.115	1.0E-05	765	0.088	0.004
KORA	0.89	0.91	1643	0.173	1.7E-15	830	0.204	1.9E-11	813	0.142	5.8E-06
MICROS	0.90	0.27	1195	0.114	3.0E-06	678	0.102	4.1E-04	517	0.182	1.6E-05
Combined**	0.90	-	4655		4.3E-24	2560		8.7E-17	2095		2.5E-11
Stage											
Colaus	0.88	1.00	5261	0.132	3.0E-13	2759	0.119	1.1E-06	2502	0.146	5.1E-08
Framingham	0.88	1.00	2220	0.072	0.003	1213	0.050	0.108	1007	0.094	0.012
GEMS	0.87	1.00	1780	0.149	2.9E-06	732	0.084	0.095	1048	0.194	2.1E-06
ALSPAC	0.92	0.37	1415	0.395	2.9E-14	691	0.453	9.4E-09	724	0.351	3.5E-07
TWINS UK	0.998	NA	1399	0.154	0.078	1399	0.154	0.078	-	-	-
InChianti	0.94	NA	1027	-0.056	0.481	562	-0.130	0.268	465	0.007	0.95
BLSA	0.92	0.61	565	0.263	0.004	266	-0.028	0.822	299	0.488	2.5E-04
Combined**	0.89	-	13667	-	5.2E-22	7622	-	2.7E-10	6045	-	8.1E-14
Stage 1 + 2											
Combined**	0.89	-	18322		1.1E-41	10182		2.8E-22	8140		7.8E-23

* EAF = effect allele frequency (i.e. frequency of G) for sex-combined analysis

† Rsqr = imputation certainty

‡ Beta estimate from linear regression adjusted for age, BMI, and (if appropriate) for sex per unit change [log(µg/mL)] for the risk allele G

** Results are provided for a beta-pooling meta-analysis using the fixed effect model weighting for the inverse variance. When a scaling-invariant p-value pooling meta-analysis using the sample size weighted z-score method was applied for sensitivity analysis, we found no major differences between both methods.

counted once), 12 for BMI and/or waist circumference, 18 for type 2 diabetes and/or glucose levels (one of them was already mentioned for BMI and is therefore only counted once), and 13 for hypertension and blood pressure. Details on these SNPs are given in Supplementary Table S4.

Only 3 out of the 73 SNPs showed p-values between 0.01 and 0.05 for example for the gender-combined analysis (with 3.65 expected under the assumption of no association). No p-value was below the Bonferroni-adjusted significance level of 0.007. Thus, our data indicated no association of these metabolic syndrome parameter SNPs with plasma adiponectin.

SENSITIVITY ANALYSES

Sensitivity analyses repeating all analyses without the adjustment for BMI showed the same results regarding the *ADIPOQ* genome-wide significant results, the lack of sex difference, the lack of other SNPs in the replication stage to show replication, and the lack of metabolic syndrome SNPs to show association with plasma adiponectin.

Table 2: Heritability and percentage of variance explained by the ADIPOQ locus SNPs: Heritability of plasma adiponectin in the family-based study MICROS and percentage of plasma adiponectin variance (KORA) explained by the ADIPOQ locus SNPs in KORA (region on chr 3, position 188.030 – 188.080kb).

	Combined	Women	Men
Heritability (%) in MICROS			
no SNP adjustment	59.6	65.1	54.0
adjusted for top hit rs17366568	58.4	64.6	51.5
adjusted for "independent "SNPs (n=9)[a]	52.9	55.1	48.1
% of variance of plasma adiponectin in KORA explained by			
top hit rs17366568	3.8	5.3	2.4
for "independent" SNPs (n=9)[a]	5.9	6.3	5.1
all SNPs with MAF >5% (n=33)[b]	6.7	6.4	5.5

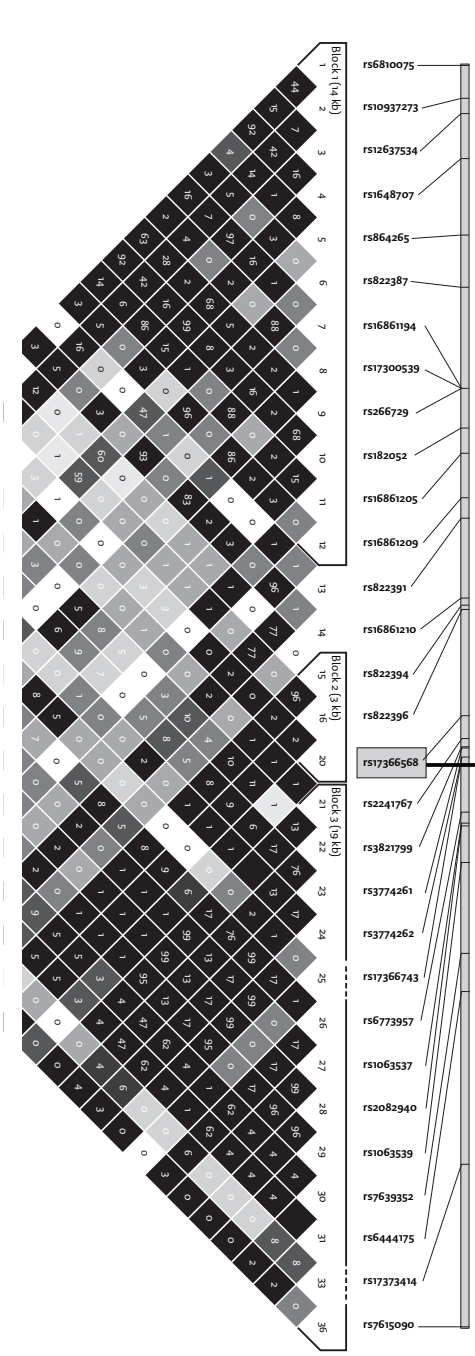
Computations were based on standardized sex-combined or sex-specific residuals of plasma adiponectin adjusted for age (and sex if applicable) and BMI without and with additional SNP adjustment; includes only SNPs with MAF>5% available in all three studies.

[a] Among the SNPs of the ADIPOQ region with MAF > 5% and available in all three GWA studies: selecting the SNP with the smallest p-value from each bin of SNPs with pairwise r_>0.2: rs1063539, rs16861194, rs17300539, rs17366568, rs17366743, rs3774261, rs6810075, rs7615090, rs822394

[b] All SNPs of the ADIPOQ region with MAF > 5% and available in all three GWA studies: rs6810075, rs10937273, rs12637534, rs1648707, rs864265, rs822387, rs16861194, rs17300539, rs266729, rs182052, rs16861205, rs16861209, rs822391, rs16861210, rs822394, rs822396, rs12495941, rs7649121, rs17366568, rs2241767, rs3821799, rs3774261, rs3774262, rs17366743, rs6773957, rs1063537, rs2082940, rs1063539, rs7639352, rs6444175, rs7628649, rs17373414, rs9860747, rs1501296, rs7615090

ADIPOQ REGION

A closer look at the *ADIPOQ* region revealed that the top SNP rs17366568 was completely independentofallother SNPs in that region. A linkage disequilibrium (LD) plot depicting D' and r^2 measures (Figure 3) revealed that for many SNPs in the *ADIPOQ* region the r^2 was weak even if they were located in the same LD block (as defined by D'). At least nine SNP groups were significantly and independently associated with plasma adiponectin. The percentage of plasma adiponectin variance explained by the top hit was 3.8% and increased to 5.9% when including an independent SNP set (selecting the SNP with the smallest p-value in each bin of pairwise r^2<0.2) and peaked at 6.7% when including all SNPs with MAF>5% in the 50 kb region covering the three LD blocks (Table 2).



A/I	z-score	P-value	group	
	6.30	3.0e-10	3	
I	4.30	1.7e-05	4	
A	3.32	0.0009	5	
I	6.24	4.3e-10	3	
A	-2.46	0.014		
I	-7.48	7.7e-14	2	
	3.50	0.0005	5	
	7.97	1.6e-15	2	
	5.17	2.4e-07	3	
A	-6.29	3.1e-10	3	
A	-3.37	0.0007	5	
	7.91	2.5e-15	2	
	-0.83	0.41		
	7.54	4.7e-14	2	
	2.70	0.007		
I	-1.45	0.15		
A	-10.13	4.3e-24	1	Top-hit
A	-2.99	0.003 7		
A	4.23	2.4e-05	6	
A	8.17	3.0e-16	6	
I	3.03	0.002 7		
	1.65	0.1		
A	8.17	3.2e-16	6	
	3.04	0.002 7		
	3.03	0.002 7		
	2.92	0.003 7		
	6.70	2.1e-116		
I	6.70	2.0e-11	6	
	6.78	1.3e-118		
I	5.02	5.3e-07	9	

Fig.3. Linkage disequilibrium (LD) plot of SNPs in the ADIPOQ region spanning 50kb (positions 188030-188080kb). The grey shading of the diamonds represent the pair-wise D' and the numbers in the diamonds represent the pair-wise r_ between the two SNPs defined by the top left and the top right sides of the diamond. The figure clearly shows that the top-hit rs17366568 is located within an own LD block and shows virtually no correlations with any other SNP in the entire 50kb region. The columns on the right side of the Figure show i) whether a particular SNP is genotyped by the Affymetrix 500K chip (A) or the Illumina HumanHap300 chip (I); all other SNPs are imputed; ii) the z-scores and iii) the p-values for each SNP-adiponection association for the combined analysis of the cohorts ERF, KORA and MICROS; iv) SNPs that are correlated with an r_>0.60 are grouped in groups 1-9.

Bioinformatic analysis revealed two main putative functional elements located in the second and the third LD block as depicted in Figure 3. Three SNPs located immediately up- and downstream of rs17366568 (for details see Supplementary Table S5) are predicted to affect 10, 6 or 4, respectively, transcription factor binding sites (using adipose tissue-specific analysis). No transcription factor binding sites or splicing regulation elements were detected for rs17366568 itself. Therefore, it is likely that rs17366568 is not the functional variant, but relates to a functional element located in the immediate vicinity (although regulatory potential was very low throughout the region).

Analysis of LD block 3 (encompassing exon 3 and a large intergenic region downstream of the *ADIPOQ* locus) revealed three putative regulatory promoter regions located approximately 5.1 kb, 6.3 kb and 15.8 kb downstream of the *ADIPOQ* locus. Interestingly, especially the proximal two regulatory regions are known to be affected by several copy number regions (see Figure 4 and Supplementary Table S6). However, no SNP in our data set was located directly in these CNVs, whose functional relevance may therefore require further investigation. More generally, the whole genomic region of ADIPOQ seems to be highly affected by copy number variations (Figure 4).

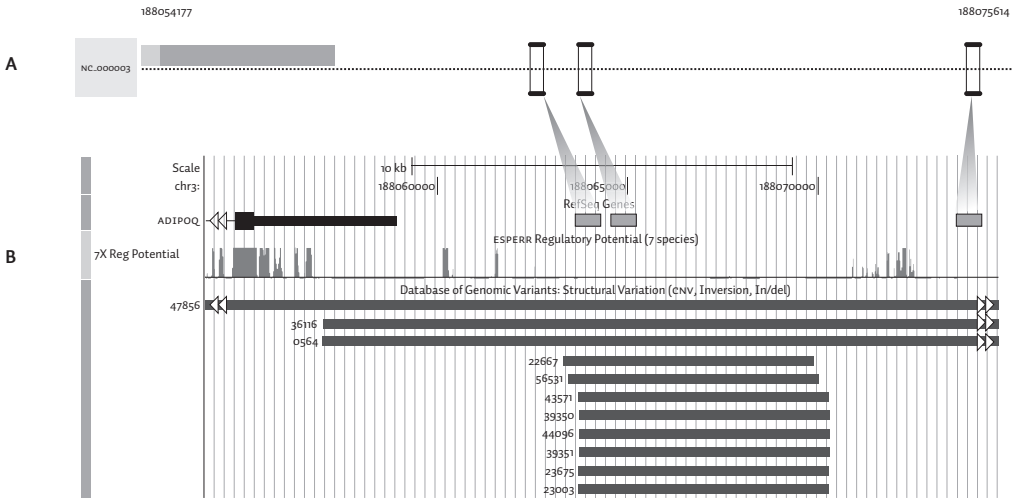


Fig.4. Predicted regulatory regions in the ADIPOQ downstream region and their affection by copy number variations. Panel A: Genomatix Software Suite: Position of the regulatory promoter regions predicted by PromoterInspector (red boxes) and their position relative the ADIPOQ gene region (green box). Panel B: UCSC Browser: Position of the predicted regulatory regions (red boxes) relative to known copy number variations in the ADIPOQ gene region (represented by bold blue lines). The numbers on the left side correspond to the accession number of the respective copy number variation in the Database of Genomic Variants.

DISCUSSION

In the meta-analysis of genome-wide SNP association with plasma adiponectin in three population-based studies including a total of 4655 subjects, we found genome-wide significant evidence for the association with the *ADIPOQ* locus, which is a known locus for plasma adiponectin[10,27]. Furthermore, we did not identify any genome-wide significant evidence for association in any other locus when replicating the other 39 most strongly associated loci in 13795 independent samples. Despite the clear sex difference in plasma adiponectin, there was no sex difference observed for the *ADIPOQ* SNP associations. Finally, we found, despite the strong association between plasma adiponectin and

the metabolic syndrome, no significant association with adiponectin for any of the chosen variants within reported loci for metabolic syndrome parameters.

Our GWA study identified only one major locus for plasma adiponectin, the *ADIPOQ* gene region. The only other GWA study on adiponectin was performed in 1845 individuals of the GEMS Study and identified also only the *ADIPOQ* locus with genome-wide significance[28]. The other top seven hits from that study could not be replicated in our GWA study, neither in the combined (all p-values >0.28) nor in the sex-specific analysis (p>0.16). In our GWA discovery stage, the power was more than 90% to detect novel loci which explain 1% of the adiponectin variance, and, including the replication stage, over 99% to show genome-wide significant evidence of the 40 SNPs in the 18454 subjects. Therefore, our data suggests a lack of a major gene locus other than *ADIPOQ*.

ADIPOQ was studied earlier as a candidate gene and the relationship to plasma levels has long been recognized. The SNP rs17366568 showing the strongest association in our GWA study explained 3.8% of the variance and this number increased to 6.7% if all analyzable SNPs in the *ADIPOQ* region were included into the model. This pronounced difference of the explained variance between the two models can be explained by a large number of SNPs independently contributing to adiponectin levels. The SNPs contributing most to the explained variance are not only located in the three different LD blocks but also several genetic variants within each of at least two of the three blocks contribute to the explained variance. In total, the explained variance was very similar to the 8% reported earlier [(10)]. Functional studies within the promoter of the *ADIPOQ* gene revealed a pronounced influence of three SNPs also investigated in our study and the corresponding haplotypes on the promoter activity which was accompanied by changes in the DNA binding activity interfering with transcription factor bindings sites[29]. Other studies showed that histone acetylation might influence the transcriptional regulation of the *ADIPOQ* gene[30] and that pioglitazone increases plasma adiponectin by posttranscriptional regulation[31]. Finally, an extensive bioinformatic analysis revealed that the *ADIPOQ* region might be a highly copy number variable region. It remains to be determined how strong the effect of these CNVs on plasma adiponectin is.

Since adiponectin has been viewed as a marker for the metabolic syndrome, we have also studied 73 SNPs that have been associated with any of the major determinants of metabolic syndrome in previous GWA studies. This candidate gene-based analysis did not yield any convincing associations with plasma adiponectin. This was surprising due to the strong link between plasma adiponectin and the metabolic syndrome or any of its components[3-5], but in-line with previous reports on a lack of association of the *ADIPOQ* SNPs with metabolic syndrome parameters[10]. Whether plasma adiponectin affects metabolic syndrome parameters or metabolic syndrome parameters modulate adiponectin is highly debated as illustrated in Supplementary Figure S2. If the association of any of these 73 SNPs had been very strong with adiponectin - stronger than with the metabolic syndrome parameters - this would have pointed towards a gene locus primarily affecting plasma adiponectin and consecutively modulating the metabolic syndrome parameters. This is not suggested by our data (panel A of Supplementary Figure S2). Our data on these 73 metabolic syndrome SNPs lacks association with adiponectin beyond that expected by chance. This would rather support the idea that genetic pathways for plasma adiponectin are different from the pathways depicted by these 73 loci (panel B), or, alternatively, that pathways depicted by these 73 loci affect plasma adiponectin via the metabolic syndrome parameter and the lack of association was due to loss of power for a parameter further down the road (panel C). Both ideas (panel B and C) would point towards the hypothesis that genetically determined adiponectin does not modulate metabolic syndrome parameters directly.

The present data suggests that the sex differences in plasma adiponectin can not be explained by any major gene. The GWA approach yielded no genome-wide significant difference between men and women for any SNP, not even the *ADIPOQ* locus. In fact, none of the variants studied in the replication or in our candidate gene approach based on metabolic syndrome loci showed a significant sex difference. Therefore, the sex-difference in plasma adiponectin might rather be explained by sex hormones[5] or sex-specific epigenetic programming that could be transmitted to subsequent generations in a sex-specific manner leading to transgenerational effects as recently suggested[32].

The heritability estimates of plasma adiponectin are high with roughly 50-60%[4,12-14]. The *ADIPOQ* locus accounts for 6.7% of the variance in our populations-based KORA Study, which is in-line with previous reports[10]. This is also in-line with 6.6% of the heritability accounted for by this locus in our family-based MICROS Study. While the *ADIPOQ* locus association with plasma adiponectin is thus among the strongest associations for quantitative phenotypes in genetic epidemiology, it explains only a small proportion of the overall heritability, a puzzle observed for many other phenotypes (e.g. lipids or obesity measures)[19,20,33]. Potential explanations of this gap between explained and estimated heritability are unknown rare variants with strong effects on adiponectin[34], unknown common loci influencing adiponectin with small effects, or deflation of association estimates due to heterogeneity between studies, uncertainties in the genotypes from imputation or uncertainties in the phenotype assessment. Our study suggests that these other genetic variants influencing plasma adiponectin are variants that explain less than 1% of the phenotypic variance. To localize these loci and to build up gene networks which identify even trans-acting quantitative trait loci, will require substantially larger data sets in combination with gene expression analysis.

STRENGTHS AND LIMITATIONS OF THE STUDY

A limitation of our study is the limited sample size for gene discovery for small genetic effects, in particular when conducting stratified analyses. Furthermore, our top hit in the *ADIPOQ* locus had limited imputation quality in two of the included GWA studies, which can be explained by the fact that KORA used a different SNP-panel (Affymetrix 500K chip) for GWAS genotyping than ERF and MICROS (Illumina HumanHap300). For most of the other SNPs followed in replication samples, the imputation quality was quite high. The relatively low imputation quality of our top-hit in two of the studies explains the lower (but still genome-wide significant) p-values in these two studies compared to KORA. This is entirely in-line with measurement error theory: a "measurement error" (like the uncertainty induced by the imputation) that does not depend on the phenotype (as the case here assuming that genotyping does not depend on adiponectin in the plasma) is expected to attenuate the precision of an underlying association yielding larger p-values. Therefore, the association in ERF and MICROS was rather underestimated than false positive. Finally, it can be considered a limitation of most GWAS studies that gonsomes are not analyzed due to technical issues not yet solved concerning the imputation of SNPs which, however, is a prerequisite to allow meta-analysis of data over various genotyping platforms used.

The strong point of our study is the population-based design, in which the participants have not been ascertained based on the presence of pathology. Hypothesizing a genetic basis of sex differences in plasma adiponectin, a further advantage is the sex-stratified analysis since a sex-combined analysis would otherwise mask an association. Further, the family-based MICROS study enables us to estimate heritability.

CONCLUSIONS

We present a genome-wide association study on adiponectin which the first time attempts to explain adiponectin sex difference by the underlying genetics. We conclude that there is no major gene involved in modulating plasma adiponectin other than the known *ADIPOQ* locus and that there is no major gene explaining the differences of plasma adiponectin between men and women.

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SUPPLEMENTARY MATERIAL

STUDY COHORTS

All participants in all studies gave informed consent and each study was approved by the appropriate Research Ethics Committees. Body-mass-index (BMI, weight divided by height^2) was assessed measuring weight and height in the study-center or by self-report. Details on adiponectin assays used for phenotyping and descriptive statistics are provided in Supplementary Table S1.

GENOME-WIDE ASSOCIATION STUDY COHORTS

Erasmus Rucphen Family study (ERF): The Erasmus Rucphen Family (ERF) study is comprised of a family-based cohort embedded in the Genetic Research in Isolated Populations (GRIP) program in the Southwest of the Netherlands. Descriptions of ERF's design have been previously published (Aulchenko et al., 2004). Briefly, twenty-two families that had a minimum of five children baptized in the community church between 1850 and 1900 were identified with the help of detailed genealogical records. All living descendants of these couples, and their spouses, were invited to take part in the study. Participants included in the current study total 2079 individuals for whom complete phenotypic and genotypic information was available. Covariates were obtained during the baseline examination.

KORA studies: The KORA cohorts (Cooperative Health Research in the Region of Augsburg, KOoperative Gesundheitsforschung in der Region Augsburg) are several cohorts representative of the general population in Augsburg und two surrounding counties that were initiated as part of the WHO MONICA Study. The KORA S3 is a survey examined in 1994/95 with standardized examinations described in detail elsewhere (Löwel et al., 2005). Ten years age-sex strata have been sampled from the 25 to 74 year old population with a stratum size of 640 subjects. 3,006 individuals participated in a follow-up examination of S3 in 2004/05 which is called KORA F3. All study participants underwent a standardized face-to-face interview by certified medical staff and a standardized medical examination including blood draw and anthropometric measurements. The 1644 subjects for the KORA GWA analysis (the KORA S3/F3 500K study) were chosen from KORA F3.

Microisolates in South Tyrol Study (MICROS): The MICROS study is part of the genomic health care program 'GenNova' and was carried out in three villages of the Val Venosta, South Tyrol (Italy), in 2001-03. It comprised members of the populations of Stelvio, Vallelunga and Martello. A detailed description of the MICROS study is available elsewhere (Pattaro et al., 2007). Information on the participant's health status was collected through a standardized questionnaire. Laboratory data were obtained from standard blood analyses. Covariates were obtained during the interview phase.

Genome-wide genotyping had been performed using the Illumina 300K array of the HumanHap300 (ERF, MICROS) or the Affymetrix 500k array (KORA-F3).

REPLICATION STUDY COHORTS

CoLaus (Caucasian Cohorte Lausannoise) Study: The CoLaus study investigates the epidemiology and genetic determinants of cardiovascular risk factors and metabolic syndrome. The 3251 females and 2937 males Caucasian participants, aged between 35 and 75 years, were selected using a simple

non-stratified random sample of the population registry of the city of Lausanne, Switzerland, as previously described (Firmann et al., 2008). Participation rate was 41%. Recruitment began in June 2003 and ended in May 2006. All participants attended the outpatient clinic of the University Hospital of Lausanne in the morning after an overnight fast. From 5435 participants genotypes are available from genotyping with Affymetrix chips (GeneChip Human Mapping 500K array and the BRLMM calling algorithm). Adiponectin levels were available for 5381 study participants and were measured by ELISA assay (R&D systems, Minneapolis, MN). Statistical analyses was conducted using Quicktest v0.94.

Framingham: The Framingham Heart Study has investigated risk factor determinants of CVD over decades in a general population (Dawber et al., 1966). It began in 1948 with the recruitment of 5209 residents aged 28-62 years in about two-thirds of the households in the town of Framingham, Massachusetts. Participants have undergone biennial examinations since the study began. In 1971, the Framingham Offspring Study (Kannel et al., 1979) was started, in part, to evaluate the role of genetic components in CVD etiology. In total, there were 5124 subjects aged 5-70 years at entry including the children of the original cohort and their spouses. The Framingham Heart Study consists almost entirely of subjects of European descent from England, Ireland, France, and Italy. Genotyping was performed using Affymetrix 500K array supplemented by the MIPS 50K array. Total adiponectin levels were available for 2228 genotyped study participants from the Offspring cohort and were measured by ELISA (R&D Systems, Minneapolis, MN) (Hivert et al., 2008). Statistical GWA analysis was performed using linear mixed effect models implemented in the function lmekin from the R kinship package (www.r-project.org), where the SNP is incorporated in the model as a fixed covariate while a familial random effect component is included to account for familial correlation.

GEMS *(Genetic Epidemiology of Metabolic Syndrome):* The study population of the Genetic Epidemiology of Metabolic Syndrome (GEMS) study consisted of dyslipidaemic cases (age 20-65 years, n=1025) matched with normolipidaemic controls (n=1008) by sex and recruitment site. Detailed information on the GEMS study design, sampling frame, and recruitment procedures has been published (Stirnadel et al., 2008). Genotyping was performed using Affymetrix GeneChip Human Mapping 500K array and the BRLMM calling algorithm. Adiponectin levels were available for 1780 study participants and were measured by ELISA assay (R&D systems, Minneapolis, MN). Statistical analyses was performed using Quicktest v0.94.

ALSPAC*:* The Avon Longitudinal Study of Parents and their Children (ALSPAC) is a population-based birth cohort study consisting initially of over 13000 women and their children recruited in the county of Avon, U.K in the early 1990s (http://www.bristol.ac.uk/alspac/). Both mothers and children have been extensively followed from the 8th gestational week onwards using a combination of self-reported questionnaires, medical records and physical examinations. Biological samples including DNA have been collected for ~10,500 of the children from this cohort. Ethical approval was obtained from the ALSPAC Law and Ethics committee and relevant local ethics committees, and written informed consent provided by all parents (Golding et al., 2001). 1518 ALSPAC individuals were genotyped using the Illumina HumanHap317K SNP chip. This chip contains 317504 SNPs and provides approximately 75% genomic coverage of the Utah CEPH (CEU) HAPMAP samples for common SNPs at r^2 >0.8. Markers with minor allele frequency <1%, SNPs with >5% missing genotypes and, any marker that failed an exact test of Hardy-Weinberg equilibrium ($p < 10^{-7}$) were excluded from further analyses and before imputation. After data cleaning, 315807 SNPs were left in the ALSPAC genome-wide association analysis (Timpson et al., 2009). Plasma adiponectin concentrations were determined in samples from 1415 individuals using ELISA (R&D Systems) with inter-assay CV being 7%. Analyses were performed using STATA and PLINK.

TwinsUK: The TwinsUK cohort (www.twinsuk.ac.uk) is an adult twin registry shown to be representative of the UK singleton population (Andrew et al., 2001). A total of 1399 (women were included in the analysis, Genotyping was performed using the Illumina HumanHap 300 Illumina HumanCNV370 Duo chips (Richards et al., 2008). Adiponectin levels were available for 1399 study participants and were measured with an in-house two-site ELISA assay using antibodies and standards from R&D Systems Europe (Abingdon, Oxford UK). The day-to-day coefficients of variation for adiponectin were 5.4% at a concentration of 3.6 μg/ml, 5.2% at 9.2 μg/ml and 5.8% at 15.5 μg/ml. Statistical analysis was conducted applying Merlin software package (Abecasis et al., 2002).

InCHIANTI: INCHIANTI is an epidemiological study of risk factors contributing to the decline in physical functioning in late life (Ferrucci et al., 2000). Individuals were selected from the population registries of two small towns in Tuscany, Italy. Participants, all of white European origin, were invited to a clinic visit for evaluation of health status as described in detail previously (Bartali et al., 2002). SNPs were genotyped on the Illumina 550k array (Melzer et al., 2008), with missing SNPs imputed using IMPUTE software. Adiponectin levels were available for 1027 study participants and were measured by RIA assay (Human Adiponectin RIA Kit, Linco Research, Inc, Missouri, USA) Statistical analyses were conducted using SNPTEST.

Baltimore Longitudinal Study of Aging (BLSA): The Baltimore Longitudinal Study of Aging (BLSA) is an observational study that began in 1958 to investigate normative aging in community dwelling adults who were healthy at study entry (Shock et al., 1984). Participants are examined every one to four years depending on their age. Currently there are approximately 1100 active participants enrolled in the study. The analysis was restricted to subjects with European ancestry. Genotyping was performed using Illumina HumanHap 550K. Adiponectin levels were available for 565 study participants and were measured by RIA (LINCO) having intra-assay and inter-assay variation of 1.8-6.2% and 6.9-9.3% respectively. Each analysis was further adjusted for the top two principal components derived from an EIGENSTRAT analysis utilizing ~10,000 randomly selected SNPs from the 550K SNP panel.

ADDITIONAL INFORMATION ON STATISTICAL METHODS

Metal-software: All combined analysis were performed using the METAL software (Abecasis and Willer, 2007, http://www.sph.umich.edu/csg/abecasis/metal). We used the METAL implemented study-wise genomic control correction as well as genomic control correction of the METAL results.

To combine the three GWA studies (stage 1), we performed a beta-pooling meta-analysis using the fixed effect model (inverse variance weighted) and a scaling-invariant p-value pooling meta-analysis (using the weighted z-score method). We found no major difference between both methods in this GWA stage. For the replication stage (stage 2) and stage 1 and stage 2 combined, we conducted the scaling-invariant p-value pooling as there were greater differences between adiponectin assays in the full set of studies. We present the weighted Z-score method results throughout the manuscript.

Test to compare gender-stratified beta-estimates from GWA analyses: Each study has provided SNP-association results for men and women separately. For each SNP, we pooled the men-specific beta-estimates across all studies (beta_men and its standard error se_beta_men) as well as the women-specific beta-estimates (beta_women and se_beta_women) using the fixed effect model. For each SNP, significant difference between gender-specific pooled beta estimates, beta_men and beta_women, was obtained by using the approximately normally distributed test statistics of beta_men – beta_women divided by the sum of their variance estimates minus the covariance of the beta-estimates

(i.e. se_beta_men² + se_beta_women² + 2 x corr (beta_men, beta_women) x se_beta_men x se_beta_women). The correlation of the beta_men and beta_women was obtained by using the empirical distribution of the beta-estimates across all SNPs under the assumption that the abundance of these SNP-associations are under the null hypothesis of no association.

BIOINFORMATIC ANALYSIS

Bioinformatic analysis for potential functional SNPs was done in two stages, using bioinformatic tools outlined in (Coassin et al., 2009). Firstly, all SNPs of the imputed data set in the *ADIPOQ* gene region have been analyzed for potential functional effects using SNPseek (http://snp.wustl.edu/cgi-bin/SNPseek/index.cgi) and SNPnexus (http://www.snp-nexus.org/) as well as FASTSNP (http://fastsnp.ibms.sinica.edu.tw/). In the second stage attempting to find potential functional variants not included in HAPMAP, all SNPs reported by Ensembl Variation v.56 in the region between rs6810075 and rs7615090 (see Supplementary Figure S2) were submitted to FASTSNP. SNPs which were predicted to affect any kind of functional element were then further investigated using the Genomatix Software Suite (Genomatix Software GmbH, Munich, Germany) and the PupaSuite for transcription factor binding site analysis as well as F-SNP (http://compbio.cs.queensu.ca/F-SNP/) for further refinement of splicing regulation effects and other kinds of functional elements. Since FASTSNP recognizes only SNPs in gene regions, all intergenic SNP both up- and downstream of the *ADIPOQ* locus were analyzed for transcription factor binding sites using the Genomatix Software Suite. All analyses in the Genomatix Software Suite were done using only transcription factors specifically expressed in the adipose tissue as well as ubiquitous ones. Additionally, the presence of general functional elements and regulatory potential (ESPERR) in the intergenic region was investigated in the UCSC Genome browser and intergenic regions were scanned for regulatory promoter elements using PromoterInspector from Genomatix. Known copy number variations were retrieved from the Database of Genomic Variants (http://projects.tcag.ca/variation/).

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BLSA: The BLSA Study was supported in part by the Intramural Research Program of the NIH, National Institute on Aging. A portion of that support was through a R&D contract with MedStar Research Institute.

Supplementary Table S1: Characteristics of Study Samples

	# of subjects	Age (yrs)			BMI (kg/m_)			Adiponectin (μg/ml)			Adiponectin
Study	(% Women)	Combined	Women	Men	Combined	Women	Men	Combined	Women	Men	Assay
Genomewide association study samples											
KORA	1644 (50.5)	62.5±10.1	62.1±10.1	63.0±10.1	28.1±4.5	28.0±5.1	28.2±3.8	10.6±4.7	12.3±4.9	9.9±3.7	ELISA [a]
ERF	1820 (57.9)	48.7± 4.4	48.0±14.4	49.6±14.4	26.9±4.6	26.5±4.9	27.4±4.2	10.7±5.7	12.5±5.9	8.1±4.2	RIA [b]
MICROS	1195 (56.7)	44.8 ± 16.7	44.8±17.1	44.9±16.1	25.4±4.8	25.0±5.3	25.8±3.9	13.5±8.4	15.6±9.6	10.7±5.3	ELISA [c]
Replication study samples											
CoLaus	5381 (52.2)	53.2±10.8	53.6±10.7	52.7±10.8	25.8±4.6	25.1±4.9	26.6±4.2	10.1±8.1	12.4±9.4	7.4±5.4	ELISA [d]
Framingham	2228 (54.6)	60.4±9.5	60.4±9.5	60.3±9.5	27.8±5.0	27.2±5.4	28.4±4.3	10.5±6.4	13.0±6.8	7.6±4.5	ELISA [d]
GEMS	1780 (41.1)	52.5±9.5	52.4±9.6	52.7±9.4	28.5±3.6	28.7±4.0	28.3±3.4	6.8±4.8	8.3±5.5	5.8±4.0	ELISA [d]
ALSPAC	1415 (48.8)	9.9 ±0.3	9.8 ±0.2	9.9 ±0.3	17.2±3.5	17.2±4.1	17.2±2.7	13.0±5.3	13.3±5.5	12.8±5.1	ELISA [d]
TwinsUK	1399 (100)	48.5±13.1	48.5±13.1	-	25.1±4.7	25.1±4.7	-	8.1±3.9	8.1±3.9	-	ELISA[d]
InCHIANTI	1027 (54.7)	67.6±15.3	68.5±15.4	66.6±15.1	27.1±4.1	27.1±4.6	27.1±3.4	13.5±9.8	15.9±10.8	10.5±7.6	RIA [b]
BLSA	565 (47.1)	67.9±13.8	65.5+14.6	69.9+12.7	26.8±4.5	26.0+4.9	27.6+4.1	13.4±8.5	15.4±8.9	11.5±7.7	RIA [b]

Values stated are n (%) or mean ± SD

Assays used for measurement of adiponectin: [a] Mercodia; [b] Linco; [c] BioVendor; [d] R&D Systems

Supplementary Table S2: SNP characteristics for the SNPs selected for replication. Numbers stated are the minor allele frequencies and the imputation certainties (R_) for each of the three stage 1 studies KORA (n=1817), ERF (n=1195), or MICROS (n=1643).

			Allele		Minor allele frequency			Rsqr		
SNP	Chr	Pos (bp)	Major	Minor	KORA	ERF	MICROS	KORA	ERF	MICROS
Combined										
rs17366568	3	188053155	G	A	0.11	0.09	0.10	0.91*	0.37	0.27
rs8058648	16	25736408	G	C	0.38	0.37	0.36	0.64	0.74	0.73
rs7735993	5	140659543	A	G	0.21	0.32	0.16	0.98	0.98*	1.00*
rs6433017	2	151554095	C	T	0.15	0.15	0.13	0.29	0.44	0.41
rs936524	19	43924986	G	A	0.25	0.25	0.20	0.98	0.96	0.95
rs17554694	19	22108988	G	A	0.21	0.17	0.14	1.00*	0.91	0.85
rs1426438	12	114024489	A	G	0.18	0.22	0.20	0.98	0.98*	1.00*
rs2804441	10	128306965	T	C	0.48	0.50	0.46	0.88	0.90	0.92
rs1272041	9	114935061	G	C	0.38	0.43	0.44	1.00*	0.99	1.00
rs13201655	6	6295138	G	T	0.38	0.41	0.42	0.96	0.91	0.83
rs7235989	18	8726346	T	G	0.45	0.50	0.37	0.61	0.85	0.89
rs2460620	15	44085750	C	T	0.26	0.23	0.20	0.96*	0.80*	0.97*
rs6448895	4	12122006	C	G	0.10	0.09	0.08	0.64	0.58	0.75
rs7221927	17	76479722	T	C	0.33	0.34	0.30	0.96*	0.68	0.62
rs476546	11	132593133	A	G	0.33	0.30	0.26	0.96	0.99	0.99
rs10041164	5	103071961	C	T	0.40	0.41	0.49	0.96*	0.99*	0.99*
rs17810558	4	147342325	G	A	0.10	0.10	0.07	0.93	0.97	0.99
rs418410	5	31722785	T	G	0.48	0.45	0.46	0.75	0.96*	0.93*
rs7128545	11	32488991	G	A	0.09	0.12	0.13	0.76	0.78	0.81
rs7921500	10	10372106	C	T	0.34	0.39	0.31	0.73	0.87	0.87
Women										
rs7544470	1	212468592	T	A	0.38	0.42	0.35	0.90	0.99	1.00
rs12617829	2	55812968	C	T	0.10	0.13	0.10	0.85	0.91	0.83
rs9928327	16	2190234	G	T	0.12	0.09	0.03	0.17	0.94*	1.00*
rs1868521	3	42268266	G	A	0.06	0.06	0.06	0.38	0.46	0.34
rs2272439	16	87474262	G	A	0.17	0.12	0.15	0.40	0.91*	0.99*
rs2271265	4	48138173	T	A	0.05	0.07	0.07	0.98	0.98	1.00
rs12206888	6	167297384	G	A	0.08	0.08	0.08	0.67	0.45	0.36
rs11599120	10	132866667	T	G	0.25	0.32	0.24	0.65	0.97	0.99
rs17332108	5	60167641	T	C	0.26	0.20	0.25	1.00	0.99	0.98
rs2005029	17	2201888	A	C	0.39	0.49	0.38	0.80	0.95	0.97
Men										
rs2169877	15	83886806	A	G	0.25	0.29	0.18	0.93*	0.75	0.68
rs13073708	3	59443394	A	G	0.19	0.17	0.28	0.83	0.84	0.79
rs6495001	15	31339385	T	G	0.12	0.20	0.17	0.67	0.97*	0.97*
rs12598394	16	9945733	T	A	0.12	0.11	0.11	0.84	0.90	0.82
rs11767869	7	22823528	T	C	0.19	0.20	0.10	0.98*	0.97	1.00
rs17310106	14	96200749	C	T	0.34	0.36	0.31	0.91	0.90	0.94
rs8096456	18	13986751	G	A	0.23	0.18	0.21	0.99*	0.80	0.91
rs2328878	6	25405664	A	G	0.50	0.45	0.47	0.84	0.98*	1.00*
rs10879888	12	73877027	A	G	0.30	0.31	0.29	0.99*	0.97	0.97
rs6811805	4	184856851	A	G	0.07	0.07	0.07	0.22	0.59	0.67

* SNPs marked were genotyped, all other SNPs were imputed as described.

Supplementary Table S3: Association results for SNPs entering replication stage selected from gender-combined or gender-stratified analyses. For the top 20 SNPs representing independent loci from the gender-combined GWA analyses and the top 10 SNPs from the men only as well as 10 SNPs from the women only GWA analysis, replication stage data was obtained. Zscores and p-values are stated from meta-analyses of the three stage 1 studies, KORA, ERF, and MICROS (n=4659, men=2097, women=2562) as well as stage 1 and stage 2 studies combined (n=18425, men=8190, women=10235). Z scores are given into the direction of effect allele A1. Study-specific results were combined using the weighted Z-score method. Results are ordered by p-values from stage 1 in the three strata of analysis (combined, women and men).

						Combined				**Women**				**Men**			
			Nearest	Allele		Stage 1		Stage 1+2		Stage 1		Stage 1+2		Stage 1		Stage 1+2	
SNP	Chr	Position (bp)	gene	A1	A2	Zscore	P	Zscore	P	Zscore	P	Zscore	P	Zscore	P	Zscore	P
Combined																	
rs17366568	3	188053155	ADIPOQ	a	g	-10.125	4.3E-24	-13.527	1.1E-41	-8.322	8.7E-17	-9.709	2.8E-22	-6.674	2.5E-11	-9.838	7.8E-23
rs8058648	16	25736408	HS3ST4	c	g	4.613	4.0E-06	2.624	0.009	3.050	0.002	0.854	0.393	3.407	0.001	2.910	0.004
rs7735993	5	140659543	SLC25A2	a	g	-4.491	7.1E-06	-0.305	0.761	-2.142	0.032	-0.126	0.900	-4.408	1.0E-05	-0.262	0.793
rs6433017	2	151554095	RBM43	t	c	-4.487	7.2E-06	-1.377	0.168	-3.181	0.001	-1.558	0.119	-3.196	0.001	-0.213	0.831
rs936524	19	43924986	CAPN12	a	g	-4.338	1.4E-05	-2.592	0.010	-3.111	0.002	-1.619	0.106	-2.631	0.009	-1.973	0.049
rs17554694	19	22108988	ZNF257	a	g	4.285	1.8E-05	2.653	0.008	3.557	3.8E-04	2.412	0.016	2.841	0.004	1.529	0.126
rs1426438	12	114024489	TBX3	a	g	-4.188	2.8E-05	-2.176	0.030	-3.717	2.0E-04	-1.452	0.146	-1.917	0.055	-1.452	0.147
rs2804441	10	128306965	C10orf90	t	c	4.138	3.5E-05	2.443	0.015	2.057	0.040	0.867	0.386	3.895	9.8E-05	2.683	0.007
rs1272041	9	114935061	SLC31A2	c	g	4.101	4.1E-05	2.568	0.010	1.525	0.127	1.442	0.149	4.316	1.6E-05	2.216	0.027
rs13201655	6	6295138	F13A1	t	g	4.098	4.2E-05	2.173	0.030	3.806	1.4E-04	2.370	0.018	1.917	0.055	0.729	0.466
rs7235989	18	8726346	KIAA0802	t	g	-4.068	4.7E-05	-2.580	0.010	-2.561	0.010	-1.316	0.188	-4.225	2.4E-05	-2.907	0.004
rs2460620	15	44085750	SQRDL	t	c	4.060	4.9E-05	2.092	0.036	2.111	0.035	1.291	0.197	3.318	0.001	1.252	0.211
rs6448895	4	12122006	RAB28	c	g	-3.999	6.4E-05	-1.383	0.167	-3.061	0.002	-0.881	0.378	-2.550	0.011	-1.038	0.299
rs7221927	17	76479722	KIAA1303	t	c	3.983	6.8E-05	2.909	0.004	3.980	6.9E-05	1.737	0.082	1.969	0.049	2.629	0.009
rs476546	11	132593133	OPCML	a	g	-3.944	8.0E-05	-1.889	0.059	-3.546	3.9E-04	-1.858	0.063	-1.575	0.115	-0.434	0.664
rs10041164	5	103071961	NUDT12	t	c	-3.788	1.5E-04	-2.008	0.045	-2.416	0.016	-0.547	0.584	-2.608	0.009	-2.145	0.032
rs17810558	4	147342325	LSM6	a	g	-3.728	1.9E-04	-1.839	0.066	-2.602	0.009	-1.339	0.181	-2.572	0.010	-1.216	0.224
rs418410	5	31722785	PDZD2	t	g	-3.723	2.0E-04	-3.406	0.001	-2.081	0.037	-1.005	0.315	-3.620	2.9E-04	-4.007	6.2E-05
rs7128545	11	32488991	EIF3M	a	g	3.646	2.7E-04	1.625	0.104	1.822	0.068	0.350	0.726	3.413	0.001	2.032	0.042
rs7921500	10	10372106	CUGBP2	t	c	-3.578	3.5E-04	-3.479	0.001	-3.250	0.001	-2.042	0.041	-1.874	0.061	-2.885	0.004

						Combined				Women				Men			
			Nearest	Allele		Stage 1		Stage 1+2		Stage 1		Stage 1+2		Stage 1		Stage 1+2	
SNP	Chr	Position (bp)	gene	A1	A2	Zscore	P	Zscore	P	Zscore	P	Zscore	P	Zscore	P	Zscore	P
Men																	
rs2169877	15	83886806	AKAP13	a	g	3.548	3.9E-04	0.970	0.332	0.611	0.541	0.115	0.909	5.038	4.7E-07	1.509	0.131
rs13073708	3	59443394	FHIT	a	g	-3.344	0.001	-3.577	3.5E-04	-1.073	0.283	-1.796	0.073	-4.892	1.0E-06	-3.896	9.8E-05
rs6495001	15	31339385	RYR3	t	g	2.533	0.011	1.072	0.284	-0.727	0.467	-0.526	0.599	4.446	8.8E-06	2.069	0.039
rs12598394	16	9945733	GRIN2A	a	t	-3.530	4.2E-04	-1.569	0.117	-1.310	0.190	-1.165	0.244	-4.441	9.0E-06	-1.319	0.187
rs11767869	7	22823528	TOMM7	t	c	-2.247	0.025	-1.955	0.051	0.863	0.388	0.509	0.610	-4.418	9.9E-06	-3.446	5.7E-04
rs17310106	14	96200749	PAPOLA	t	c	3.323	0.001	1.322	0.186	1.060	0.289	0.772	0.440	4.384	1.2E-05	1.422	0.155
rs8096456	18	13986751	MC2R	a	g	2.317	0.020	0.525	0.599	-0.372	0.710	-0.348	0.728	4.121	3.8E-05	1.306	0.192
rs2328878	6	25405664	LRRC16A	a	g	2.269	0.023	0.784	0.433	-0.110	0.913	-1.026	0.305	4.103	4.1E-05	2.548	0.011
rs10879888	12	73877027	KCNC2	a	g	-3.596	3.2E-04	-2.480	0.013	-1.700	0.089	-0.245	0.807	-4.102	4.1E-05	-3.819	1.3E-04
rs6811805	4	184856851	C4orf41	a	g	2.008	0.045	1.960	0.050	-1.236	0.216	0.135	0.893	3.990	6.6E-05	2.816	0.005
Women																	
rs7544470	1	212468592	SMYD2	a	t	4.440	9.0E-06	2.116	0.034	5.188	2.1E-07	3.134	0.002	1.654	0.098	0.314	0.753
rs12617829	2	55812968	PNPT1	t	c	-3.814	1.4E-04	-2.052	0.040	-4.942	7.7E-07	-2.773	0.006	-0.558	0.577	-0.066	0.947
rs9928327	16	2190234	CASKIN1	t	g	-2.960	0.003	-4.063	4.9E-05	-4.520	6.2E-06	-3.941	8.1E-05	0.980	0.327	-1.337	0.181
rs1868521	3	42268266	CCK	a	g	-2.736	0.006	-1.806	0.071	-4.313	1.6E-05	-2.463	0.014	0.148	0.883	-0.166	0.868
rs2272439	16	87474262	CBFA2T3	a	g	2.944	0.003	1.253	0.210	4.227	2.4E-05	1.739	0.082	0.613	0.540	0.280	0.779
rs2271265	4	48138173	SLAIN2	a	t	4.045	5.2E-05	2.243	0.025	4.174	3.0E-05	2.404	0.016	2.219	0.026	1.235	0.217
rs12206888	6	167297384	RNASET2	a	g	-2.935	0.003	-1.659	0.097	-4.084	4.4E-05	-2.212	0.027	-0.658	0.511	-0.542	0.588
rs11599120	10	132866667	TCERG1L	t	g	-2.147	0.032	-1.096	0.273	-3.931	8.4E-05	-2.573	0.010	0.599	0.549	0.870	0.384
rs17332108	5	60167641	ELOVL7	t	c	2.835	0.005	1.121	0.262	3.880	1.0E-04	2.020	0.043	0.109	0.913	-0.413	0.679
rs2005029	17	2201888	SGSM2	a	c	-2.767	0.006	0.437	0.662	-3.753	1.8E-04	-0.395	0.693	-0.359	0.720	1.146	0.252

Supplementary Table S4: Metabolic syndrome parameter SNPs and their association with adiponectin. SNPs were selected as the most strongly associated SNP (according to p-value) in published genome-wide association studies (GWAS) for HDL cholesterol, triglycerides, waist circumference or BMI, type 2 diabetes mellitus or glucose concentrations, and hypertension. Stated are number of subjects, the p-value and the effect estimate (if available) from the published GWAS (stage 1 and stage 2 results combined if not stated otherwise) analysis for the respective trait. The p-value of these SNPs with adiponectin in the present study is computed from linear regression on log-transformed adiponectin concentration adjusted for age, sex, and BMI in the three meta-analyzed stage 1 studies (KORA, ERF, MICROS) (n=4655, women=2560, men=2095).

							Results from literature	**p-values for adiponectin**		
Gene	Chr	Position	rsnumber	Alleles	f (%)	Reference	n / p / effect	combined	women	men
HDL cholesterol										
LIPG	18	45421212	rs4939883	T (C)	17	(Kathiresan et al., 2009)	19,785 / 7.0x10-15 / -0.14 SD	0.72	0.73	0.80
CETP	16	55562980	rs1532624	C (A)	57	(Aulchenko et al., 2009)	19,674 / 9.4x10-94 / 8.24 z-sc	0.23	0.041	0.60
PLTP	20	44009909	rs7679	C (T)	19	(Kathiresan et al., 2009)	40,248 / 4.0x10-9 / -0.07 SD	0.49	0.02	0.11
NR1H3	11	47242866	rs7120118	C (T)	42	(Sabatti et al., 2009)	4,525 / 3.6x10-8 / 0.04 mmol/l	0.10	0.03	0.67
LPL	8	19888502	rs12678919	G (A)	10	(Kathiresan et al., 2009)	19,794 / 2.0x10-34 / 0.23 SD	0.53	0.78	0.50
LIPC	15	56470658	rs1532085	G (A)	59	(Aulchenko et al., 2009)	19,736 / 9.7x10-36 / 5.03 z-sc	0.70	0.99	0.56
ABCA1	9	106696891	rs3905000	G (A)	86	(Aulchenko et al., 2009)	17,913 / 8.6x10-13 / -4.37 z-sc	0.33	0.82	0.05
LCAT	16	66459571	rs2271293	A (G)	11	(Kathiresan et al., 2009)	31,946 / 9.0x10-13 / 0.07 SD	0.44	0.72	0.14
APOA1C3A4A5	11	116154127	rs964184	G (C)	14	(Kathiresan et al., 2009)	19,794 / 1.0x10-12 / -0.17 SD	0.55	0.80	0.16
APOB	2	21059688	rs6754295	C (A)	25	(Aulchenko et al., 2009)	17,915 / 4.4x10-8 / 2.63 z-sc	0.38	0.44	0.61
CTCF-PRMT8	16	66459571	rs2271293	G (A)	87	(Aulchenko et al., 2009)	17,910 / 8.3x10-16 / 4.99 z-sc	0.44	0.72	0.14
MADD-FOLH1	11	48475469	rs7395662	G (A)	61	(Aulchenko et al., 2009)	17,917 / 6.0x10-11 / 2.82 z-sc	0.69	0.11	0.35
GALNT2	1	228362314	rs4846914	G (A)	40	(Kathiresan et al., 2009)	19,794 / 4.0x10-8 / -0.05 SD	0.44	0.76	0.07
MVK/MMAB	12	108379551	rs2338104	C (G)	45	(Kathiresan et al., 2009)	19,793 / 1.0x10-10 / -0.07 SD	0.55	0.24	0.57
CLPTM1	19	50169221	rs16979595	A (G)	16	(Wallace et al., 2008)	1,636 / 6.1x10-3 / NA	0.74	0.79	0.21
FADS1-2-3	11	61327359	rs174547	C (T)	33	(Kathiresan et al., 2009)	40,330 / 2.0x10-12 / -0.09 SD	0.75	0.19	0.41
TTC39B	9	15279578	rs471364	C (T)	33	(Kathiresan et al., 2009)	40,414 / 3.0x10-10 / -0.08 SD	0.88	0.41	0.68
HNF4A	20	42475778	rs1800961	T (C)	3	(Kathiresan et al., 2009)	30,714 / 8.0x10-10 / -0.19 SD	0.98	0.57	0.38
ANGPTL4	19	8375738	rs2967605	T (C)	16	(Kathiresan et al., 2009)	35,151 / 1.0x10-8 / -0.12 SD	0.54	0.71	0.61
no name	17	2375258	rs9891572	T (C)	16	(Sabatti et al., 2009)	4,525 / 2.3x10-7 / 0.05 mmol/l	0.70	0.45	0.92
GRIN3A	9	103402758	rs1323432	A (G)	88	(Willer et al., 2008)	8,656 / 2.5x10-8 / 1.93 mg/dL	0.45	0.44	0.80

							Results from literature	p-values for adiponectin		
Gene	Chr	Position	rsnumber	Alleles	f (%)	Reference	n / p / effect	combined	women	men
Triglycerides [e]										
TOMM40-APOE	19	50106291	rs439401	G (A)	32	(Aulchenko et al., 2009)	17,913 / 1.8x10-09 / NA	0.15	0.04	0.97
NCAN, CILP2, PBX4	19	19523220	rs17216525	T (C)	7	(Kathiresan et al., 2009)	19,840 / 4.0x10-11 / -0.11 SD [a]	0.61	0.22	0.87
XKR6-AMAC1L2	8	11082571	rs7819412	G (A)	48	(Kathiresan et al., 2009)	33,336 / 3.0x10-08 / -0.04 SD [a]	0.86	0.89	0.97
LOC440069	11	116112647	rs1558861	T (C)	18	(Kooner et al., 2008)	≈12,000 / 1.6x10-23 / 0.08 SD	0.64	0.81	0.64
TRIB1	8	126560154	rs2954029	T (A)	44	(Kathiresan et al., 2009)	19,840 / 3.0x10-19 / -0.11 SD [a]	0.04	0.01	0.86
GCKR	2	27584444	rs1260326	T (C)	45	(Kathiresan et al., 2009)	19,840 / 2.0x10-31 / 0.12 SD [a]	0.34	0.92	0.19
NR1H3	11	47242866	rs7120118	A (G)	42	(Sabatti et al., 2009)	4,525 / 3.6x10-08 / 0.04 mmol/l	0.10	0.03	0.67
no name	15	36935941	rs2624265	C (T)	42	(Sabatti et al., 2009)	4,526 / 4.3x10-07 / 0.07 mmol/l	0.44	0.85	0.44
MLXIPL	7	72502805	rs714052	G (A)	12	(Kathiresan et al., 2009)	19,840 / 3.0x10-15 / -0.16 SD [a]	0.49	0.34	0.82
ANGPTL3, DOCK7	1	62704280	rs1167998	C (A)	32	(Aulchenko et al., 2009)	17,913 / 2.0x10-12 / NA	0.19	0.34	0.27
Body mass index										
FTO	16	52378028	rs9939609	A (T)	41	(Willer et al., 2009)	113,204/ 4.9x10-74/ 0.33 kg/m_ [b]	0.17	0.37	0.36
MC4R	18	56002077	rs17782313	C (T)	21	(Willer et al., 2009)	110,567 / 1.1x10-20 / 0.20 kg/m_ [b]	0.87	0.39	0.59
TMEM18	2	624905	rs6548238	C (T)	84	(Willer et al., 2009)	114,643 / 3.2x10-26 / 0.26 kg/m_ [b]	0.76	0.80	0.63
KCTD15	19	39013977	rs11084753	G (A)	45	(Willer et al., 2009)	101,526 / 4.5x10-12 / 0.06 kg/m_ [b]	0.98	0.86	0.91
GNPDA2	4	45023455	rs10938397	G (A)	41	(Willer et al., 2009)	81,758 / 3.4x10-16 / 0.19 kg/m_ [b]	0.98	0.78	0.62
SH2B1	16	28790742	rs7498665	G (A)	34	(Willer et al., 2009)	116,497 / 2.2x10-14 / 0.15 kg/m_ [b]	0.85	0.90	0.73
MTCH2	11	47619625	rs10838738	G (A)	67	(Willer et al., 2009)	110,737 / 1.9x10-11 / 0.07 kg/m_ [b]	0.38	0.47	0.93
NEGR1	1	72524461	rs2815752	A (G)	62	(Willer et al., 2009)	113,319 / 6.0x10-8 / 0.10 kg/m_ [b]	0.32	0.08	0.80
Waist circumference [f]										
TFAP2B	6	50911009	rs987237	G (A)	16	(Lindgren et al., 2009)	118,691/ 1.9x10-11/ 6.72 z-sc [c]	0.21	0.11	0.72
MSRA	8	9897480	rs7826222	G (C)	18	(Lindgren et al., 2009)	80,210/ 8.9x10-9/ 5.75 z-sc [c]	0.03	0.39	0.03
LYPLAL1	1	217710,837	rs2605100	G (A)	69	(Lindgren et al., 2009)	47,633/ 2.6x10-8 / 5.57 z-sc	0.16	0.40	0.17
NRXN3	14	79014915	rs10146997	G (A)	21	(Heard-Costa et al., 2009)	70,014 / 5.3x10-8 / 0.0498 z-sc	0.82	0.87	0.67

to be continued on page 98-99

Supplementary Table S4 Continue

							Results from literature	p-values for adiponectin		
Gene	Chr	Position	rsnumber	Alleles	f (%)	Reference	n / p / effect	combined	women	men
Type 2 diabetes mellitus [g]										
PPARG	3	12368125	rs1801282	C (G)	90	(Zeggini et al., 2007)	14,586+17,968 [d] / 1.7_10-6 / OR: 1.14	0.91	0.85	0.64
KCNJ11	11	17365206	rs5215	C (T)	40	(Zeggini et al., 2007)	14,586+17,968 [d] / 5.0_10-11 / OR: 1.14	0.08	0.06	0.63
TCF7L2	10	114744078	rs7901695	C (T)	28	(Zeggini et al., 2007)	14,586+17,968 [d] / 1.0_10-48 / OR: 1.37	0.64	0.83	0.75
IGF2BP2	3	186994389	rs4402960	T (G)	30	(Zeggini et al., 2007)	14,586+17,968 [d] / 8.6_10-14 / OR: 1.14	0.07	0.88	0.02
CDKN2(A)/2B	9	22124094	rs10811661	T (C)	80	(Zeggini et al., 2007)	14,586+17,968 [d] / 7.8_10-15 / OR: 1.20	0.20	0.49	0.29
CDKAL1	6	20769013	rs10946398	A (C)	66	(Zeggini et al., 2007)	14,586+17,968 [d] / 4.1_10-11 / OR: 1.12	0.24	0.10	0.96
SLC30A8	8	118253964	rs13266634	C (T)	76	(Zeggini et al., 2007)	14,586+17,968 [d] / 5.3_10-8 / OR: 1.12	0.46	0.19	0.96
HHEX/IDE	10	94455539	rs5015480	C (T)	58	(Zeggini et al., 2007)	14,586+17,968 [d] / 5.7_10-10 / OR:1.13	0.42	0.80	0.08
NOTCH2	1	120230001	rs10923931	T (G)	11	(Zeggini et al., 2008)	58,667 / 4.0x10-08 / OR: 1.13	0.17	0.14	0.85
ADAMTS9	3	64686944	rs4607103	C (T)	76	(Zeggini et al., 2008)	62,387 / 1.2x10-08 / OR: 1.09	0.68	0.66	0.09
THADA	2	43644474	rs7578597	T (C)	90	(Zeggini et al., 2008)	60,832 / 1.1x10-09 / OR: 1.15	0.29	0.09	0.63
TSPAN8 / LGR5	12	69949369	rs7961581	C (T)	27	(Zeggini et al., 2008)	62,301 / 1.1x10-09 / OR: 1.09	0.03	0.01	0.78
CDC123, CAMKID	10	12368016	rs12779790	G (A)	18	(Zeggini et al., 2008)	62,366 / 1.2x10-10 / OR: 1.11	0.72	0.44	0.72
JAZF1	7	27953796	rs864745	T (C)	50	(Zeggini et al., 2008)	59,617 / 5.0x10-14 / OR: 1.10	0.44	0.98	0.28
KCNQ1	11	2796327	rs2237892	C (T)	92	(Yasuda et al., 2008)	1,612+1,424 [d] / 6.7x10-13 / OR: 1.49	0.45	0.62	0.28
Glucose [h]										
G6PC2	2	169471394	rs560887	A (G)	30	(Bouatia-Naji et al., 2008)	9,353 / 4.0x10-23 / -0.06 mmol/l	0.39	0.38	0.67
MTNR1B	11	92348358	rs10830963	G (C)	30	(Prokopenko et al., 2009)	36,610 / 3.2x10-50 / 0.072 mmol/l	0.27	0.05	0.83
Hypertension and blood pressure										
MTHFR	1	11797044	rs17367504	G (A)	14	(Newton-Cheh et al., 2009)	82,973 / 2.0x10-13 / -0.85 mmHg SBP	0.19	0.16	0.38
CYP17A1	10	104836168	rs11191548	T (C)	91	(Newton-Cheh et al., 2009)	132,552 / 7.0x10-24 / 1.16 mmHg SBP	0.62	0.66	0.87
PLCD3	17	40563647	rs12946454	T (A)	28	(Newton-Cheh et al., 2009)	77,690 / 1.0x10-08 / 0.57 mmHg SBP	0.73	0.32	0.54
MDS1	3	170648590	rs1918974	T (C)	54	(Newton-Cheh et al., 2009)	87,891 / 8.0x10-08 / -0.27 mmHg DBP	0.85	0.80	0.97
PRDM8 / FGF5	4	81541520	rs16998073	T (A)	19	(Newton-Cheh et al., 2009)	101,623 / 1.0x10-21 / 0.50 mmHg DBP	0.27	0.54	0.18
C10orf107	10	63194597	rs1530440	T (C)	19	(Newton-Cheh et al., 2009)	87,273 / 1.0x10-09 / -0.39 mmHg DBP	0.10	0.30	0.15
SH2B3 / ATXN2	12	110470476	rs653178	T (C)	53	(Newton-Cheh et al., 2009)	79,661 / 3.0x10-18 / -0.46 mmHg DBP	0.67	0.20	0.55

							Results from literature	p-values for adiponectin		
Gene	Chr	Position	rsnumber	Alleles	f (%)	Reference	n / p / effect	combined	women	men
CYP1A1	15	72864420	rs1378942	C (A)	37	(Newton-Cheh et al., 2009)	134,258 / 1.0x10-23 / 0.43 mmHg DBP	0.11	0.79	0.11
ZNF652	17	44795465	rs16948048	G (A)	39	(Newton-Cheh et al., 2009)	82,441 / 5.0x10-09 / 0.31 mmHg DBP	0.72	0.29	0.18
ATP2B1	12	88533090	rs2681472	NA	NA	(Levy et al., 2009)	29,136 / 1.7x10-08 / -0.16 mmHg	0.24	0.79	0.07
ITGA9	3	37571809	rs7640747	NA	NA	(Levy et al., 2009)	29,136 / 4.8x10-07/ 0.12 mmHg	0.98	0.41	0.41
CACNB2	10	18748804	rs11014166	NA	NA	(Levy et al., 2009)	29,136 / 7.8x10-07/ -0.11 mmHg	0.26	0.94	0.07
CDH13	16	81200160	rs11646213	A (T)	41	(Org et al., 2009)	3557 / 5.3x10-08 / OR: 0.67	0.10	0.34	0.15

Alleles: Effect allele (Non-effect allele)

f (%), Frequency of effect allele (%)

Abbreviations: z-sc, z-score units; SD, standard deviation; NA, not applicable; SBP, systolic blood pressure; DBP, diastolic blood pressure; OR, odds ratio; Chr, chromosome

[a] Sample size and p value are provided for the combined stage 1 and stage 2 samples.

[b] Sample size and p value are provided for the combined stage 1, stage 2 and DECODE sample, estimate is taken from the stage 2 population-based cohorts

[c] Sample size and p value are provided for the combined stage 1, stage 2 and CHARGE sample.

[d] Number of cases + controls

[e] Genes already mentioned for HDL cholesterol are no longer mentioned for triglycerides (e.g. PLTP, LPL, LIPC, APOA1C3A4A5, APOB, GALNT2, FADS1-FADS2-FADS3)

[f] Genes already mentioned for BMI are no longer mentioned for waist circumference (e.g. FTO, MC4R)

[g] FTO was already mentioned for BMI and is no longer mentioned for type 2 diabetes

[h] GCKR was already mentioned for triglycerides and is no longer mentioned for glucose

Supplementary Table S5: Bioinformatic analysis of all SNPs in the proximity of rs17366568 (between rs822396and rs2241767).

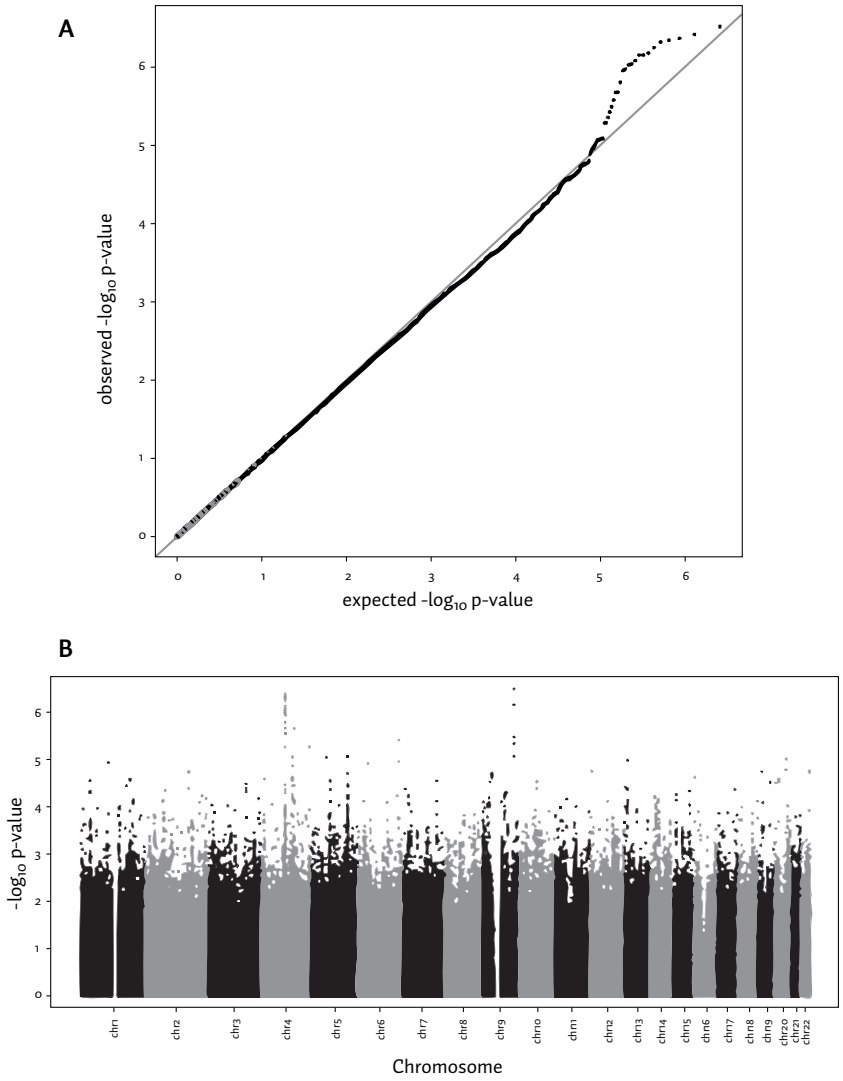
SNP	Distance to rs17366568	FASTSNP Prediction	FASTSNP Risk Score [min. 1 - max. 5]	Region	Genomatix Adipose-TFBS	PupaSuite	F-SNP	Visual SNP	HAPMAP
rs34265972	-2751	Intronic enhancer	1-2	intronic	0	Conserved	n.a.	n.a.	no
rs13066093	-2410	Intronic enhancer	1-2	intronic	0	No effect	n.a.	n.a.	no
rs12495941	-2273	Intronic enhancer	1-2	intronic	0	No effect	n.a.	n.a.	yes
rs36219755	-1675	Intronic enhancer	1-2	intronic	1	No effect	n.a.	n.a.	no
rs7649121	-1668	Intronic enhancer	1-2	intronic	0	No effect	n.a.	n.a.	yes
rs7627128	-1654	Intronic enhancer	1-2	intronic	1	No effect	n.a.	n.a.	yes
rs36219760	-1261	Intronic enhancer	1-2	intronic	0	No effect	n.a.	n.a.	no
rs9877202	-846	Intronic enhancer	1-2	intronic	0	No effect	n.a.	n.a.	yes
rs36219762	-791	Intronic enhancer	1-2	intronic	10	No effect	n.a.	n.a.	no
rs17366568	**0**	**Intronic enhancer**	**1-2**	**intronic**	**0**	**No effect**	**n.a.**	**n.a.**	**yes**
rs34046054	41	Intronic enhancer	1-2	intronic	6	No effect	n.a.	n.a.	no
rs34513325	178	Intronic enhancer	1-2	intronic	4	No effect	n.a.	n.a.	no
rs2241766	439	Sense/synonymous; Splicing regulation	2-3	coding	0	No effect	Splicing regulation	No effect	no
rs62622816	490	Sense/synonymous; Splicing regulation	2-3	coding	0	No effect	n.a.	splicing regulation	no
rs13061862	555	Missense (non-conservative); Splicing regulation	3-4	coding	0	Splicing regulation	splicing, protein damaging	protein damaging	yes
rs1501299	670	Intronic enhancer	1-2	intronic	0	No effect	No effect	No effect	yes

n.a.; not analyzed

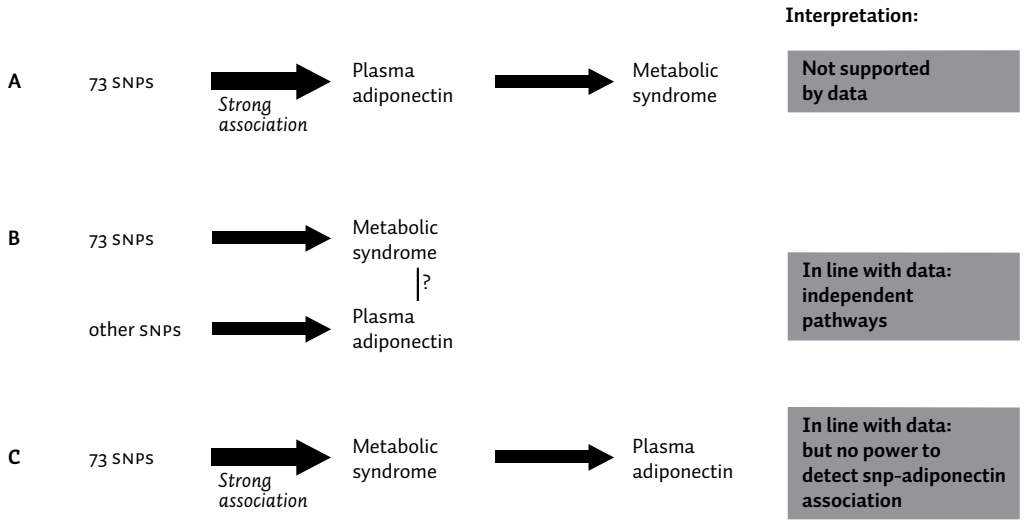
A description of the programs used and mentioned in the column headers of this table is provided in the chapter "Bioinformatic Analysis" on page 6 of the Supplementary Material.

Supplementary Table S6: Position of the predicted promoter regions downstream of ADIPOQ gene region.

		Position		
	Size [bp]	Start	End	Distance from ADIPOQ
Region 1	331	188.063.725	188.064.056	4.779 bp
Region 2	379	188.064.893	188.065.272	5.947 bp
Region 3	319	188.074.441	188.074.760	15.495 bp



Supplementary Figure S1: Differences between the gender-specific beta-estimates. Panel A: the quantile-quantile (QQ) plot of SNPs for the respective p-values shows some observed gender difference of SNP effects beyond the expected by chance. Expected p-values are plotted on X-axis against the observed p-values plotted on the Y-axis. P-values derived from the 200 kB region around ADIPOQ (position ranging from 187950 to 188150 Kb) are depicted as red dots. Panel B: Manhattan plot showing p-values for the difference between men and women of association of each SNPs in the meta-analysis with plasma adiponectin levels. SNPs are plotted on the X-axis to their position on each chromosome against p-values for the gender difference in the SNP association with plasma adiponectin on the Y-axis (shown as –log10 P-value).



Supplementary Figure S2: Illustration on the debate whether plasma adiponectin affects metabolic syndrome parameters or metabolic syndrome parameters modulate adiponectin (for explanation, see Discussion section of the main paper). The 73 SNPs refer to the SNPs selected from previous genome-wide association studies on metabolic syndrome parameter loci (see Supplementary Table S4).

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Chapter 9

General Discussion & Future Perspectives

CLINICAL RELEVANCE OF METS

The first MetS definition was formulated in the 1920s, based on the observation that several individual CVD/T2D risk factors tend to cluster. As is the case with every syndrome, it is difficult to determine boundaries. Currently, four MetS definitions are commonly used. These are the definitions formulated by (1) the World Health Organization (WHO) (2) the European Group for the study of Insulin Resistance (EGSIR) (3) the National Cholesterol Education Program-Adult Panel III (NCEP ATPIII) and (4) the International Diabetes Federation (IDF).

These various definitions overlap with regard to individual risk factors and threshold values. However, the overlap of diagnoses between the different MetS definitions is far from absolute. For example, Figure 1 illustrates the overlap of MetS diagnoses using the NCEP ATP III and the IDF MetS definition in the ERF population (see **chapter 2**). The overlap between individuals diagnosed according to the IDF and ATP III MetS criteria was 65.4% in women and 61.5% in men. This overlap is unexpectedly small, since the NCEP ATP III and IDF definition use exactly the same components. However, an important difference between the two definitions is the obligatory minimal waist circumference in the IDF definition. In addition, the two definitions differ with regard to inclusion of participants using medication and in the slightly lower threshold values for each component in the IDF definition. This threshold difference also contributes to the substantial non-overlap between the IDF and NCEP ATPIII definition. An attempt to unify MetS criteria was recently presented by the IDF, the American Heart Association (AHA), National Heart, Lung and Blood institute (NHLBI), the World Heard Federation (WHF) and the International Atherosclerosis Society (IAS)[2]. The harmonizing measures involved that three abnormal findings out of five should qualify a person for the MetS. Furthermore, it was proposed to use single cutoff points for all components, with the exception of obesity for which further research is required. In addition, obligatory minimal waist circumference was rejected. Whether this definition will be uniformly adopted by the scientific and clinical communities remains to be observed.

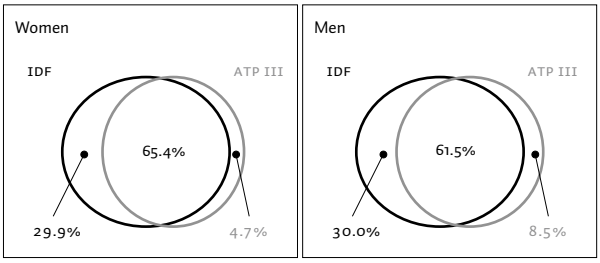

Fig. 1: Overlap in diagnosis between two commonly used MetS definitions, NCEP ATP III and IDF in Erasmus Rucphen Family (ERF) study.

Threshold values for the individual trait abnormalities in the various MetS definitions are lower than the threshold values used by clinicians as indication for treatment. For example, the fasting plasma glucose threshold value in the IDF MetS definition is far below the threshold defining T2D (Table 1). Similarly, the treatment threshold values for hypertension and hypertriglyceridemia are higher than the threshold values of hypertension and hypertriglyceridemia according to MetS. Thus, MetS diagnosis *per se* is not an indication for pharmaceutical intervention, but does reveal that the metabolic homeostasis is in misbalance.

Once specific components of the MetS have exceeded the treatment values, the general strategy used by clinicians is the prescription of individual drugs for each clinical outcome. T2D is treated with plasma glucose lowering medication like metformin, sulfonylurea derivates (SU) and thiazolidinedione

Table 1 Threshold values individual MetS components versus indication clinical intervention

	IDF (2006)[a]	Clinical threshold
FPG (mmol/L)	> 5.6	> 7.0
SBP (mm Hg)	≥ 130	> 140
DBP (mm Hg)	≥ 85	> 100
♂HDL-C (mmol/L)	< 1.03	na
♀HDL-C (mmol/L)	< 1.29	na
TG (mmol/L)	≥ 1.7	> 5.0

IDF; International diabetes federation. a Europids. FPG; fasting plasma glucose, SBP; systolic blood pressure, DBP; diastolic blood pressure, HDL-C; HDL-cholesterol, TG; total plasma triglycerides. na: no clear threshold available.

(TZD). An elevated plasma level of TG is treated with fibrates. Hypertension is a complicated disorder to treat, which is also reflected by the great number of different types of medication which are available (diuretics, β-blockers, ACE-inhibitors, calcium antagonists). Although life style advice on weight control and physical activity is generally also given, it is the most difficult intervention for patients to embrace. Perhaps it should be emphasized more specifically that lifestyle intervention trials have shown that many patients can drastically reduce and even discard their medication once weight loss is achieved[6].

The predictive value of diagnosing an individual as having MetS is limited[8]. Several studies have shown that multiple variable prediction analyses of the individual MetS components perform much better in the accuracy of prognosis for CVD and diabetes than the diagnosis MetS does[9]. The development of quantitative risk scores, like the Framingham risk scores (CVD and stroke www.framinghamheartstudy.org), are based on such multiple variable prediction analysis. These particular scores are focused on dyslipidemia and include age and smoking as predictors. In contrast to MetS, these risk scores are used by clinicians for the indication of pharmaceutical treatment (predominantly statins). Since hypercholesterolemia is particularly resistant to lifestyle intervention, statin treatment has proven its clinical value. Thus, although the diagnosis MetS *per se* may not predict outcome, it is at the same time an opportunity for the patient to modify his or her lifestyle to prevent an ever increasing risk of CVD and diabetes[10,8].

Information about the prevalence of MetS in the general population can provide information about public health perspectives. Such information can contribute to an alternative governmental policy. For example, many countries, indeed promote exercise and reduction of caloric intake to limit the increase of MetS prevalence.

SCIENTIFIC RELEVANCE OF THE METS

The most important finding in **chapter 2** was the limited heritability of MetS as compared to the heritability of the individual MetS components. MetS may thus not be a promising trait for finding novel loci in genetic research. The limited heritability of MetS is likely to be associated with the fact that the MetS is a heterogeneous disorder that encompasses multiple sets of different abnormalities with different etiologies. This is illustrated by the observation of specific clusters of components within the MetS. For example, low HDL-cholesterol and high TG versus low HDL-cholesterol and hypertension are both distinct clusters within MetS, but these two clusters may represent a different origin of metabolic impairment. The combination of low HDL and high TG indicates an impaired lipoprotein metabolism[11] that may be due to increased VLDL production and increased cholesterol-ester transfer activity (that reduces HDL). In contrast, the combination of low HDL and hypertension indicates an impaired vascular function[12]. The genetics of susceptibility for each of these two clusters within the MetS will be distinct. At the same time there is increasing evidence for genetic overlap

between TG and HDL genes, as shown by recently reported genome wide studies, involving the lipid metabolism and including over 100,000 persons[13]. Since the MetS is likely to include many more sub clusters of specific components, this implies that the genetics of the entity MetS is complex.

Despite the fact that the MetS as a singular trait has little value in gene discovery research, it can still contribute to the search for novel loci involved in metabolic impairments. By recognizing the fact that MetS involves a collection of multiple impaired pathways, the challenge lies in the recognition of the clusters that define these specific pathways. MetS therefore serves as an umbrella or starting point for further research into sub-phenotyping or defining sub clusters within MetS. Genetic research of novel traits or endo-phenotypes associated with particular clusters of MetS will provide insight into the pathology of MetS.

An example of genetic correlation between all MetS components and the related trait adiponectin was described in **chapters 7 and 8**. **Chapter 7** described a significant genetic overlap between adiponectin and plasma insulin and insulin sensitivity which was confirmed by genetic association using genetic variants in the adiponectin gene conform a mendelian randomization approach. **Chapter 8** described a genome wide association analysis of plasma adiponectin. This study concluded that the adiponectin gene is the major gene in explaining variation in plasma adiponectin. Larger studies will be necessary to detect the remaining genes with only a small effect.

It is clear that the MetS is the consequence of interaction between genes and the environment. Thus, it is imperative to gain insight into the role of specific genes or loci in the context of specific environments. Examples of the intricate relationship between MetS and environmental factors such as female hormone status and menopause were described in **chapters 4, 6 and 7**. **Chapter 4** showed that female hormonal status interacts with genetic variants in the *APOC3/A5* cluster causing hypertriglyceridemia. In **chapter 6** it was shown that postmenopausal women have a high risk of MetS, independently of age and body mass index. **Chapter 7** concluded that although plasma adiponectin levels differ considerably between the genders, the genetic component explaining the plasma adiponectin variance is similar in men and women.

ASSOCIATION OF METS WITH OTHER COMMON DISEASES

Interestingly, MetS or particular MetS components have been associated with several common disorders, other than CVD and T2D. Examples of such association are the common disorders: obstructive sleep apnea (OSA), addiction, migraine and dementia.

OSA is characterized by pauses in breathing during sleep due to low muscle tone or soft tissue around the airway, which is often caused by obesity. This form of apnea causes a disturbance in diurnal rhythm, which in itself may be associated with aggravation of the MetS. Thus, apnea may be a consequence of obesity, while at the same time aggravating the MetS by disturbing the diurnal rhythm. Also other sleep disturbances may lead to MetS. For example, some disturbed diurnal rhythm derivates are associated with shift work and, interestingly, also with the MetS[14]. Genome wide significance was reported recently with regard to plasma glucose and a SNP in the Melatonin Receptor 1B (*MTNR1B*) gene. This gene is involved in the melatonin related diurnal rhythm mechanism[15], which implicates that the genetics of diurnal rhythm is involved in the MetS.

Compulsive overeating is an addiction that is obviously strongly associated with obesity and the MetS. Compulsive overeating may be caused by specific sociological and psychological factors, which are poorly understood. It has also been shown that alcohol dependency and obesity are well

correlated[16]. In addition, compelling similarities between compulsive overeating and drug addiction were recently reviewed[17]. Furthermore, compulsive overeating clearly has a genetic component, since it has recently been shown that BMI is associated with genes involved in the susceptibility to addiction[18].

Migraine is a common neurological disorder that occurs in 35% of adult women and 20% of adult men of the general population. Migraine is characterized by attacks of severe headache and can be accompanied by visual distortions that are called auras. The current view is that the headache component of migraine has a neurovascular origin. Vasodilatation in specific brain regions indirectly triggers the higher order pain centers. The auras are thought to be caused by a slowly spreading depolarization over the cortex[19]. Hypertension, hyperglycemia and, in particular, obesity overlap with both migraine-susceptibility and MetS. A common link may be systemic inflammation, which could also affect the brain. This meningual inflammation may result in impaired vascular function in the brain and subsequent susceptibility to migraine[20,21].

A common form of dementia is Alzheimer's disease (AD). AD is a progressive neurological degenerative disease and mainly diagnosed in the elderly. AD is characterized by the deposition of amyloid-β plaques in the brain. Animal studies it has been shown that high fat diet promoted the AD-type of amyloidosis, while caloric restriction showed the opposite[22]. Even more intriguing is the role of insulin on cognitive function and AD. Not only can T2D lead secondary to dementia but there is also increasing evidence for increased insulin levels leading to AD[23]. Thus, AD may be a co-morbidity of the MetS caused by unfavorable dietary habits.

The second most common form of dementia is vascular dementia caused by multiple cerebral infarcts. The MetS is associated with the development of premature atherosclerosis which is a common mechanism underlying both (multiple) cerebral and cardiovascular infarcts. Unstable atherosclerotic plaques might rupture which in turn, triggers the development of a clinical sequence leading to thrombosis. When thrombi resulting from plaque rupture are not cleared, they will be transported down stream, which results in the temporary or permanent obstruction of blood vessels in the heart or the brain. This results in an infarct that is characterized by damaged or necrotic tissue[24].

To conclude, the application of the complex entity MetS as a single disease in genetic research for finding novel susceptible loci is unlikely to be successful. Despite its complexity, MetS functions as a common denominator in the search for connections between distinct traits.

GENETICS OF DYSLIPIDEMIA

The MetS lipid components hypertriglyceridemia and low HDL-cholesterol are clearly associated and their co-occurrence has been termed metabolic dyslipidemia (ref). Thus far, this specific abnormality has not been recognized by the traditional classification of different types of dyslipidemia that is used in the lipid clinic[25]. The so-called Frederickson classification is presented in Table 2 and involves 6 different types of hyperlipoproteinemia (HLP). The sub-classification of dyslipidemias or HLPs is relatively strict. However, it can be hypothesized that for example the hypertriglyceridemia that is observed in Type III, which involves an APOE2 homozygote background, and Type IV is affected by the same proteins and, thus by the same genetic variants[26].

An example of a shared modifier between HLP types III and IV was described in **chapters 3 and 5**. The HTG patients described in **chapter 3** suffer from elevated plasma triglyceride levels. The

Table 2 Frederickson classification of hyperlipoproteinemia (HLP) / dyslipidemia.

Phenotype	Involved (lipo)proteins	Total plasma cholesterol	Total plasma triglycerides
Type I	LPL deficient / chylomicrons	na	↑↑↑↑
Type II[a]	LDL	↑↑	na
Type II[b]	LDL / VLDL	↑↑	↑↑
Type III	IDL	↑↑	↑↑↑
Type IV	VLDL	na	↑↑
Type V	VLDL / chylomicrons	↑↑	↑↑↑↑

LPL: lipoprotein lipase; LDL: low density lipoprotein; VLDL: very low density lipoprotein; IDL: intermediate density lipoprotein. na: not affected.

clearance of TG from the plasma is primarily due to the action of lipoprotein lipase (LPL), which itself is subject to complex regulation by multiple cofactor and modifier proteins. **Chapter 3** described the association analysis of genetic variants in the apolipoprotein A5 (*APOA5*) which is a modifier of LPL. The contribution of these genetic variants was found to be relatively small, but significant.

Recently it has been described that APOE2 homozygosity can explain up to 30% of the genetic component for the expression of HLP Type III. Other genetic factors should explain a substantial part of the genetics of HLP Type III[27]. The HTG patients described in **chapter 5** suffer from HLP Type III. We hypothesized that the genetic modifiers found in **chapter 3** play a role in the expression of HLP type III (**chapter 5**). The main finding of **chapter 5** was that genetic variation in *LPL* and two *LPL* modulators, *APOC3* and *APOA5*, indeed contributed to the expression of HLP III.

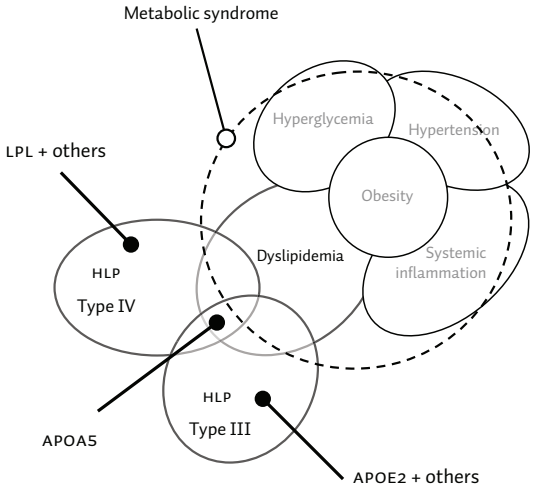

Fig. 2: Schematic overview of a genetic overlap between HLP type IV –and type III with regard to the MetS and its dyslipidemic cluster. Both HLP type IV –and III share the triglyceride phenotype and both HLP types were associated with genetic variants in the LPL modifying apoAV.

The hypertriglyceridemia that is part of the MetS definition is undoubtedly affected by the same proteins that affect the TG component of HLP type III and type IV (Figure 2). The challenge lies in the recognition and sub-clustering of the MetS patients that suffer from HTG and are carriers of specific genetic variants in TG metabolism. More detailed phenotyping of MetS patients in combination with the analysis of a panel of candidate genes could provide the basis for this sub-clustering.

GENETICS AND GENDER IN METS

Gender plays an important role in the expression of the MetS. This is illustrated by the well documented observation that women, especially before menopause, are in some way protected from the expression of MetS or related metabolic impairments[28,29,30,31,32,33]. In this thesis, this is illustrated in **chapter 2** by the significant difference in prevalence of MetS between men and women in the age range of 30 to 50 years old. Furthermore, in **chapter 6**, an increased risk of MetS in post menopausal women was shown. Interestingly, other disorders also show gender differences with regard to their prevalence. For example, migraine is more prevalent in women than in men[34] and obstructive

sleep apnea is more prevalent in men than in women[35]. In general, gender should be included as covariate in the epidemiological studies of MetS, MetS components or MetS related traits. However, **chapter 4** clearly showed that the susceptibility to high TG levels is strongly influenced not only by oral contraceptives use but also by pregnancy. In this context, one could argue that also oral contraceptives use and menopause should also be included as covariates in statistical analysis, while pregnant women should be excluded from studies in the general population.

However, the inclusion of gender, menopause and oral contraceptives use as covariates in genetic epidemiological studies results in ignoring gender specific genetics and thus, by definition, cannot explain the gender specific variance of the trait. Information on gender specific genetics can only be obtained by stratification of the cohort with regard to gender and/or with regard to female hormonal parameters.

Although differences in prevalence of disease across sexes do not imply that the genes involved are different for men than for women, the role of sex specific hormones in disease, such as MetS, asks for sex specific gene discovery. Since gender is a standard parameter in genetic epidemiological studies, stratification according to gender is not an issue. However, age at menopause and the life time exposure to contraceptives are difficult to determine at retrospect. Unfortunately, the major problem with stratification is the loss of statistical power. Table 3 illustrates a hypothetical power estimation for applying gender stratification in a study involving MetS (http://pngu.mgh.harvard.edu/~purcell/gpc). The stratification results in a strong reduction of the sample size in each statistical test and, thus, a reduction of statistical power of the study. The recessive model illustrated in Table 3 is strongly simplified and assumes certain genotypic risks for MetS of the alleles. Table 3 illustrates, that in the total group, with a relatively small sample size of 1000, the power to detect a genetic variant (minor allele frequency 25%, genotypic risk Aa=1.3, genotypic risk AA=1.8) within P=5% is 90%. In the stratified group however, of which the sample size is reduced by a half, the power is only 63%. Such stratified design allows the detection of gender-specific and not-gender-specific associations, but lacks, in this example, sufficient statistical power for both associations. Therefore, in genetic analyses stratified for gender, it is essential that the sample size is large enough. Fortunately, the present day genome wide association studies do involve large sample sizes. Gender specific loci can be located on the X and Y chromosome, but, it is also possible that autosomal loci show a gender-specific association.

Table 3: Statistical power estimation of MetS, N=1000

	PhenotypeTotal	Gender stratified
Case-control ratio	333 : 999	166 : 333
80% Power recessive model α=0.05 (%)	0.90	0.63

Power estimation model (80%) based on the following assumptions: Prevalence MetS: 35%; Minor allele frequency = 25%; genotypic risk Aa=1.3, genotypic risk AA=1.8.

GENETIC ASSOCIATIONS IN METS

In this thesis, considerable attention is paid to the adipocyte hormone adiponectin. The association of plasma adiponectin levels with obesity and insulin sensitivity is well established. This has led to the detailed investigation of the genetic architecture of adiponectin (Figure 3). A variety of associations between plasma adiponectin, adiponectin SNPs, the MetS and MetS traits were described in **chapters 6, 7 and 8**.

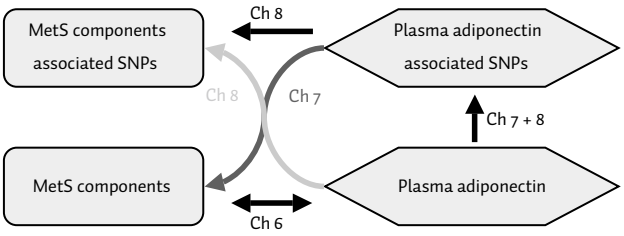

Fig. 3: Overview of research targets described in chapter 6, 7 and 8.

The genetic epidemiological studies described in **chapters 7 and 8** showed that the heritability of adiponectin is high, ranging between 55% and 60%. Similar estimates were found, considering women and men together, but also stratified by gender. **Chapter 7** replicated findings of others with regard to the association of genetic variants in the adiponectin gene (*ADIPOQ*) with plasma adiponectin levels. This *ADIPOQ* is one of the genes that were successfully identified by candidate gene approach and was replicated by two sets GWAS. The GWAS described in **chapter 8** did not reveal any other locus than *ADIPOQ* to be strongly associated with plasma adiponectin. As discussed above, the detection of more loci affecting plasma adiponectin, will require larger collaborative studies, using pooled results from the present consortia.

Many of the *ADIPOQ* associated variants described in **chapter 8** were also covered by the variants described in **chapter 7**. Although the genetic part of the studies in **chapters 7 and 8** involved association analysis of plasma adiponectin and MetS traits, they differed in design. The study in **chapter 7** primarily questioned whether genetic variants in *ADIPOQ* affect MetS and related traits, while the study in **chapter 8** questioned whether a series of loci associated with MetS traits affected plasma adiponectin. The meta -and replication analyses described in **chapter 8** are discussed in more detail in the next section.

In **chapter 7** the study of genetic overlap between adiponectin and MetS, MetS components and MetS related traits was described. The estimates of genetic overlap indicated that waist circumference, HDL-C, plasma insulin, HOMA-IR and BMI share a genetic component with plasma adiponectin. The association of an *ADIPOQ* SNP with plasma insulin and HOMA-IR was described in **chapter 7** and partly confirmed this genetic overlap. However, in **chapter 8** no associations were found between SNPs known to affect MetS traits and plasma adiponectin. This lack of association may be explained by the long chain of metabolic steps and interactions between genes affecting MetS traits, which indirectly affect adiponectin. Moreover, despite the large sample size, the statistical power was still too low to be able to detect such MetS related genetic variants involved in plasma adiponectin.

Further research into the overlap and thus exact mechanism, between adiponectin and MetS (traits) is necessary. This research could focus on the association of novel endo-phenotypes of obesity and the reverse cholesterol pathway. However, since adiponectin has already been thoroughly investigated in both mechanistic directions using association analysis without much success, it seems likely that alternative approaches may be more successful. Such approaches could involve *in vitro* and *in vivo* research to determine the exact mechanism of action of adiponectin in metabolic pathways.

GENOME WIDE ASSOCIATION IN METS

Genome wide association studies on a wide range of traits have been designed in accordance with the "Common Disease Common Variant" (CDCV) hypothesis. In this hypothesis, common genetic variation is usually defined as individual variants with a minor allele frequency (MAF) of more than 5%. Study designs in association analyses of MetS and related traits have involved quantitative

traits such as WC[4] and qualitative traits such as diabetes[36]. Thus far, the CDCV approach has yielded some 20 loci that are associated with the binary trait T2D and 15 loci that are associated with the quantitative trait body mass index[4]. Furthermore, the genes that were found to be associated with lipid levels, jointly, also determined which person end up in the extreme[13]. However, the effect sizes of the genetic variants in these loci were found to be small. In addition, even when the genetic variants of such loci were used in a combined analysis, such as a "risk allele score analysis", generally, a very small increase of total effect size was observed.

Thus far, for most traits only a small percentage of the variance can be genetically explained[3,4,5,7]. The extent of variance of plasma adiponectin which can be explained by the SNPs in the adiponectin gene itself ranges from 2% explained by the strongest *ADIPOQ* SNP to 8% explained by 9 *ADIPOQ* associated SNPs (**chapter 8**). Even though this percentage of explained variance seems low, it is high compared to the values for other traits (Figure 4). For example, the heritability of human stature is estimated to be approximately 80% of which only 6% can be explained by genetic variants in multiple genes.

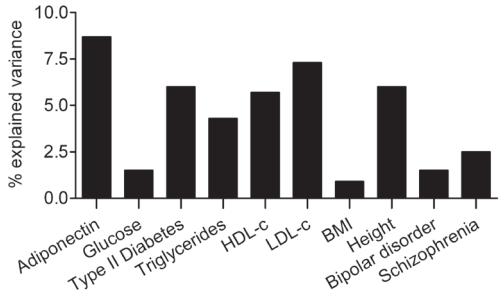

Fig. 4: Overview of percentages of the genetic component explaining the variance of Adiponectin (chapter 8), glucose and Type II diabetes[1], plasma lipid[3], BMI[4]. Height[5], schizophrenia and bipolar disorder[7].

Interestingly, the *ADIPOQ* SNPs, described in **chapter 8,** that were associated with plasma adiponectin, were not similar to the findings of others. Moreover, the recently reported association of the *ARL15* gene, influencing plasma adiponectin levels, was not observed in the study described in **chapter 8**[37].

The relatively limited contribution of the CDCV GWAS approach to gaining insight in the genetics of complex diseases has resulted in the questioning of the relevance of the CDCV hypothesis itself. First, it is questioned whether common variance itself is not subject to epigenetic, gene-gene and/or environmental interactions. However, for each of these explanations no experimental proof has been found, but neither these explanations can be excluded. Second, it is hypothesized that common disorders may not be caused by common genetic variation, but by large amounts of more or less rare variation present in the general population[38]. Finally, it is questioned whether the use of binary traits, which have their origin in the clinic, is the most appropriate approach in genetic epidemiology. Figure 5 illustrates such binary stratification in "healthy" and "diseased" using a threshold value or cut off point in association analysis according to the "common disease common variant" (CDCV) hypothesis. Such threshold values are based on clinical cut off points and/or threshold values determined by means of epidemiological studies. However, it is unlikely that these threshold values define the exact biological relevant set point of impairment, which definitely compromises the statistical power of association analyses.

The hypothesis that quantitative traits are determined by multiple forms of regulation is currently being addressed in various ways. The role of epigenetics and especially parental imprinting is being investigated by parent-of-origin specific association analyses. This technique addresses the question whether a specific genetic variant has different effects depending on the parent who

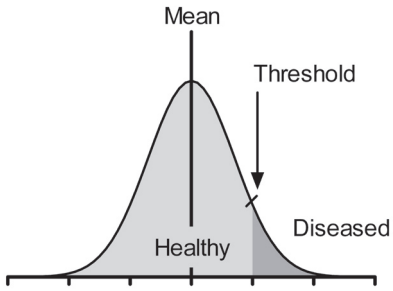

Fig. 5: Schematic illustration of stratification towards healthy and diseased using a threshold value or cut off point in binary association analysis.

donated the allele[39,40]. Genome wide gene-gene interaction analyses are being performed, but are computationally challenging[41,42,43]. However, gene-gene interaction studies using a limited number of genetic variants are feasible. For example, the interaction between the two candidate genes cholesteryl ester transferase protein (CETP) and hepatic lipase (HL) was shown to be associated with HDL-cholesterol[44].

The second question with regard to the validity of the CDCV hypothesis is addressed by the common disease rare variant (CDRV) hypothesis[38,1]. Two types of rare variation are thought to play a role in the genetic basis of disease. The first type of rare genetic variants includes coding SNPs, promoter SNPs or SNPs affecting microRNAs. The latter two variants are involved in transcriptional regulation. The currently used genotyping platforms were not designed to include such rare variants but, contain as many common tagging SNPs as possible. The second type of variation involves rare or *de novo* copy number variation (CNV) in the form of duplications or deletions of relatively large regions of a chromosome[38,1]. Thus far, rare genetic variants covered by the first category can only be discovered using linkage analysis followed by sequencing. Deep sequencing will be discussed in section 9.6 below. CNVs, however, can be detected with the current platforms using the intensities of the genotypes of each individual. Large association analyses of CNV and MetS traits are, at present, still limited in number and size.

Finally, the question with regard to the appropriateness of binary traits in GWAS is addressed by simply banishing binary traits. This approach is covered by the infinitesimal hypothesis[38]. The infinitesimal hypothesis poses that the use of threshold values defining the disorder precludes the finding of thousands of common variants with minor effect size. It was estimated that redefining traits like T2D towards a more continuous trait for particular genetic epidemiological studies would instrumentally improve the power to detect novel loci. However, it should be recognized that the infinitesimal approach implies that a disease like T2D is a continuum, and does not fully take into account the possibility that multiple distinct underlying disease mechanisms may have the same outcome.

FUTURE PERSPECTIVES

STEADY STATE VERSUS DYNAMIC PHENOTYPICAL PARAMETERS

Thus far, GWAS have been predominantly performed on static plasma parameters such as glucose, lipids and hormones. Plasma levels are, however, the net result of production and clearance and these processes may be affected without a net effect on plasma levels. Moreover, plasma levels of for example glucose and lipids are generally measured after an overnight fast, whereas it may actually be the response to feeding (and not fasting) that is different for a specific parameter.

Thus, a more promising alternative to improve the discovery of novel loci in GWAS is to define dynamic phenotypes. For example, studies on T2D or insulin sensitivity could be more successful when using parameters from hyperglycemic clamp and hyperinsulinemic euglycemic clamp analysis[45]. Both clamping methods provide specific data on the sensitivity and flux with regard to

glucose metabolism. Using both these specific insulin sensitivity parameters would definitely reduce the heterogeneity in GWAS with regard to traits like plasma glucose or T2D. The reduction of the heterogeneity of the fasting plasma glucose trait has recently been described[46]. This report described the inclusion of HOMA-B, which is a measure for pancreatic β-cell function, and HOMA-IR, which is a measure for peripheral insulin sensitivity, in GWA and meta-analysis. The levels of insulin sensitivity and β-cell function both affect plasma glucose levels but, through distinct biological pathways. The authors clearly showed that the inclusion of the heterogeneity lowering traits HOMA-B and HOMA-IR indeed contributed to the detection of 9 novel loci which were not detected by GWAS using fasting glucose. The determination of dynamic parameters other than HOMA-B and HOMA-IR is, however expensive, time-consuming and often more burdening for the participants.

Thus, the challenge for the generation of dynamic parameters lies in the development of novel less time consuming and less expensive methods.

SEX CHROMOSOMAL AND MITOCHONDRIAL DNA

The currently reported GWAS have largely ignored the X and Y chromosomes in their analyses. In addition, the mitochondrial DNA is also absent in virtually all GWAS reported over the last years. Since the MetS and several of its individual components show large differences in prevalence or level between genders, the absence of both sex chromosomes is a missed opportunity to discover novel loci. Mitochondria play a key role in energy metabolism and thus the inclusion of mitochondrial genetic variants in GWA could contribute to the discovery of new loci. The absence of both the sex chromosomes and mitochondrial variants in present GWAS is, however, not without reason.

For the X chromosome, one reason is that recombination occurs in females and only partly in males. This latter partial recombination involves the pseudo-autosomal regions (PARs) and is described below. All genotyping platforms cover the X chromosome well. Nevertheless, inclusion of the X chromosome in GWAS is not yet standard.

For the Y chromosome, one reason is that recombination can only occur at the PARs. Thus, with the exception of these PARs, the main part of this chromosome shows very strong LD. The region PAR1 is localized on the short arm of the X or Y chromosome and the PAR2 region is located on the long arm of X or Y chromosome. To date, 24 genes have been reported in the PAR1 region and only 5 genes have been reported to be located in the PAR2 region. The limited number of genes and the fact that the remaining part of the Y chromosome is in strong LD, might explain why companies like Affymetrix and Illumina have virtually ignored the Y chromosome in their SNP array platforms. The Illumina arrays in particular, only included none or a small amount of genetic PAR variation on their arrays. It should be noted, though, that the most recently released arrays of both companies show an improved PAR and Y chromosome coverage. Nevertheless, the GWAS which are now or have recently been reported are, in many cases, based on the older SNP array versions[47,48].

Similar to the Y chromosomes, with the exception of the PARs, the mitochondrial DNA also shows no recombination. Mitochondria are passed down maternally, completely unchanged except for occasional spontaneous mutations. Thus, when mutations occur, they are in complete linkage with all other present variants in that mitochondrial DNA. Therefore, mitochondrial haplotypes are an important resource for studying the maternal history of populations[49,50]. In the course of evolution, the mitochondria have donated several genes to the autosomes. This group of mitochondrial genes is thus covered by all genotyping platforms. However, the formerly used Illumina and Affymetrix arrays

did not properly cover the mitochondria themselves. In particular Illumina 300K did not include any mitochondrial variants. To conclude, the limited coverage or absence of the X, Y and mitochondrial genetic variation on the former versions of, in particular the Illumina, SNP arrays is a missed opportunity to discover novel loci associated with gender specific traits like MetS or some of its components and related traits. Re-genotyping of the whole genome using more recently developed arrays is not cost-effective, because, it would involve mainly redundant genotypic information. Therefore, genetic information on the PARS, Y chromosome and mitochondria should rather be obtained by genotyping on other platforms, such as the Sequenom using MALDITOF technology (http://www.sequenom.com)[50] or sequencing.

COMMON DISEASE, COMMON VARIANTS VS. COMMON DISEASE, RARE VARIANTS

Since the CDRV hypothesis requires the identification of rare mutations in many individuals, this calls for a totally different genotyping technique than the one used in the CDCV hypothesis, namely large scale DNA sequencing. Ideally, the whole genome sequence of each individual of a particular cohort is determined.

Over the last decades, the sequencing technique based on the DNA chain terminating method, developed by Frederick Sanger has been most extensively used[51]. However, this method is not suitable for genome wide sequencing of whole cohorts. Recently, several companies have developed novel methods for large scale and high throughput sequencing, which is illustrated in Table 4[52]. These novel sequencing techniques are called second or next generation sequencing. In theory, these techniques are capable of determining whole genome sequences with a high throughput.

However, there are problems with regard to cost, storage and analysis of next generation sequencing data, which makes the implementation of these new techniques more challenging. First, the next generation sequencing techniques are not flawless in nucleotide calling. Therefore, sequences have to be retyped several times, which means that the analysis will consume more time and, importantly, increase the expenses. In addition, the coverage -or reading length- of all next generation sequencing techniques is limited. Furthermore, sequencing whole genomes of each individual in a particular cohort will generate an enormous amount of data. Even for a small cohort, analyzing such an amount of data is a challenging task. More importantly, genome wide association scans using rare variants obtained from whole genome sequence analysis will result in a tremendous increase in the number of variables to test. The normal controls, computational methods and technical power necessary for such complex analyses are simply not yet available and this is not likely to change in the near future. To date, only genome wide sequencing of coding regions, called exome sequencing, can be performed and implemented in association analysis. Thus, on the short term, the proper testing of the CDRV hypothesis on a large and truly genome wide scale requires novel approaches that remain to be developed.

Thus far, a limited test of the CDRV hypothesis has been performed by analyzing the coding regions of candidate genes, which were identified using CDCV techniques. This approach has yielded some interesting results. For example, it was recently shown that rare mutations in two novel T2D CDCV associated loci, the *KCNJ11* and *ABCG8* genes, were associated with an increased risk of T2D[53]. These mutations were shown to be in strong linkage disequilibrium with the common variants formerly discovered in a genome wide association of T2D. In addition, this report proposed a clear biological mechanism which may lead to the expression of T2D. However, for other disease associated loci,

Table 4: Overview of 2nd (next) generation sequence technology

Company	Technique	Maximum length / strain	Maximum Strains / run
Illumina - SOLEXA	Array based - parallel sequencing	100 bp	8 * 15.10^6
Applied Biosystems -SOLiD	Array / bead based - Multiplex polony sequencing	100 bp	16 * 15.10^6
Roche - 454	Single bead / well based - pyro sequencing	500 bp	16 * 5.10^5
Helicos *Single molecule sequencing*	Array based - Single molecule incorporation	35 bp	50 * 15.10^6

rare causal variants might be located in regions far apart from their coding region or even on distinct chromosomes. Thus, whether this limited implementation of next generation sequencing is a fruitful approach in general is not clear yet.

APPLICATION OF BIOINFORMATICS AND SYSTEMS BIOLOGY

The technology to measure a large number of biological components with very high throughput is covered by the term "omics" and has boomed over the past decade. The current SNP and DNA sequence assays technologies are examples of such omic approach and is covered by the term "genomics". More or less simultaneously, technologies to perform genome-wide analyses of mRNA levels, proteins and metabolites have been developed. Obviously, the handling, storage and analyses of these often very large data sets require biostatistics and bioinformatics expertise.

At present, the tools to analyze single data sets, for example, sets of SNP or gene-expression data, are well developed. Moreover, SNP and gene expression data sets can be relatively easily combined, because they share a common reference; genomic position[54]. A polymorphism in a gene associated with its own expression is called a *cis* association, while a polymorphism that explains an altered mRNA expression of a distinct chromosomal region is called a *trans* association. This method in an extended form, using full omic data sets, is called eQTL analysis[55,56,57]. Recently, several tissue specific eQTL data sets have been made publicly available. This public availability makes the eQTL analysis a feasible approach, since the tissue specific determination of expression levels in the currently used epidemiological study cohorts is unrealistic (http://eqtl.uchicago.edu).

The major challenge in the combination of different "omics" data sets lies in the data sets where the common reference (i.e. the gene) is more difficult to define. For example, metabolites are notoriously difficult to assign to a single specific enzyme or gene and combining metabolomics and transcriptomics therefore requires an alternative approach. A suitable approach could be the mapping of the up or down regulated genes and metabolites to a common literature-based concept database[58]. Finding the overlap between the data sets then requires finding the overlap in the common reference concepts. No matter what approaches are taken to combine data sets, an essential requirement for further development of this field, is the availability of data sets that are publicly available.

It should also be recognized that some of the "omic" technologies may not be developed far enough for implementation in bioinformatics and biostatistics approaches. For example, a reproducible and qualitative "protein fingerprint" method[59,60,61] is currently in development, while others, such as epigenomics and metabolomics approaches, are likely to become available at a much later stage.

It should be clear that, similar to the full scale testing of the CDRV hypothesis, combinatorial genomics analysis is a challenge for the computational power of the currently used bioinformatics platforms[62,63].

The bioinformatics toolbox has been extended to so-called pathway analyses which determine whether specific biological components that belong to a pathway are enriched under the one condition rather than under the other[64,65]. The origins of these pathways are found in often manually cured databases (KEGG: www.genome.jp/kegg/pathway.html). The current pathway analyses generally make calls based on the expected/observed number of components of a specific pathway and do not take into account that some steps in a pathway may be rate limiting. The novel field of mathematical Systems Biology aims to quantify the role of components in pathways. This requires the development and analysis of mathematical models that describe pathways and, eventually, the biological systems in cells, organs and organisms. The ultimate goal would be an analysis that combines all omic approaches, using genome-wide mathematical models, in order to describe the patho-physiology of the system under study.

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SUMMARY

The primary causes of death among both males and females in developed countries with a western lifestyle are vascular diseases including CVD and stroke. In the last decade, approximately 30% of all deaths in the Netherlands were due to stroke, CVD or related vascular diseases. Metabolic risk factors for CVD, stroke and T2D are obesity, hyperglycemia, dyslipidemia and hypertension. These risk factors often cluster together. The metabolic syndrome (MetS) takes into account such clustering. **Chapter 1** introduces the MetS definition and its individual components and their corresponding morbidities, prevalence and suggested biological mechanisms. In addition, several feasible genetic epidemiological study approaches for finding novel loci for the MetS or related traits are described.

The main question in the study described in **chapter 2** was whether MetS is a promising feasible phenotype in itself or that its individual components are more promising phenotypes to find novel loci in future genetic association studies. To answer this question, we used the Erasmus Rucphen Family (ERF) study. The ERF population is a genetically isolated population and involves large interrelated families. The latter made it possible to obtain extended pedigree information about this population. The power of such pedigree information is that the heritability of complex diseases, like MetS, can be easily estimated without genotyping. In **chapter 2** a study of heritability estimates of MetS and of the individual MetS components is described. This study found that the individual components of MetS, in particular waist circumference and HDL-cholesterol, are markedly more heritable than MetS itself. It was therefore concluded that the individual components of MetS are more promising with regard to finding novel loci.

The studies described in **chapters 3 and 4** were focused on the genetics of plasma triglyceride. Hypertriglyceridemia (HTG) is a component of the MetS. In general, HTG involves an impairment of VLDL and/or chylomicron production or clearance. Lipoprotein lipase (LPL) is involved in the clearance of VLDL and chylomicrons. The apoAV is thought to be a modulator of LPL and is present on chylomicrons and VLDL. ApoAV is thought to have a stimulatory effect on the clearance of TG by LPL. The main question in the study described in **Chapter 3** was whether genetic variants in the *APOA5* gene contributed to the HTG phenotype. This study concluded that two genetic variants located in the *APOA5* gene, S19W and SNP3 did indeed contribute to the expression of HTG.
In the survey of the genetic association between *APOA5* variants and HTG, described in **chapter 3**, we found one individual who lacked apoAV. Interestingly, during an extended period of time, plasma TG levels in this female individual showed to be highly variable. In the study described in **Chapter 4**, we aimed to determine the genetic variant causing this particular apoAV deficiency and to elucidate the interrelationship between apoAV, female hormones and plasma TG. From this study it was concluded that the apoAV deficiency involved a novel discovered splice variant which resulted in impaired splicing causing the complete absence of the apoAV protein on chylomicrons and VLDL. In addition, it was shown that pregnancy or oral contraceptive in combination with this apoAV deficiency dramatically increased the plasma TG levels in this patient, whereas without pregnancy or oral contraceptive, diet intervention resulted in a normalized plasma TG level.

The study described in **chapter 5** involved a genetic association study of HLP type III. This metabolic disease is characterized by elevated levels of both TG and (total) cholesterol, caused by inefficient

clearance of the LDL, VLDL and chylomicrons by the liver. This inefficient clearance is caused by the diminished binding capacity of the apoE2 variant to the LRP receptor. Patients suffering from HLP type III are homozygote carriers of the *APOE2* genetic variant. However, the penetrance of this homozygosity to expression of HLP type III is low, which indicates that multiple genetic factors contribute to the expression of HLP type III. In this study we aimed to determine whether genetic variants in the Hepatic lipase (*HL*), lipoprotein lipase (*LPL*), apolipoprotein C3 *(APOC3)* and apolipoprotein A5 (*APOA5)* genes contribute to the manifestation of hyperlipidemia type III. HL and LPL are both involved in the clearance of TG from VLDL and chylomicrons to the liver or periphery. ApoCIII and apoAV are thought to be modulators of LPL. Genotypic -and allelic frequency differences as well as odds ratios between HLP type III patients and normolipidemic homozygote carriers of apoE2 were determined. The study concluded that genetic variation in the LPL, apoC3 and apoA5 indeed contributed to the expression of HLP type III.

Adiponectin is a hormone which is secreted by adipose tissue. It has been shown to be strongly associated with obesity and T2D and has been suggested as a biomarker for the MetS. MetS depends on factors like lifestyle, age and gender or related factors. The study described in **chapter 6** was particularly aimed at elucidating the effect of menopause and low plasma adiponectin levels and the risk for MetS. In addition, we investigated whether plasma adiponectin shows a better discriminative accuracy for the presence of MetS than traits more easily obtained, such as BMI and age. This study concluded that, in particular, low levels of plasma adiponectin in postmenopausal women are a risk for MetS, independently of age and BMI. However, the discriminating accuracy of adiponectin for the presence of MetS is exceeded by BMI in men and pre -and postmenopausal women. These conclusions imply that the clinical value of adiponectin is limited.

The mechanisms and genetics underlying the correlations between adiponectin and MetS, obesity and T2D and related traits have not been explained yet. The study described in **chapter 7** aimed to determine the genetic overlap between plasma adiponectin and MetS, its individual components and MetS related traits like BMI insulin resistance (HOMA-IR) and systemic inflammation. Furthermore, we aimed to validate such genetic overlap with regard to the adiponectin gene (*ADIPOQ*). In this study we used data from the ERF study. The genetic correlation was determined using bivariate heritability analyses and the genetic association study was PERFormed with 10 *ADIPOQ* tagging variants. This study concluded that adiponectin shared a genetic component with HDL-cholesterol and plasma insulin. Furthermore, it was concluded that two *ADIPOQ* variants were independently associated with plasma adiponectin and that one of these variants was associated independently of plasma adiponectin with plasma insulin and HOMA-IR. The latter conclusion suggests there is indeed a genetic overlap between adiponectin and insulin and HOMA-IR with regard to the *ADIPOQ* gene itself.

Chapter 8 describes the search for novel loci associated with plasma adiponectin using genome-wide association (GWA) in the ERF cohort. These ERF results were used in a meta-analysis with two other cohorts: (1) a German cohort - Kooperative Gesundsheidsforshung in der Region Ausburg (KORA) and (2) an Italian cohort - Microisolates in South Tyrol Study (MICROS). Since plasma adiponectin levels show large differences between the genders, the analyses were PERFormed in a combined set and in women and men separately. Meta-analysis results were submitted for replication to 7 other

large cohorts. The study described in **chapter 8** concluded that variants in *ADIPOQ* showed a highly significant association with plasma adiponectin in both the combined and the two gender stratified analyses. Other hits in the meta-analyses were found not to be genome wide significant, but were nevertheless submitted for replication (threshold $P < 10^{-6}$). The *ADIPOQ* variants were found to explain a relative large percentage of the adiponectin variance.

Chapter 9 starts with a discussion of the relevance of MetS in a clinical setting, a social context and in research. Secondly, the chapters in this thesis are reviewed, both individually and in relation to each other. Thirdly, limitations of study methods and designs are discussed, in particular the study design concerning the common variants common disease (CVCD) hypothesis. Finally, suggestions are made for the improvement of existing methods and for alternative approaches for future analyses towards unraveling the genetic and environmental factors that play a role in metabolic risk factors.

SAMENVATTING

Vasculaire ziekten vormen de primaire doodsoorzaak bij mannen en vrouwen in ontwikkelde landen met een Westerse leefstijl. In de afgelopen tien jaar werden 30% van alle sterfgevallen veroorzaakt door beroerten, CVD of andere gerelateerde vasculaire ziekten. Metabole risicofactoren voor CVD, beroerte en TD2 zijn obesitas, hyperglycemia, dylipidemia en hypertensie. Deze risicofactoren zijn vaak geclusterd. De definitie van het Metabool Syndroom (MetS) is gebaseerd op zulke clustering. **Hoofdstuk 1** introduceert de definitie van MetS en de individuele componenten en hun respectievelijke sterftecijfers, prevalentie en veronderstelde onderliggende biologische mechanismen. Vervolgens worden verschillende haalbare genetisch-epidemiologische onderzoeksbenaderingen om nieuwe loci voor Mets-gerelateerde eigenschappen te vinden beschreven.

De belangrijkste vraag in het onderzoek dat in **hoofdstuk 2** wordt beschreven was of MetS op zichzelf een veelbelovend fenotype is of dat de afzonderlijke componenten van MetS geschikter zijn als fenotypen om nieuwe loci te vinden in toekomstige genetische associatiestudies. Om deze vraag te beantwoorden is gebruik gemaakt van het Erasmus Rucphen Familie (ERF) onderzoek. Deze ERF-populatie is een genetisch geïsoleerde populatie met veel grote, nauw met elkaar verbonden families. Dit laatste gegeven maakte het mogelijk om uitgebreide stamboominformatie over deze populatie te verkrijgen. De kracht van dergelijke uitgebreide genealogische informatie is dat de erfelijkheid van complexe ziekten, zoals MetS, gemakkelijk kan worden geschat zonder dat daar genotypering voor nodig is. In het in **hoofdstuk 2** beschreven onderzoek werd de erfelijkheid van zowel Mets als van de afzonderlijke componenten van Mets bekeken. Uit dit onderzoek kwam naar voren dat de erfelijkheid van de afzonderlijke componenten van MetS, in het bijzonder middelomvang en HDL-cholesterol, aanzienlijk groter is dan die van MetS zelf. Hieruit werd geconcludeerd dat de afzonderlijke componenten van MetS veelbelovend zijn met betrekking tot Mets voor het vinden van nieuwe loci.

De onderzoeken die beschreven worden in **hoofdstukken 3 en 4** waren beide gericht op de genetica van plasma triglyceride. Hypertriglyceridemia (HTG) is een afzonderlijke Mets component In het algemeen wordt HTG gekenmerkt door een defect in de productie of de klaring van VLDL en/of chylomicronen. Lipoproteine lipase (LPL) speelt een belangrijke rol in de klaring van VLDL en chylomicronen. Chylomicronen en VLDL bevatten beide apoAV, een proteïne dat wordt gezien als een modulator van LPL. Verondersteld wordt dat apoAV een stimulerend effect heeft op de klaring van TG door LPL. De hoofdvraag in het onderzoek dat beschreven wordt in **hoofdstuk 3** was of genetische varianten in het APOA5-gen bijdroegen aan het HTG fenotype. De conclusie van dit onderzoek was dat twee genetische varianten in het APOA5 gen, te weten S19W en SNP3, inderdaad bijdragen aan de expressie van HTG.
In het onderzoek naar de genetische associatie tussen APOA5 varianten en HTG, zoals beschreven in **hoofdstuk 3**, werd 1 individu gevonden bij wie apoAV volledig ontbrak. Een interessant resultaat was dat, over een langere periode gemeten, de plasma TG niveaus van deze vrouw sterk varieerden. Het in **hoofdstuk 4** beschreven onderzoek was erop gericht om vast te stellen welke genetische variant deze specifieke apoAV deficiëntie veroorzaakte en om het veronderstelde verband tussen apoAV, vrouwelijke hormonen en plasma TG te verhelderen. Uit dit onderzoek werd geconcludeerd dat de apoAV deficiëntie werd veroorzaakt door een nieuw ontdekte splice variant, resulterend in

gemankeerd mRNA waardoor het apoAV proteïne op chylomicronen en VLDL volledig ontbreekt. Bovendien werd aangetoond dat deze apoAV deficiëntie in combinatie met zwangerschap of gebruik van een oraal anticonceptiemiddel een dramatische toename in de plasma TG niveaus bij deze patiënt tot gevolg had, terwijl zonder zwangerschap of oraal anticonceptiemiddel aanpassingen in het dieet leidden tot een genormaliseerd plasma TG niveau.

Het onderzoek dat in **hoofdstuk 5** beschreven wordt betrof een genetische associatiestudie van hyperlipoproteinemia type III (HLP type III). Deze metabole ziekte wordt gekenmerkt door verhoogde niveaus van zowel TG als (totaal) cholesterol welke veroorzaakt worden door de inefficiënte klaring door de lever van LDL, VLDL en chylomicronen. Deze inefficiënte klaring wordt veroorzaakt door een verminderde bindingscapaciteit van de apoE2 variant met de LRP receptor. Patiënten die lijden aan HLP type III zijn homozygote dragers van de *APOE2* genetische variant. Echter, de penetrantie van deze homozygositeit naar expressie van HLP type III is laag, wat erop duidt dat meerdere genetische factoren bijdragen aan de expressie van HLP type III. In dit onderzoek werd getracht vast te stellen of de genetische varianten in de Hepatische lipase (HL), lipoproteine lipase (LPL), apolipoproteine C3 (*APOC3*) en apolipoproteine A5 (*APOA5*) bijdragen aan de manifestatie van HLP type III. HL en PL zijn beide betrokken bij de klaring van TG uit VLDL en chylomicronen naar de lever of periferie. ApoCIII en apoAV worden beschouwd als modulators van LPL. Er werden zowel genotypische- en allelische frequentieverschillen als het genetisch risico tussen HLP type III patiënten en normolipidemische homozygote dragers van apoE2 vastgesteld. De conclusie van het onderzoek was dat genetische variatie in *LPL*, *APOC3* en *APOA5* inderdaad bijdraagt aan de expressie van HP type III.

Adiponectine is een hormoon dat wordt uitgescheiden door vetweefsel.
Adiponectine is sterk geassocieerd met obesitas and TD2 en is geopperd als een biomarker voor het MetS. MetS is afhankelijk van factoren als leefstijl, leeftijd, gender en gerelateerde factoren. Het onderzoek dat in **hoofdstuk 6** wordt beschreven is vooral gericht op het ophelderen van het effect van de menopauze en lage adiponectine niveaus en het risico voor MetS. Daarnaast werd onderzocht of plasma adiponectine nauwkeuriger discrimineert voor de aanwezigheid van MetS dan eenvoudiger te meten kenmerken, zoals BMI en leeftijd. Dit onderzoek concludeerde dat vooral lage niveaus van plasma adiponectine in postmenopauzale vrouwen een risico op MetS vormen, onafhankelijk van leeftijd of BMI. Echter, de discriminerende nauwkeurigheid van adiponectine voor de aanwezigheid van MetS wordt overtroffen door BMI bij mannen en pre- en postmenopauzale vrouwen. Deze conclusies impliceren dat de klinische waarde van adiponectine beperkt is.

De onderliggende mechanismen en genetica van de correlaties tussen adiponectine en MetS, obesitas en TD2 en verwante kenmerken zijn nog niet verklaard. Het doel van het in **hoofdstuk 7** beschreven onderzoek was om de genetische overlap tussen plasma adiponectine en MetS, de afzonderlijke componenten van Mets en Mets-gerelateerde kenmerken zoals BMI, insuline resistentie (HOMA-IR) en systemische inflammatie vast te stellen. Daarnaast werd getracht een dergelijke genetische overlap te valideren voor wat betreft het adiponectine gen (*ADIPOQ*). Voor dit onderzoek zijn gegevens uit de ERF-studie gebruikt. De genetische correlatie werd bepaald door middel van bivariate ERFelijkheidsanalyses en de genetische associatiestudie werd uitgevoerd met *ADIPOQ* "tagging" varianten. De conclusie van dit onderzoek was dat adiponectine een genetische component deelt met HDL-cholesterol en plasma insuline. Bovendien bleek dat twee *ADIPOQ*

varianten onafhankelijk geassocieerd zijn met plasma adiponectine en dat één van deze varianten onafhankelijk geassocieerd is met plasma insuline en HOMA-IR. Deze laatste conclusie suggereert dat er inderdaad een genetische overlap bestaat tussen adiponectine en insuline en HOMA-IR in relatie tot het *ADIPOQ* gen zelf.

In **hoofdstuk 8** wordt de zoektocht beschreven naar nieuwe loci die geassocieerd zijn met plasma adiponectine middels "genome-wide association" (GWA) binnen het ERF-cohort. Deze resultaten werden gebruikt in een meta-analyse met twee andere cohorten: (1) een Duits cohort – Kooperative Gesundheidsforshung in der region Ausburg (KORA) en (2) een Italiaans cohort – Microisolates in South Tyrol Study (MICROS). Aangezien plasma adiponectine niveaus grote verschillen laten zien tussen de genders, werden de analyses zowel in een gecombineerde set als afzonderlijk in mannen en vrouwen uitgevoerd. De meta-analyse resultaten zijn vervolgens voor replicatie aangeboden aan 7 andere grote cohorten. Uit het in **hoofdstuk 8** beschreven onderzoek werd geconcludeerd dat bepaalde varianten in *ADIPOQ* zeer significant waren geassocieerd met plasma adiponectine, zowel in de gecombineerde als in de twee gender gestratificeerde analyses. Andere hits in de meta-analyses bleken niet genoombreed significant te zijn, maar werden desondanks wel ter replicatie (drempel $P < 10\text{-}6$) aangeboden. De *ADIPOQ* varianten bleken een relatief groot percentage van de adiponectine variantie te verklaren.

Hoofdstuk 9 begint met een discussie over de relevantie van het MetS in de context van de kliniek, maatschappelijk en in wetenschappelijk onderzoek. Vervolgens worden alle voorgaande hoofdstukken in dit proefschrift zowel afzonderlijk als in relatie tot elkaar in ogenschouw genomen. Daarna worden de beperkingen ten aanzien van onderzoeksmethoden en onderzoeksontwerp besproken, in het bijzonder voor het onderzoeksontwerp met betrekking tot de "common variants common disease" (CVCD) hypothese. Ten slotte worden suggesties gedaan voor verbetering van bestaande methoden en ook voor alternatieve benaderingen van toekomstige analyses gericht op het ontrafelen van de genetische en omgevingsfactoren die een rol spelen in metabole risicofactoren.

ABBREVIATIONS

Apo	Apolipoprotein
AUC	Area Under the receiver operating characteristic Curve
BC	Body Composition
BMI	Body Mass Index
CDCV	Common Disease Common Variant (hypothesis)
CDRV	Common Disease Rare Variant (hypothesis)
CRP	C-Reactive Protein
CVA	Cerebral Vascular Accident
CVD	Cardio Vascular Disease
DBP	Diastolic Blood Pressure
DNA	DeoxyriboNucleic Acid
ERF	Erasmus Rucphen Family (study)
FPG	Fasting Plasma Glucose
GWA	Genome Wide Association
h^2	heritability
HDL-C	High Density Lipoprotein Cholesterol
HL	Hepatic Lipase
HLP	HyperLipoProteinemia
HMW	High Molecular Weight
HOMA-IR	Homeostatic Model Assessment-Insulin Resistance
HTG	HyperTriGlyceridemia
LA	Linkage Analysis
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
LPL	LipoProtein Lipase
LUMC	Leiden University Medical Center
MAF	Minor Allele Frequency
MetS	Metabolic Syndrome
mRNA	messenger RiboNucleic Acid
OR	Odds Ratio
S	Sibship effect
SBP	Systolic Blood Pressure
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
T2D	Type-2 Diabetes mellitus
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
WC	Waist Circumference
_E	Environmental correlation estimate
_G	Genetic correlation estimate

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CURRICULUM VITAE

Peter Henneman werd op 27 oktober 1975 geboren in Katwijk aan Zee. In 1994 behaalde hij het HAVO diploma aan het Da Vinci College te Leiden. In hetzelfde jaar startte hij met een chemische laboratoriumopleiding aan de Hogeschool Leiden. In 1996 werd het propedeutische examen behaald. In het jaar 1998 heeft hij stage gelopen bij het Forensisch Laboratorium voor DNA Onderzoek (FLDO) van de Universiteit Leiden, afdeling Humane en Klinische Genetica, onder leiding van dr E. Bakker en dr P. de Knijff. Eind 1998 werd het bachelor examen met goed gevolg afgelegd.

Peter Henneman was vanaf oktober 1998 tot en met december 2004 als analist werkzaam bij het FLDO. Naast de werkzaamheden bij het FLDO, begon Peter Henneman in 1999 met de studie biochemie aan de Universiteit Leiden. Het propedeutische examen werd in 2001 behaald. In datzelfde jaar startte Peter de *master* stage bij het FLDO onder leiding van dr P de Knijff. In 2003 werd het doctoraal examen behaald.

Vanaf januari 2005 tot en met maart 2009 was Peter Henneman werkzaam als assistent in opleiding bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum. Dit promotietraject stond onder leiding van prof. dr R.R. Frants en dr Ir J.A.P. Willems van Dijk van de afdeling Humane Genetica van het Leids Universitair Medisch Centrum en prof. dr C.M. van Duijn en dr. Y.S. Aulchenko van de afdeling Genetische Epidemiologie van het Erasmus Medisch Centrum te Rotterdam. De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift. Sinds april 2009 is hij aangesteld als postdoctoraal onderzoeker bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum.

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