Components of the metabolic syndrome

Clustering and genetic variance

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Abstract

Background

Abdominal obesity, hyperglycemia, hypertriglyceridemia, low HDL cholesterol levels and hypertension frequently co-occur within individuals. The cluster of these features is referred to as metabolic syndrome (MetS). The aim of this thesis was to investigate which metabolic endpoints should be studied in order to explain the clustering of MetS features best. Furthermore, genetic association studies were conducted to get more insight into the pathophysiology underlying the clustering of MetS features.

Methods

We conducted two studies to investigate which metabolic endpoints should be studied in order to best explain the clustering of MetS features. In the EPIC-NL case-cohort study we used confirmatory factor analyses to study the model fit and predictive ability for type 2 diabetes (T2D) and cardiovascular diseases (CVD) of several MetS models, including traditional and novel MetS features. Furthermore, we reviewed twin and family studies, which presented genetic correlation coefficients between different traditional and novel MetS features and between MetS and novel MetS features. We conducted four studies investigating which single nucleotide polymorphisms (SNP's) were associated with clustering of MetS features. First, we systematically reviewed published candidate gene studies on MetS. Second, we analyzed whether SNP's associated with inflammatory biomarkers, waist circumference, insulin resistance, HDL cholesterol or triglycerides in genome wide association studies (GWAS) were also associated with MetS and MetS-score in a random sample of the EPIC-NL study. Third, in the Doetinchem cohort, we determined if SNP's of genes located in transcriptional pathways of glucose and lipid metabolism were associated with multiple MetS features simultaneously. Fourth, we evaluated the interaction between these SNP's and BMI in relation to glucose levels.

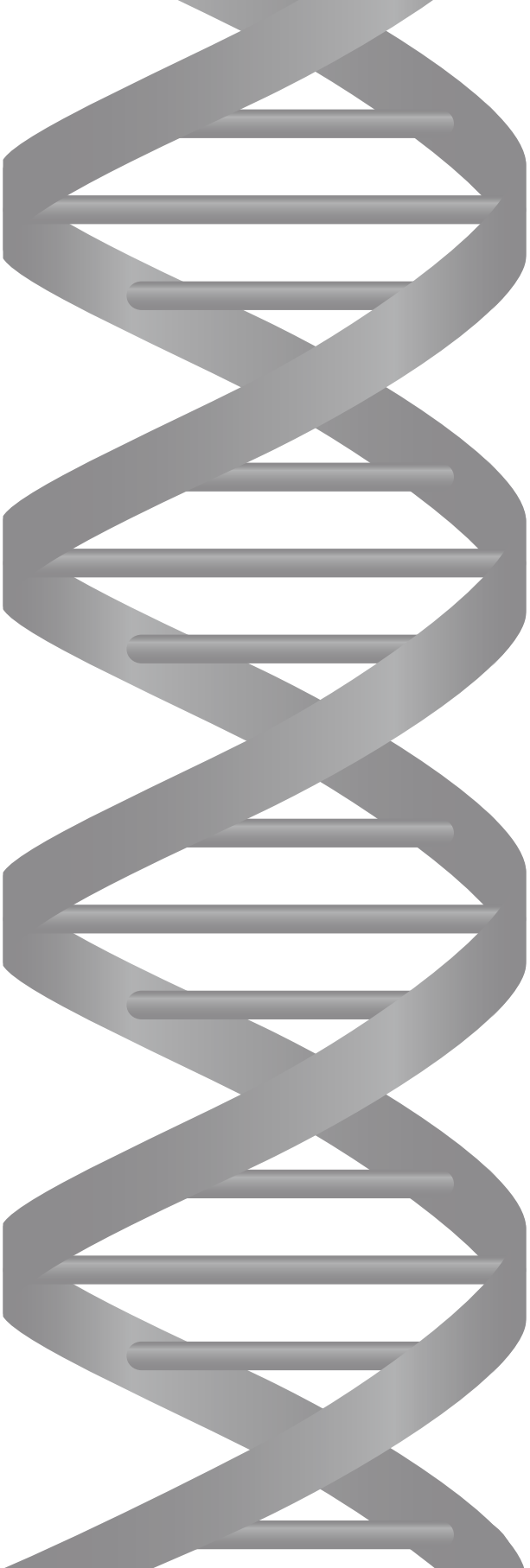
Results

A MetS model composed of the traditional MetS features and high sensitive C-reactive protein (hsCRP) optimally predicted T2D and CVD, while still representing a single entity. Our review of 9 twin and 19 family studies showed that genetic correlations were strongest, i.e. genetic pleiotropy was highest, between waist circumference and HOMA-IR, HDL cholesterol and triglycerides, and between adiponectin and MetS. After having systematically reviewed 25 genes in 88 candidate gene studies, we found evidence for an association of *FTO* rs9939609, *TCF7L2* rs7903146, *APOA5 C56G* (rs3135506), *APOA5 T1131C* (rs662799), *APOC3 C482T* (rs2854117), *Il6 174G>C* (rs1800795) and *CETP Taq-1B* (rs708272) with MetS. SNP's associated with waist circumference in GWAS were on a group level significantly associated with MetS in a random sample of EPIC-NL, whereas a group of SNP's associated with insulin resistance was significantly associated with MetS-score. On the individual level *MC4R* rs17782312 and *IRS1* rs2943634 were associated with MetS. In the Doetinchem cohort *CETP Ile405Val* (rs5882) and *APOE Cys112Arg* (rs429358) were associated with both the prevalence of low HDL cholesterol levels and with abdominal obesity. In this cohort, two highly correlated SNP's in the *PPARGC1A* gene, *Gly482Ser* (rs8192678) and *Thr528Thr* (rs3755863), showed a significant interaction with BMI on glucose levels.

Conclusion

One MetS factor with or without hsCRP, can be used to study the clustering of MetS and MetS related features, because this factor can be represented as one statistical entity. However, in order to fully explain the clustering of MetS features, specific combinations of MetS features should be studied. Our results indicate that genetic pleiotropy is highest for the combination of HOMA-IR and waist circumference and the combination of HDL cholesterol and triglycerides. Therefore these combinations are good candidate endpoints for studies on genetic variants pleiotropic to several MetS and MetS related features. SNP's associated with the clustering of MetS features are involved in mechanisms traditionally believed to underlie MetS development, i.e. glucose metabolism and weight regulation, but also in other mechanisms, i.e. lipid metabolism and inflammation. This suggests that, although the MetS features may represent a statistical entity, there are multiple, related mechanisms explaining the clustering of MetS features.

Contents



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Povel CM

General Introduction

Metabolic syndrome

Abdominal obesity, hyperglycemia, hypertriglyceridemia, low serum high density lipoprotein (HDL) cholesterol levels and hypertension frequently co-occur within individuals. The cluster of these features is generally referred to as the metabolic syndrome (MetS).

The metabolic syndrome was first recognized in 1923. At that time, Kylin [1], a Swedish physician, described a syndrome involving hypertension, hyperglycemia and hyperuricaemia. Afterwards, in 1965 Avogaro and Crepaldi [3] described a syndrome which consisted of hypertension, hyperglycemia and obesity. In 1988, the MetS was brought to the attention of a wider audience by Reaven [4]. He described "Syndrome X", which increases the risk of type 2 diabetes (T2D) and cardiovascular diseases (CVD) and was composed of insulin resistance, hyperglycemia, dyslipidemia and hypertension. According to Reaven the mechanism underlying this syndrome was insulin resistance [4].

After all these years, the precise definition of MetS is still under debate and several expert groups from e.g. WHO and NHLBI are reconsidering its definition. However, for international comparisons and to facilitate research on MetS etiology, it is important that a commonly agreed set of criteria exist which defines MetS [2]. According to the most recent consensus statement a person is defined to have MetS when he/she has three or more of the following five features (see text box): increased waist circumference, elevated serum triglycerides (≥1.7 mmol/L) or drug treatment for elevated triglycerides; reduced serum HDL cholesterol (in men: <1.0 mmol/L; in women: <1.3 mmol/L) or drug treatment for reduced HDL cholesterol; increased blood pressure (systolic ≥130 and/or diastolic ≥85 mm Hg) or antihypertensive drug treatment; increased fasting plasma glucose (>5.6 mmol/L) or glucose lowering drug treatment [2]. Regarding increased waist circumference, it is recommended to use ethnic specific cut-off points. However, recommendations on cut-off points for Caucasians differ considerably; either a waist circumference ≥94 cm for men and ≥80 cm for women [5], corresponding with the BMI cut-off point for overweight [2], or a waist circumference ≥102 cm for men and ≥88 cm for women, corresponding with the BMI cut-off point for obesity [2], is recommended [6, 7].

Criteria for diagnosis of the metabolic syndrome [2]
The presence of at least any 3 of the following 5 risk factors

Measures	Categorical Cut Points
Elevated waist circumference	Population and country specific definitions [a]
Elevated triglycerides or drug treatment [b]	≥1.7 mmol/L (150 mg/dL)
Reduced HDL cholesterol or drug treatment [b]	<1.0 mmol/L (40 mg/dL) in males <1.3 mmol/L (50 mg/dL) in females
Elevated blood pressure or drug treatment	Systolic ≥130 and/or diastolic ≥85 mmHg
Elevated fasting glucose or drug treatment	≥5.6 mmol/L (100 mg/dL)

[a] It is recommended that the IDF cut points be used for non-Europeans and either the IDF or AHA/NHLBI cut points used for people of European origin until more data are available.

[b] The most commonly used drugs for elevated serum triglycerides and reduced serum HDL cholesterol are fibrates and nicotinic acid. A patient taking 1 of these drugs can be presumed to have high triglycerides and low HDL cholesterol. High-dose ω-3 fatty acids presume high triglycerides.

The prevalence of MetS is relatively high and rising [8]. In Western societies the prevalence among adults is 25-30% [8-10]. In the Netherlands, the prevalence of MetS among people aged 30 to 40 years is approximately 20% in men and 9% in women. Among people between 50 and 60 years the prevalence is approximately 50% in men and 60% in women [11].
People with MetS are at a twofold increased risk for developing coronary heart disease (CHD) and at a fivefold increased risk for developing T2D in the next five to ten years [2, 12]. In CHD or diabetes patients, the MetS is often present, and the number of components of the MetS is associated with disease progression and risk [2]. Thus, given its high prevalence and severe consequences, MetS is a phenomenon of high public health relevance.
There are multiple risk factors for MetS including genetic factors, physical inactivity, over nutrition and obesity. Also older people and people of Asian origin are at increased risk of developing MetS [7]. The obesity epidemic and the ageing population are therefore important causes for the rising prevalence of MetS [6, 8].

Metabolic syndrome under debate

Most metabolic diseases are characterized by one unifying pathophysiological factor. For example, myocardial infarction is characterized by myocardial necrosis, osteoporosis by a low bone mass and micro-architectural deterioration of bone tissue, and lung cancer by the accumulation of abnormal cells in the lungs [13]. For MetS, the question is whether it represents a distinct clinical entity characterized by a unifying pathophysiological factor, like these other diseases, or whether it is loosely associated cluster of risk factors for T2D and CVD [14, 15]. According to Reaven the unifying pathophysiological factor for MetS is insulin resistance [4]. However, more recently it has been proposed that not one, but multiple interrelated causal mechanisms, underlie MetS development [7].
If a metabolic disease it a clinical entity, characterized by a unifying pathophysiological factor, the symptoms characterizing the disease are most likely highly correlated and represent a statistical entity. Thus, when a unifying pathophysiological factor for MetS exists, the MetS features are likely to represent a statistical entity [15]. This can be tested with confirmatory factor analysis, a hypothesis driven data reduction technique, which can be used to combine data. Studies using confirmatory factor analysis on MetS features so far suggest that the MetS features included in the current definition indeed represent one statistical entity [16-22]. This makes it more likely that one unifying pathophysiological factor is responsible for MetS development, and explains the clustering of its features, but by no means proves this. It could for example also be that two distinct pathophysiological factors both cause a similar pattern of disease symptoms.
Not only the concept of MetS is under debate, but also its definition . Several changes to the current MetS definition have been suggested in scientific literature. First, the features currently included in the MetS definition are under debate. In order to increase the predictive ability of MetS for T2D and CVD, it has been proposed to add features to the definition of MetS. These features include amongst others circulating adiponectin, C-reactive protein (CRP), albumin, APOB and free fatty acid levels (FFA) or a fatty liver [5]. However, it is unclear if MetS represents one statistical entity after addition of one or more of these features. Second, some favor a continuous MetS definition, as in the current bivariate MetS definition information is lost. For example, a minor change in

triglyceride levels from 1.70 mmol/L to 1.64 mmol/L, could result in an individual no longer being classified as having MetS [15, 23]. However, this change in triglyceride levels has only a minor effect on the metabolic profile and the risk for T2D and CVD of this individual. Furthermore, when plotted against the number of positive features the risk for CVD raises continuously, with no suggestion of a threshold effect [24]. A continuous definition of MetS does not exist yet. Such a definition could for example be based on a MetS factor derived from confirmatory factor analysis.

Clustering of metabolic syndrome features – pathophysiology –

A good understanding of the mechanism behind the clustering of MetS features is important, because such understanding may eventually facilitate the development of preventive or treatment strategies which can target multiple MetS features simultaneously. Currently it is not yet fully understood why the features of MetS cluster [14]. Most likely the clustering of these features is caused by multiple underlying, interrelated causal mechanisms [7]. These mechanisms are described below and depicted in figure 1.

Adipose tissue dysfunction may be important in MetS development. The efflux of FFA from the adipose tissue is determined by the balance between lipolysis and lipogenesis [25]. In obese or insulin resistant individuals this balance is often disturbed. Plasma FFA levels are increased, due to increased endogenous lipolysis and a reduced FFA uptake by adipose tissue. Endogenous lipolysis is increased due to defects in adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). FFA uptake is decreased due

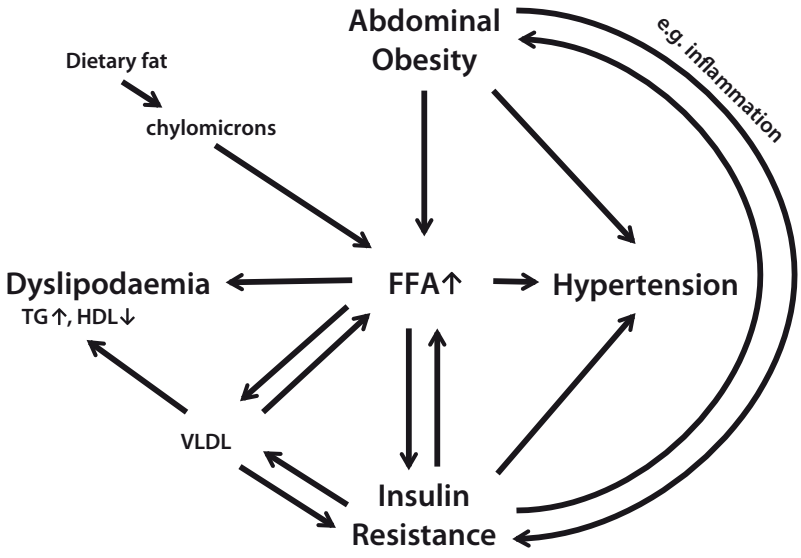

Figure 1 | Possible interrelationships between the features of metabolic syndrome
TG, triglycerides; HDL, HDL cholesterol; VLDL, very low density lipoprotein; FFA, free fatty acids

to insufficient lipogenesis and a defective regulation of adipose tissue lipoprotein lipase (LPL) in response to insulin. The defective regulation of LPL may result in a decreased uptake of chylomicrons in the adipose tissue and an increased spillover of fatty acids in the plasma FFA pool [25, 26].
The inability of adipose tissue to trap FFA leads to increased lipid accumulation in non-adipose tissue, i.e. muscle, liver and β-cells, resulting in many metabolic abnormalities. Elevated FFA uptake into muscle increases intramyocyte triglyceride content with concurrent insulin resistance [25]. The FFA flux to the liver is mainly influenced by visceral and only to a lesser extent by subcutaneous fat. This is related to the fact that the visceral fat depot drains directly in the portal vein, which represents 80% of the total hepatic blood flow. An increased FFA flux to the liver may increase the production of very low density lipoprotein (VLDL) and stimulate insulin resistance of the liver. Hepatic insulin resistance may aggravate overproduction of VLDL even further. Increased VLDL levels may, through hydrolysis of VLDL, lead to increased FFA concentrations in the blood stream. Furthermore, increased VLDL levels may, through an increased transfer of triglycerides in VLDL to LDL or HDL by cholesteryl ester transfer protein (CETP), lead to increased small dense LDL cholesterol and increased triglyceride-enriched HDL cholesterol levels. As triglyceride-enriched HDL cholesterol is a better substrate for hepatic lipase, it is cleared rapidly from the circulation, resulting in decreased HDL cholesterol levels. Furthermore, as in the liver FFA serve as a substrate for synthesis of triglyceride, an increased FFA flux to the liver increases triglyceride levels [25-30]. In the β-cells, a chronic increased FFA flux from adipose tissue (>48 h) decreases glucose stimulated insulin secretion, resulting in insulin resistance [25]. Finally, increased FFA levels in the blood stream can mediate vasoconstriction, therewith causing hypertension [28].

Insulin resistance may also explain part of the clustering of the MetS features, independent of plasma FFA levels. Due to the lower response to insulin, some metabolic effects of insulin may be diminished. First, glucose uptake by tissues is diminished, and glucose levels are increased. Second, hyperinsulinemia may result in enhanced sodium reabsorption and increased sympathic nervous system activity, which both contribute to the development of hypertension [4, 28]. Third, insulin resistance leads to a reduction in endothelial nitric oxide (NO) production, leading to endothelial dysfunction and atherosclerosis [27]. Fourth, early literature suggested that insulin resistance may cause weight gain due to the inhibition of lipolysis, reduced thermic effect of food, increased appetite or increased efficiency of fat storage. However, more recent literature also suggests that insulin resistance serves as a homeostatic mechanism to protect against further weight gain, especially in those with diabetes [31].

In obese people insulin resistance, dyslipidemia and hypertension may develop through an increased efflux of FFA [28]. Furthermore, obesity may lead to the activation of the renin angiotensin system, resulting in the development of hypertension and possibly insulin resistance [27]. Finally, due to the increase in adipose tissue size the production of adipokines, such as TNF-α, leptin or IL-6 is increased in obese people. [27]. Elevated levels of these adipokines may have several metabolic effects [28, 32]. For example, there are indications that IL-6 increases insulin resistance, FFA levels, triglyceride levels and blood pressure [25, 32, 33]. Furthermore, IL-6 induces the production of CRP by the liver [25, 32]. In contrast to other adipokines for which production increases, production of adiponectin decreases with obesity. The suppressed adiponectin production may increase plasma FFA, glucose and hsCRP levels [25].

Clustering of metabolic syndrome features – genetic variance –

The pathophysiology behind the clustering of MetS features is currently not fully understood. Genetic association studies on the clustering of MetS features may contribute to a better understanding. As genetic variants affects gene functioning, the association between genetic variants and a disease may identify genes relevant for the development of this disease. Identification of disease genes may eventually increase our understanding of disease mechanisms. The advantage of genetic association studies is, that according to the principles of Mendelian randomisation, they are not affected by reverse causality and are largely unaffected by socioeconomic and behavioural confounders [34], resulting in the potential for clear study interpretation [35]. Compared to mechanistic animal experiments advantages of genetic association studies are the cost-effectiveness of genotyping and the relevance to humans [35].
In order to unravel the pathophysiology of clustering of MetS features, the main interest is in genetic variants associated with multiple MetS features. However, up till now most genetic association studies focussed on a single MetS feature, whereas fewer studies focussed on genetic variants associated with combinations of MetS features, of with the prevalence of MetS itself. Probably more genetic variants associated with a combination of MetS features of with MetS remain to be discovered.
Below we first describe studies on genetic variance and MetS. Second, we describe studies on the genetic variance and specific combinations of MetS features.

A heritability estimate represents the amount of variation in a phenotype that is influenced by genetic variation. Heritability estimates of MetS range from 10% to 30% [36-38]. These estimates are generally lower than the heritability estimates for most individual MetS features, which range from 8%-37% for blood pressure [39] to 28%-81% for lipid levels [40].
Actual genetic variants explaining the heritable component of MetS can either be detected in family studies, or in populations of unrelated individuals. In family studies, linkage analyses aim to find a chromosomal locus or genetic variant which is associated with the segregation of the disease in a pedigree. Initially, linkage analyses were based on a large number of short tandem repeats (STRs) or micro-satellites. In two genome-wide linkage studies based on these markers, an association between the 3q27 locus and MetS was shown [41, 42]. On this locus, one of the candidate genes for MetS, the *ADIPOQ* gene, which encodes for adiponectin, is located.
Most studies on genetic determinants of MetS were, however, not conducted in family studies, but in populations of unrelated individuals. These genetic association studies mainly focused on single nucleotide polymorphisms (SNP's). Many candidate gene studies on MetS have been conducted. However, at the start of the research described in this thesis no clear overview of these studies was available [43, 44]. SNP-MetS associations were also studied in two genome wide association studies (GWAS). In these studies six different SNP's, all involved in lipid metabolism, were associated with MetS. In the first GWAS among 4 Finish population-based cohorts (N~8000) rs964184 in the *ZNF259* gene was associated with MetS [43]. The other GWAS was conducted among 7 independent studies, comprising 22 161 participants from European ancestry [44]. In this study, 5 SNP's (*BUD13* rs10790162, *ZNF259* rs2075290, *APOA5* rs2266788, *LPL* rs295 and *CETP* rs173539) were associated with MetS.

Studying specific combinations of MetS features is another way to get insight into the genetic variance responsible for the clustering of MetS features. The amount of genetic variance which is shared between two MetS features, i.e. the genetic pleiotropy between two MetS features, can be estimated by means of a genetic correlation coefficient. Together with the environmental correlation coefficient, a genetic correlation coefficient determines the phenotypic correlation coefficient between two MetS features (formula 1) [45].

Formula 1 | Genetic pleiotropy

$$\rho(phenotype) = (heritability\_x)(heritabilty\_y) * \rho(genotype) + (1\text{-}heritability\_x)(1\text{-}heritabilty\_y) * \rho(environment)$$

In several studies, genetic correlation coefficients between MetS features have been calculated [46]. The effect estimates of the genetic correlation coefficients varied widely across studies and at the start of the research described in this thesis a good overview was lacking.
Up until now, a few SNP's associated with multiple MetS or MetS related features have been discovered. For example, *FADS1* rs174550 is associated both with plasma glucose and serum HDL cholesterol levels [47]. In most studies, either candidate gene [48-54] or GWAS [47, 55, 56], the association with individual MetS features was analysed first; afterwards it was determined which SNP's were associated with multiple MetS features. In only a few genetic association studies a specific combination of two MetS features has been studied directly [44, 57-59]. In a GWAS among 7 independent studies, including 22 161 participants from European ancestry, genetic associations with any combination of two MetS features were studied [44]. In this study 27 SNP's in 16 genes were associated with a combination of two MetS features [44]. Most of these SNP's were involved in lipid metabolism and except for two, all combinations of MetS features contained either serum triglycerides or HDL cholesterol, whereas seven contained both triglycerides and HDL cholesterol. Of the 27 SNP's associated with a combination of two MetS features, only four were also associated with the prevalence of MetS. This exemplifies that not all genetic variants which explain part of the clustering of MetS features are also associated with MetS itself.

The SNP's discovered in GWAS on MetS and on specific combinations of MetS features could only explain a small fraction of the correlation among the MetS features [44]. Many more genetic variants explaining the clustering of MetS features remain to be discovered. These genetic variants may be discovered by studying genetic associations with MetS as endpoint. Alternatively, they may be discovered by studying genetic associations with specific combinations of MetS features.

There are several ways in which a genetic variant can contribute to the co-occurrence of two MetS features. The three models presented below illustrate this.
According to the first model, the effect of a genetic variant on MetS feature 1 is completely mediated by the effect of a genetic variant on MetS feature 2 (figure 2a). In this case, the association between the genetic variant and MetS feature 2 would completely disappear after adjustment for MetS feature 1. A good example of this model is the association of the *FTO* rs9939609 SNP with glucose, insulin and lipid levels. The associations with these endpoints disappeared after adjustment for BMI and were consistent

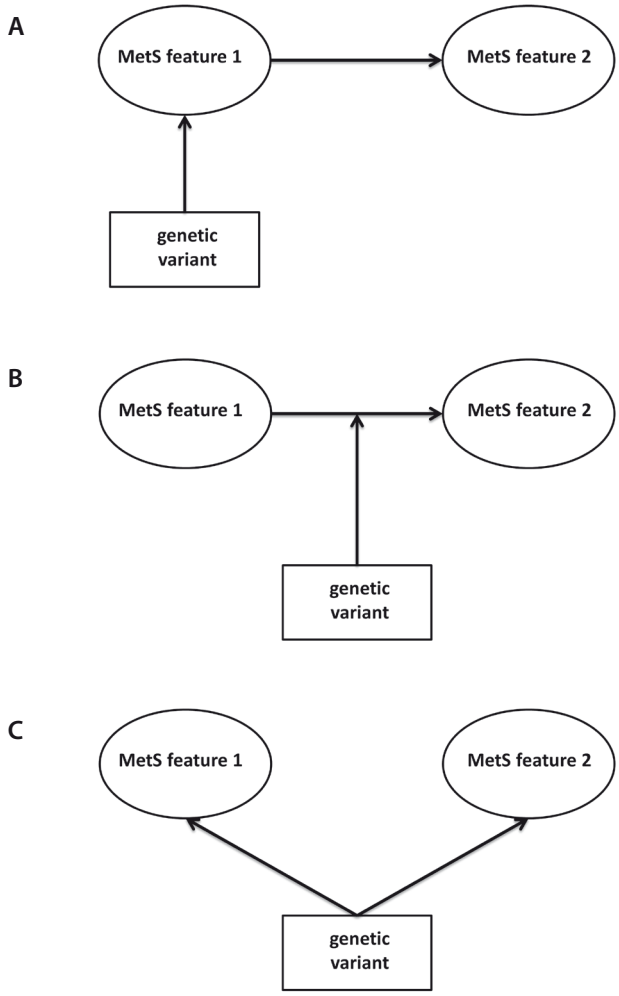

Figure 2 A The effect between the genetic variant and MetS feature 2 is mediated by the effect between the genetic variant and MetS feature 1 (model 1)
B The genetic variants affects MetS feature 1 and MetS feature 2 via independent pathways (model 2)
C The genetic variant modifies the association between MetS feature 1 and MetS feature 2 (model 3)

with those predicted given the *FTO*-BMI and BMI-endpoint associations [54]. According to the second model, a genetic variant is associated with two MetS features through two independent pathways (figure 2b). For example, the *GCKR* rs780094 SNP increases triglyceride levels via increased VLDL-triglyceride synthesis, but decreases glucose levels via decreased hepatic glucose production and increased glucose utilization [60]. It may be the case, as for *GCKR* rs780094, that the pathways leading to distinct phenotypes initially overlap, e.g. *GCKR* rs780094 induces an overexpression of GCK but separates afterwards, e.g. GCK overexpression affects lipid and glucose levels via distinct mechanisms. According to the third model, a genetic variant affects the clustering of MetS features by changing the strength of an association existing between two MetS features

(figure 2c). For example, the association between adiposity and blood pressure is stronger in carriers of the 350G, 825T OR 1429T allele in the *GNB3* gene [61].
Application of the three models described above in research on genetic variants and several combinations of MetS features may give a deeper insight in how genetic variants may affect the clustering of MetS features. To the best of our knowledge, these models have not been used previously with the underlying aim to explain the clustering of MetS features.

Research question and outline of the thesis

Currently, it is unclear how the clustering of MetS features can be best studied, i.e. which endpoints, features or combinations of features should be investigated. Furthermore, the pathophysiology behind the clustering of MetS features is not fully understood. Therefore, the research questions of this thesis are:

1 *Which metabolic endpoints should be studied in order to explain the clustering of MetS features best?*

2 *Which pathophysiology is underlying the clustering of MetS features?*

To investigate the first question we have posed two sub-questions:

1^a *Which MetS model predicts T2D and CVD best, while still representing one statistical entity?*

1^b *How much genetic variance is shared between the different MetS and MetS related features?*

To investigate the second question we have defined two more sub-questions:

2^a *Which genetic variants are associated with MetS?*

2^b *Are genetic variants located in transcriptional pathways of glucose and lipid metabolism associated with the clustering of MetS features through model 1, 2 or 3?*

In the study described in chapter 2 (question 1a) we have designed several one-factor MetS models, consisting of traditional and non-traditional MetS features, with confirmatory factor analysis in the EPIC-NL case-cohort study [62]. For those MetS models with a good model fit, we have determined which predicted incident T2D and CVD best.
In chapter 3 (question 1b), we have reviewed studies describing genetic correlation coefficients between MetS and MetS related features, therewith aiming to identify MetS features which have much, and MetS features which have little, genetic variation in common.In chapter 4 and 5, we have focused on genetic variants associated with MetS (question 2a). In chapter 4, we have systematically reviewed available candidate gene studies on MetS. In chapter 5, we have studied whether SNP's associated with waist circumference, insulin resistance, HDL cholesterol, triglycerides or inflammatory

biomarkers in GWAS, are also associated with MetS in a random sample of the EPIC-NL study [62].
In chapter 6 and 7 we have described the association between specific combinations of MetS features and SNP's in genes, located in transcriptional pathways of glucose and lipid metabolism in the Doetinchem cohort [63] (question 2b). In chapter 6 we have described if some of these SNP's were associated with multiple MetS features, either independently (model 2) or through a mediating effect (model 1). In chapter 7 we have studied whether interaction effects existed between these SNP's and BMI on glucose levels (model 3).
In chapter 8 we have discussed the findings of this thesis, their public health relevance and methodological considerations. Furthermore, recommendations for further research have been given.

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Metabolic syndrome model definitions predicting type 2 diabetes and cardiovascular diseasen

Submitted

Abstract

Objective

Metabolic syndrome (MetS) is a cluster of abdominal obesity, hyperglycemia, hypertension and dyslipidemia, which increases the risk for type 2 diabetes and cardiovascular diseases (CVD). Some argue that MetS is not a single disorder, because the traditional MetS features do not represent one entity. They would like to exclude features from MetS. Others would like to add additional features in order to increase predictive ability of MetS. The aim is to identify a MetS model which optimally predicts type 2 diabetes and CVD, while still representing a single entity

Research, Design and Methods

In a random sample (n=1928) of the EPIC-NL cohort and a subset of the EPIC-NL MORGEN study (n=1333), we tested the model fit of several one-factor MetS models using confirmatory factor analysis. We compared predictive ability for type 2 diabetes and CVD of these models within the EPIC-NL case-cohort study of 545 incident type 2 diabetes, 1312 incident CVD cases, and a random sample, using survival analyses and reclassification.

Results

The standard model, representing the current MetS definition (EPIC-NL CFI=0.95; MORGEN CFI=0.98), the standard model excluding blood pressure (EPIC-NL CFI=0.95; MORGEN CFI=1.00), and the standard model extended with hsCRP (EPIC-NL CFI=0.95) had an acceptable model fit. Compared to the standard model, the model extended with hsCRP predicted type 2 diabetes (IDI: 0.34) and CVD (IDI: 0.07) slightly better.

Conclusions

It seems valid to represent the traditional MetS features by a single entity. Extension of this entity with hsCRP slightly improves predictive ability for type 2 diabetes and CVD.

Introduction

Metabolic syndrome (MetS) is a cluster of multiple correlated metabolic features, that is associated with a fivefold increased risk of type 2 diabetes and a twofold increased risk of cardiovascular disease (CVD) [1]. According to the joint interim statement of IDF and AHA/NHLBI, MetS is defined as the presence of three or more of the following five features: abdominal obesity, hyperglycaemia, hypertension, hypertriglyceridemia and low HDL cholesterol levels [1].

Nevertheless, the debate around the definition of MetS is still ongoing. Firstly, several expert groups including WHO, NHLBI and IDF are considering the inclusion of additional features in the definition of MetS [1, 2] such as markers of sub-clinical inflammation [2], markers of liver function [3], uric acid [4] and albumin [2]. This could increase the predictive ability of MetS for type 2 diabetes and CVD. Secondly, as MetS could be regarded as a single disorder, all features included in the definition of MetS, should represent a single entity, i.e. should be captured in a single factor. Currently, it is unclear whether MetS can still be considered a single entity after inclusion of additional features. Some even argue that under the current definition MetS does not represent a single disorder, and favour exclusion of blood pressure from MetS [5].

Whether the current MetS definition or MetS definitions extended with additional features represents a single entity, can be tested with factor analysis. Factor analysis is a data reduction technique, which can be used to amalgamate data. Two factor analyses techniques exist: explanatory factor analysis (EFA), which is a data driven technique and confirmatory factor analysis (CFA), which is hypothesis driven. In most studies EFA has been used [6]. However, due to the explorative and subjective nature of EFA, results of EFA studies on MetS are inconsistent [7]. In contrast, conclusion of CFA studies were very so far quite consistent, suggesting that the MetS features included in the current definition represent one entity [4, 7-12]. However, as CFA MetS models including additional features, such as hsCRP [8], uric acid [4], albumin and liver enzymes, have rarely been studied, it is unknown whether they represents one entity. Furthermore, to the best of our knowledge, the different one-factor CFA MetS models, have never been compared with respect to their predictive ability for the development of type 2 diabetes and CVD. Once a MetS model, that optimally predicts type 2 diabetes and CVD, while still representing one disorder, has been identified, future research should focus on the pathophysiology behind this MetS model. A deeper understanding of this pathophysiology may eventually lead to development of treatment strategies targeting the mechanism responsible for the co-occurrence of MetS features.

The first aim of this paper was to test if the traditional MetS features represent a single factor, and if so, if this was still the case after inclusion of novel MetS features. The second aim was to identify a MetS model that best predicts type 2 diabetes and CVD, while still representing a single factor.

Research, Design and Methods

EPIC-NL: Study design

The EPIC-NL cohort consists of the two Dutch contributions to the European Investigation into Cancer and Nutrition (EPIC) project: the Prospect and the Monitoring Project on Risk Factors for Chronic Diseases (MORGEN) cohorts. Both cohorts were initiated in 1993 and the study design of this combined cohort is described in detail elsewhere [13]. In brief, Prospect is a prospective cohort study among 17 357 women aged 49-70 who participated in the breast cancer screening between 1993 and 1997. The MORGEN-project consists of 22 654 men (n = 10 260) and women (n = 12 394) aged 20-59 years recruited from three Dutch towns (Amsterdam, Doetinchem, and Maastricht). From 1993 to 1997, each year a new random sample of approximately 5000 individuals were examined for the MORGEN-project. Both studies complied with the Declaration of Helsinki. The Prospect-EPIC study was approved by the Institutional Review Board of the University Medical Center Utrecht and the MORGEN project was approved by the Medical Ethical Committee of TNO, The Netherlands [13].

Study Population

Analyses were performed in two subsets composed of EPIC-NL participants, in whom all MetS features were measured: the EPIC-NL case-cohort study [13] and a subset of EPIC-NL MORGEN participants [14]. The EPIC-NL case-cohort study consist of a 6.5% baseline random sample of the total EPIC-NL study (n=2604), all incident diabetes cases (n=924) and all incident CVD cases (n=2030). In the EPIC-NL case-cohort study, we compared the model fit and predictive ability for type 2 diabetes, CHD and stroke of different MetS models. Blood status was non-fasting and glucose status was assessed with haemoglobin A1C (HbA1C). In addition to information on traditional MetS features, also information on non-traditional MetS features, such as high sensitive C-reactive protein (hsCRP), was available.

The EPIC-NL MORGEN subset was used as a replication sample for the analysis on model fit. This subset consists of 1379 non-diabetic participants, who indicated that their last meal was on the day before blood sampling. In contrast to the EPIC-NL case-cohort study, plasma glucose was measured instead of HbA1C, while information on non-traditional MetS features was not available.

Participants with missing blood samples (157 participants in the random sample; 174 incident CVD cases; and 66 incident type 2 diabetes), participants who were taking glucose lowering or blood pressure lowering medication (282 participants in the random sample; 709 incident CVD cases; and 279 incident type 2 diabetes; and 45 participants of the MORGEN subset) or participants with missing values for one of the MetS or MetS related features (237 participants in the random sample; 34 incident diabetes cases; and 165 incident CVD cases) were excluded. Subjects with missing blood samples were on average 2.4 years older and had a 1.1 kg/m^2 higher BMI, than those without missing blood samples. Age and BMI were similar between subjects with and without missing values for one of the MetS or MetS related feature. Finally, the EPIC-NL case-cohort study consisted of 1928 participants in the random sample, 545 incident diabetes cases and 1312 incident CVD cases. For the risk prediction analyses prevalent type 2 diabetes and CVD cases were excluded from the random sample. Of the random sample participants, 53 were incident type 2 diabetes cases and 88 were incident CVD cases. The EPIC-NL MORGEN subset consisted of 1333 participants. Of the EPIC-NL participants 133 were included both in the EPIC-NL case-cohort study and in the EPIC-NL MORGEN subset.

Ascertainment of diabetes in EPIC-NL

The ascertainment and verification of diabetes cases has been described in detail elsewhere [15]. In short, diabetes was ascertained via self-report, a urinary glucose strip test and linkage to registers of hospital discharge diagnoses. Self-reported diabetes status was assessed in the baseline questionnaire and in 2 follow-up questionnaires, which were sent out within regular intervals of three to five years. For Prospect participants only, a urinary glucose strip was sent out with the first follow-up questionnaire. Follow up by linkage to registers of hospital discharge diagnoses was completed on the 1st of January 2006. Potential cases were verified against participants' general practitioner or pharmacist information. Only verified type 2 diabetes cases were included.

Ascertainment of cardiovascular disease in EPIC-NL

Data on CVD morbidity were obtained through linkage with the national medical registry. For CVD mortality, vital status was obtained through linkage with the municipal population registries. Subsequently, primary and secondary causes of death were obtained through linkage with Statistics Netherlands. Follow up was completed on 1st of January 2006. Coronary heart disease (CHD) was coded with ICD-9 codes 410-414 and Cerebro Vascular Accident (CVA) with ICD-9 codes 430-434, 436 [16]. CVD was defined as the presence of CHD, CVA or both.

Baseline measurements in EPIC-NL

At baseline, a physical examination was performed and non-fasting blood samples were drawn. Furthermore, a general questionnaire and an food frequency questionnaire (FFQ) were filled out by each participant [13].

During the physical examination, systolic and diastolic blood pressure measurements were performed twice in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany) (Prospect) or twice on the left arm using a random zero sphygmomanometer (MORGEN). The mean of the two measurements was taken. Waist circumference and height were measured. Body weight was measured with light indoor clothing without shoes. Blood levels of established biochemical parameters were measured in EDTA or citrate plasma. HbA1c was measured in erythrocytes using an immunoturbidimetric latex test. High-density lipoprotein (HDL) was measured with a homogeneous assay with enzymatic endpoint. Trigycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), uric acid and glucose were measured using enzymatic methods, whereas hsCRP was measured with a turbidimetric method. Albumin was measured using a colorimetric method [13, 14].

Data on smoking habits, educational level, self-reported medication use, physical activity, and alcohol intake were obtained by general questionnaires and a FFQ. Physical activity was categorized by calculating the Cambridge Physical Activity Score [17].

Statistics

Triglycerides, hsCRP, ALT, AST and GGT were log transformed to improve normality. Using confirmatory factor analysis (CFA), we designed several second-order one factor MetS models based on the MetS model of Shen *et al.* [9]. These models consisted of three levels: a single MetS factor; several first-order factors (e.g. lipids), which defined the single MetS factor; and some second-order factors (e.g. triglycerides and HDL cholesterol), which defined the first-order factors. We designed the following one-factor MetS models: model 1, a standard MetS model, based on the current defini-

tion of MetS [1], including the traditional MetS features, i.e. waist circumference, triglycerides, HDL cholesterol, systolic blood pressure, diastolic blood pressure and as marker of glucose status, either HbA1C or glucose (figure 1a, 1b); model 2, a standard MetS model excluding the blood pressure factor; and model 3, a standard MetS model extended with either a hsCRP factor (figure 1c), a albumin factor, a uric acid factor or a liver enzymes factor. The liver enzymes factor was a first-order factor defined by the second-order factors ALT, AST en GGT. In all models, the factor variance of the

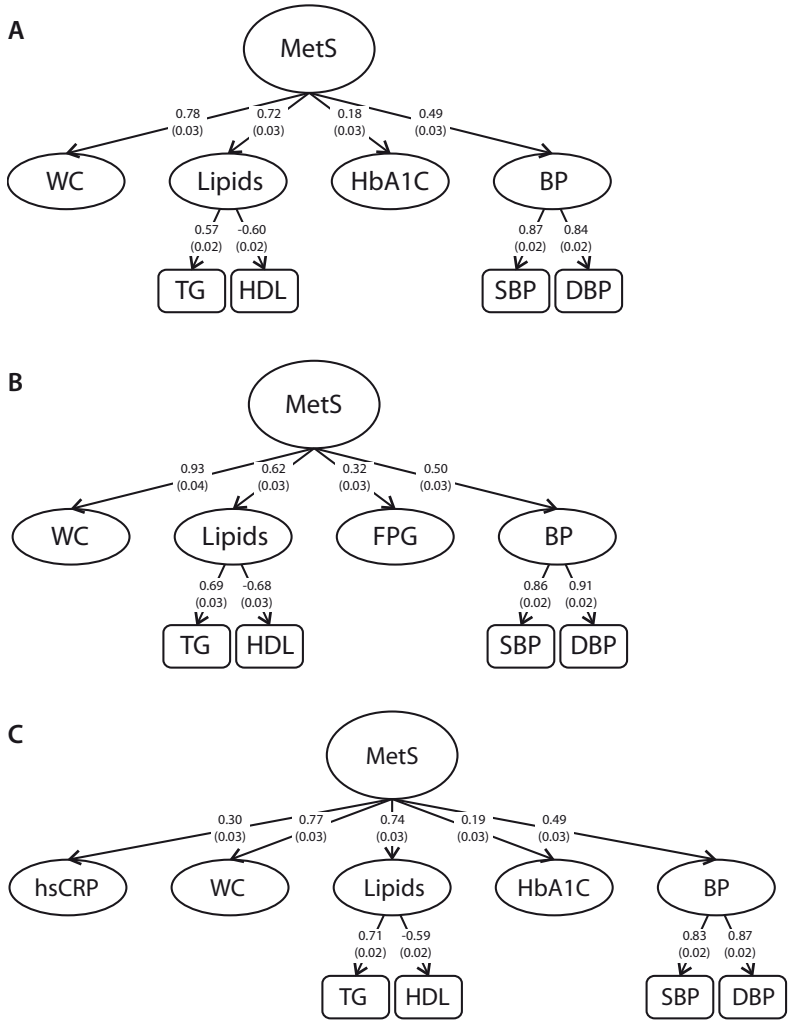

Figure 1 | A The standard second-order one-factor MetS model in the random sample of EPIC-NL
B The standard second-order one-factor MetS model in the subset of the MORGEN study
C The standard second-order one-factor MetS model extended with hsCRP in the random sample of EPIC-NL

Data are presented as factor loading (standard error); All factor loadings are significant (P<0.05); The first-order factors are: WC, lipids, HbA1C, FPG and BP; The second-order factors are: TG, HDL, SBP and DBP; Mets, Metabolic syndrome; WC, waist circumference; TG, triglycerides; HDL, HDL-cholesterol; HbA1C, haemoglobin A1c; FPG, fasting plasma glucose; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure

MetS factor, the factor loading of triglycerides, and the factor loading of systolic blood pressure were fixed to 1. For model 3, the MetS model excluding blood pressure, not enough degrees of freedom (df) were available to calculate model fit. Therefore, the error variance of the factor with the highest factor loading (waist circumference) was fixed to 1 for the model fit calculations of this model.

Model fit of MetS models composed of traditional MetS features was calculated both in the random sample of EPIC-NL and in the EPIC-NL MORGEN subset. Model fit of MetS models including non-traditional features was calculated only in the random sample of EPIC-NL. We compared the model fit of all alternative MetS models with the model fit of the standard one-factor MetS model (model 1). Factor loadings and standard errors were obtained using the maximum likelihood method. The χ^2 test, the comparative fit index (CFI), the standardized root means square residual (SRMR) and the root mean square error of approximation (RMSEA) were used to assess model fit. Models with RMSEA >0.10, CFI <0.95 or SRMR >0.08 were rejected [18]. The χ^2 difference test was used to compare model fit across different models.

For MetS models with an acceptable model fit, we compared the predictive ability of the factor scores for incidence of type 2 diabetes, CVD, CHD and CVA in the EPIC-NL case-cohort study. We calculated the factor scores using the factor score coefficients of the different MetS features extracted by the regression method from the random sample. All factor scores coefficients were standardized to the means and standard errors of the MetS features in the random sample. For all factor scores, we calculated cox proportional hazard ratio's (HR's), integral discrimination indices (IDI's) and C-statistics for incidence of type 2 diabetes, CVD, CHD and CVA. For the one factor MetS models, HR were calculated per SD of factor score. The change in C-statistics and the IDI's were used to compare predictive ability of the standard MetS model, model 1, with the alternative models. The C-statistic is equivalent to the probability that the predicted risk is higher for a case than for a non-case [19].

The IDI can be viewed as the difference in the proportion of variance explained by two models [20]. Model calibration was tested by the Hosmer-Lemeshow chi-square test. IDI's and C-statistics were adjusted for the overrepresentation of cases in the case-cohort study by inverse probability weighing. HR's were adjusted with a pseudo likelihood method [21].

CFA analyses were performed in MPLUS sixth edition (Los Angeles, CA: Muthén & Muthén). HR's, C-statistics and IDI's were calculated in SAS version 9.2 (SAS Institute, INC., Cary, North Carolina).

Results

Baseline characteristics of the study population are provided in table 1. Participants in the EPIC-NL case-cohort study were on average 51.8 years and 28.5% was male. MetS prevalence was higher in incident diabetes cases (77.4%) and incident CVD cases (46.0%) than in the random sample (26.7%). In the MORGEN subset, participants were on average 39.1 years and 50.5% was male. MetS prevalence was somewhat lower (14.9%) than in the random sample of EPIC-NL (26.7%).

The standard one-factor MetS model (model 1; figure 1a, b), which is based on the current definition of MetS, had an acceptable model fit with a CFI of 0.95 (table 2). Other one-factor MetS models with a good model fit were the MetS model excluding blood pressure (model 2) and the MetS model extended with hsCRP (model 3; figure 1c). The

Table 1 | Baseline characteristics of the EPIC-NL study

	EPIC-NL case-cohort			Subset of EPIC-MORGEN
	Random sample (*n*=1928)	Type 2 diabetes (*n*=545)	CVD (*n*=1312)	(*n*=1333)
Sex (% men)	25.9 (500)	27.2 (148)	33.5 (440)	50.5 (673)
Age (yr)	48.9 (11.7)	55.7 (7.3)	55.0 (8.9)	39.1 (10.6)
Waist circumference (cm)	85.5 (11.4)	96.7 (11.5)	89.6 (12.0)	86.5 (12.6)
BMI (kg/m^2)	25.8 (3.9)	29.5 (4.5)	26.6 (4.0)	25.2 (4.0)
HbA1C (%)	5.40 (0.61)	6.33 (1.30)	5.64 (0.83)	-
Plasma glucose (mmol/L)	-	-	-	5.31 (0.97)
HDL-cholesterol (mmol/L)	1.28 (0.34)	1.05 (0.26)	1.18 (0.32)	1.32 (0.36)
Triglyceride (mmol/L)	1.31 (0.92-1.94) [a]	2.01 (1.40-2.74) [a]	1.54 (1.12-2.27) [a]	0.94 (0.69-1.40)
Systolic Blood Pressure (mm Hg)	124.8 (17.7)	137.3 (22.0)	134.8 (21.1)	118.7 (15.6)
Diastolic Blood Pressure (mm Hg)	77.3 (10.2)	82.9 (10.9)	81.4 (11.3)	77.5 (10.3)
MetS prevalence(%) [a,b]	26.7 (515)	77.4 (422)	46.0 (603)	14.9 (199)
hsCRP (mmol/L)	1.22 (0.57-2.67)	2.50 (1.15-4.75)	1.74 (0.81-3.53)	-
Alanine Aminotransferase (IU/L)	14.5 (11.9-18.4)	16.8 (13.2-22.7)	14.6 (11.9-18.8)	-
Aspartate Aminotransferase (IU/L)	20.0 (17.4-23.5)	20.9 (17.5-25.5)	20.1 (17.5-24.1)	-
Gamma Glutamyltransferase (IU/L)	20.7 (16.5-28.1)	28.5 (22.6-40.2)	24.4 (19.2-33.1)	-
albumin (g/L)	38.9 (4.9)	37.7 (4.8)	38.2 (4.9)	-
uric acid (mmol/L)	258.5 (67.7)	284.9 (70.4)	269.0 (70.9)	-
Cambridge Physical Activity Index	2.8 (1.0)	2.7 (1.1)	2.7 (1.1)	2.6 (1.1)
Current smokers (%)	32.2 (620)	29.3 (158)	42.7 (556)	51.9 (686)
Alcohol abstainers (%)	9.5 (176)	11.2 (59)	6.5 (83)	13.9 (184)
Alcohol (g/d) [c]	12.3 (15.5)	9.5 (13.8)	12.6 (16.5)	17.9 (23.0)
Highly educated (%) [d]	21.6 (412)	9.5 (51)	14.5 (189)	18.6 (248)

Data are presented as means (standard deviation), median (p25-p75) or %(n); Subjects with blood pressure lowering or glucose lowering medication are excluded; CVD, cardiovascular disease; BMI, body mass index; HbA1C, haemoglobin A1c; MetS, metabolic syndrome; hsCRP, high sensitive C-reactive protein

[a] Non-fasting values

[b] MetS is defined as having 3 or more of the following features: Hyperglycemia: HbA1C ≥5.7% or fasting plasma glucose ≥5.6 mmol/L; Abdominal obesity: ♂ ≥102 cm; ♀≥ 88 cm; Low HDL: ♂ <1.0; ♀<1.3 mmol/L; Hypertriglyceridemia: ≥1.7 mmol/L ;Hypertension: ≥130/85 mm Hg;

[c] Among alcohol users

[d] People who completed higher vocational education or university.

Table 2 | Model fit indices of several MetS models in the random sample of the EPIC-NL study and in a subset with participants of the MORGEN study

	random sample EPIC-NL (*n*=1928)					
Model	χ^2	df	P	RMSEA	SRMR	CFI
Standard one factor model (1)	150.2	7	<0.001	0.10	0.045	0.95
Model 1 – blood pressure (2)	43.1	2	<0.001	0.10	0.040	0.95
Model 1 + hsCRP (3)	163.3	12	<0.001	0.08	0.040	0.95
Model 1 + uric acid (4)	289.4	12	<0.001	0.11	0.059	0.92
Model 1 + liver enzymes (5)	440.5	24	<0.001	0.10	0.056	0.92
Model 1 + albumin (6)	665.2	12	<0.001	0.09	0.168	0.80
Difference (model 2 – model 1)	-107.1 [a]	5	<0.001			
Difference (model 3 – model 1)	13.1	5	0.03			
Difference (model 4 – model 1)	139.2	5	<0.001			
Difference (model 5 – model 1)	290.3	17	<0.001			
Difference (model 6 – model 1)	515.0	5	<0.001			

[a] Absolute values are used to calculate significance of χ^2

CFI, comparative fit index; SRMR, standardized root means square residual; RMSEA, mean square error of approximation

CFI's of one-factor MetS models extended with uric acid, liver enzymes or albumin were below 0.95, indicating that their model fit was unacceptable. These extended MetS models did also not fit well, after the exclusion of the blood pressure factor (data not shown). Compared to the standard MetS model (model 1), the standard MetS model excluding blood pressure (model 2) fitted better, whereas the model extended with hsCRP (model 3) fitted worse.

HR's for type 2 diabetes (HR 2.71, 95%CI 2.30-3.18) and CVD (HR 1.25, 95%CI 1.13-1.39) were lowest in the MetS model excluding blood pressure (model 2), whereas they were highest in the MetS model extended with hsCRP (HR type 2 diabetes 3.94, 95%CI 3.28-4.74; CVD 1.28, 95%CI 1.16-1.42)(table 3). Off all MetS models, the model extended with hsCRP (model 3) predicted type 2 diabetes, CHD, CVA and CVD the best (type 2 diabetes C-index 0.8013; CVD C-index 0.6352). For all models the the Hosmer-Lemeshow test was not-significant, indicating a good calibration.

Discussion

We have examined the factor structure of MetS using confirmatory factor analysis (CFA) in two population based study samples. The good model fit of the standard one-factor MetS model, representing the current definition, indicated that it is valid to compose one entity out of the traditional MetS features. If the standard MetS model was extended with hsCRP predictive ability for type 2 diabetes and CVD increased slightly, while model fit was still acceptable.

In line with the results of previous CFA studies [7-9], we found that it is valid to compose out of the five traditional MetS features one entity, i.e. one factor. The model fit of a one-factor MetS model, composed of the traditional MetS features, was even better after exclusion of the blood pressure factor. This is consistent with other studies indicating that blood pressure is distinct from the other traditional MetS features, both from a physiological [22] and a statistical point of view. For example, blood pressure generally has the lowest factor loading in CFA MetS models [7-11]. Furthermore, blood

Subset of MORGEN (*n*=1333)					
χ^2	**df**	**P**	**RMSEA**	**SRMR**	**CFI**
56.0	7	<0.001	0.07	0.039	0.98
2.2	2	0.34	0.01	0.01	1.00
-53.8 [a]	5	<0.001			

Table 3 | Predictive ability of several MetS models for type 2 diabetes and CVD

	Standard MetS model (reference)		Standard MetS model minus blood pressure		Standard MetS model extended with hsCRP	
Included MetS features	TG, HDL, HbA1C, WC, SBP, DBP		TG, HDL, HbA1C, WC		TG, HDL, HbA1C, WC, SBP, DBP, hsCRP	
Diabetes (*n*=545)						
HR (95% CI) [a]	3.58	(3.05; 4.20)	2.70	(2.37; 3.11)	3.77	(3.21; 4.42)
HR (95% CI) [b]	3.70	(3.09; 4.42)	2.71	(2.30; 3.18)	3.94	(3.28; 4.74)
C-index	0.7949		0.7539		0.8013	
C-index change (P-value)	-		-0.0411	(<0.0001)	0.0064	(0.0001)
IDI (95% CI)	-		-1.58	(-1.89; -1.28)	0.34	(0.25; 0.44)
P-value Hosmer-Lemeshow	0.36		0.21		0.87	
CVD (*n*=1312)						
HR (95% CI) [a]	1.31	(1.20; 1.43)	1.28	(1.17; 1.39)	1.33	(1.22; 1.46)
HR (95% CI) [b]	1.26	(1.14; 1.40)	1.25	(1.13; 1.39)	1.28	(1.16; 1.42)
C-index	0.6315		0.6153		0.6352	
C-index change (P-value)	-		-0.0162	(<0.0001)	0.0037	(0.01)
IDI (95% CI)	-		-0.22	(-0.28; -0.20)	0.07	(0.04; 0.09)
P-value Hosmer-Lemeshow	0.76		0.94		0.95	
CHD (*n*=956)						
HR (95% CI) [a]	1.40	(1.27; 1.55)	1.37	(1.24; 1.52)	1.42	(1.29; 1.57)
HR (95% CI) [b]	1.35	(1.20; 1.51)	1.30	(1.16; 1.47)	1.36	(1.21; 1.53)
C-index	0.6496		0.6336		0.6519	
C-index change (P-value)	-		-0.0161	(<0.0001)	0.0022	(0.16)
IDI (95% CI)	-		-0.19	(-0.23; -0.15)	0.04	(0.02; 0.06)
P-value Hosmer-Lemeshow	0.88		0.95		0.96	
CVA (*n*=375)						
HR (95% CI) [a]	1.11	(0.98 ; 1.25)	1.07	(0.95; 1.21)	1.13	(1.00; 1.29)
HR (95% CI) [b]	1.08	(0.93; 1.25)	1.04	(0.90; 1.19)	1.10	(0.95; 1.28)
C-index	0.5760		0.5603		0.5828	
C-index change (P-value)	-		-0.0157	(<0.0001)	0.0068	(0.004)
IDI (95% CI)	-		-0.04	(-0.05; -0.02)	0.02	(0.01; 0.03)
P-value Hosmer-Lemeshow	0.68		0.83		0.90	

HR, hazard ratio; IDI, Integrated discrimination improvement; hsCRP, high sensitive C-reactive protein; Hazard ratios are presented per standard deviation, IDI's are presented as % improvement

[a] adjusted for age, sex and cohort

[b] adjusted for age, sex, cohort, smoking (current, former, none), education level, Cambridge physical activity index and alcohol intake

pressure is identified as a separate factor in most EFA studies [6]. Although omitting the blood pressure factor improved model fit, it also considerably decreased predictive ability for type 2 diabetes, CVA and CHD. Since this predictive ability is of clinical relevance, removal of blood pressure from the MetS definition is questionable.

Of the one-factor MetS models extended with non-traditional MetS features only the MetS model extended with hsCRP had an acceptable model fit. In 645 non-Hispanic whites or African Americans, an essentially similar MetS model also had a good model fit [8]. In our data the MetS model extended with hsCRP predicted type 2 diabetes, CVA and CHD slightly better than the standard MetS model. In two large prospective cohort studies hsCRP added substantial prognostic information to MetS [23, 24]. Therefore, in contrast to our study, the addition of hsCRP to MetS was clinically relevant in these previous studies. Part of the added predictive power of hsCRP may be explained by the

association of hsCRP with insulin resistance and fibrinolysis. Both increase risk of type 2 diabetes and CVD, but are not included in the current definition of MetS [25].

In our study, the model fit of other one-factor MetS models extended with additional features, i.e. albumin, liver enzymes or uric acid was not acceptable. To the best of our knowledge models extended with albumin or liver enzymes have not been previously studied. Our results with respect to these models are, however, in line with several EFA studies [23-26]. Contrary to our results, among 410 Spanish participants, a one-factor MetS model extended with uric acid had a very good model fit (CFI 0.99) [4]. The relatively low factor loading of MetS features strongly associated with uric acid, such as glucose [27], may explain the bad model fit of model extended with uric acid in our data.

Strength of our study was the hypothesis driven CFA approach, which we used to compare the model fit of a standard MetS model with several modified MetS models. Results of CFA studies are generally much more reproducible, than results of EFA studies. Furthermore, we tested the model fit of the MetS models in two relatively large study samples, and results were very similar. A limitation of our study was the absence of glucose measurements in the EPIC-NL case-cohort study. HbA1C levels were used instead. HbA1C levels were highly correlated with fasting plasma glucose levels (r^2=0.75) in 160 participants with both measurements available. Despite this, the correlation between HbA1C and waist circumference was much weaker than the correlation between fasting plasma glucose and waist circumference. This weak correlation, which has also been observed by others [28], resulted in a relative low factor loading for the glucose factor (based on HbA1C) in the random sample of EPIC-NL. This can perhaps explain why the model fit was generally worse for the MetS models in the random sample of EPIC-NL, compared to the models of the EPIC-NL MORGEN subset. A second limitation was that triglycerides levels were non-fasting in the EPIC-NL case-cohort study. As postprandial lipid levels are more strongly correlated with MetS features than fasting lipid levels, we may have overestimated the factor loadings of the lipid factor in the EPIC-NL case-cohort [29]. This is confirmed by the fact that the factor loading of the lipid factor was higher the EPIC-NL random sample than in the MORGEN subset, in which fasting sample were used. The participants (~7%) we excluded from the EPIC-NL case-cohort due to missing blood samples, had on average a 1.1 kg/m^2 higher BMI. As the correlations between waist circumference and other MetS features were slightly higher in the group with missing blood samples, exclusion of these participants may have resulted in somewhat lower factor loadings for the waist circumference factor. Additionally, the two datasets we used were not completely independent, as 133 subjects were present both in the EPIC-NL case-cohort study and in the MORGEN subset. However, when we excluded these 133 participants from the MORGEN subset, results were essentially similar. Finally, in order to be able to estimate model fit of the MetS model excluding blood pressure, we fixed the error variance of waist circumference in this model to one. This fixation has probably worsened the model fit. Therefore, we may have underestimated the improvement in model fit obtained by deleting the blood pressure factor.

In conclusion, it is valid to compose out of the traditional MetS features one entity and consequently to view MetS as a single disorder. A model additionally including hsCRP still represented a single entity and predicted type 2 diabetes and CVD somewhat better than a MetS model with only the traditional features. Therefore inclusion of hsCRP in future MetS definitions may be considered.

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Conflict of interest

The authors declare no conflict of interest

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Shared genetic variance between the features of the metabolic syndrome: heritability studies

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Abstract

Heritability estimates of MetS range from approximately 10%–30%. The genetic variation that is shared among MetS features can be calculated by genetic correlation coefficients. The objective of this paper is to identify MetS feature as well as MetS related features which have much genetic variation in common, by reviewing the literature regarding genetic correlation coefficients. Identification of features, that have much genetic variation in common, may eventually facilitate the search for pleitropic genetic variants that may explain the clustering of MetS features.

A PubMed search with the search terms "(metabolic syndrome OR insulin resistance syndrome) and (heritability OR genetic correlation OR pleiotropy)" was performed. Studies published before July 7th 2011, which presented genetic correlation coefficients between the different MetS features and genetic correlation coefficients of MetS and its features with adipose tissue-, pro-inflammatory and pro-thrombotic biomarkers were included.

Nine twin and 19 family studies were included in the review. Genetic correlations varied, but were strongest between waist circumference and HOMA-IR (r^2: 0.36 to 0.79, median: 0.50), HDL cholesterol and triglycerides (r^2: –0.05 to –0.59, median –0.45), adiponectin and MetS (r^2: –0.32 to –0.43; median –0.38), adiponectin and insulin (r^2: –0.10 to –0.60; median –0.30) and between adiponectin and HDL-cholesterol (r^2: –0.22 to –0.51, median –0.29).

In conclusion, heritability studies suggest that genetic pleiotropy exist especially between certain MetS features, as well as between MetS and adiponectin. Further research on actual genetic variants responsible for the genetic pleiotropy of these combinations will provide more insight into the etiology of MetS.

Introduction

Metabolic syndrome (MetS) refers to the clustering of abdominal obesity, hypertriglyceridemia, low HDL-cholesterol levels, hypertension and hyperglycemia [1]. People with three or more of these features are defined to have MetS according to the consensus statement of IDF and NCEP ATP III [1]. People with MetS are at increased risk of coronary heart disease (CHD) and type 2 diabetes (T2D) [1]. Besides the conventional MetS features, adipose tissue-, pro-inflammatory- and pro-thrombotic biomarkers, such as adiponectin and CRP, are important MetS related factors that play a role in the onset of CVD and T2D [2]. If added to the definition of MetS, these biomarkers may improve the predictive power of MetS for these conditions [2].

Heritability estimates of MetS range from approximately 10 to 30% [3-5]. This indicates that MetS is influenced both by environmental and genetic factors. The amount of additive genetic variation which is shared between two MetS features can be estimated from family and twin studies and is expressed by a genetic correlation coefficient. A genetic correlation expresses the extent to which two measurement reflect the same genetic character [6]. Research on genetic correlations can facilitate the search for pleiotropic genetic variants. MetS features which are highly genetically correlated have much genetic variation in common. For those features it will be easier to identify common genetic variants [6]. Identification of, for example, common genetic variants for dyslipidemia and insulin resistance may help to understand why some diabetic patients will develop dyslipidaemia, while others will not. Eventually, this understanding may affect treatment strategies.

Genetic correlation coefficients of the MetS features with each other and with MetS related biomarkers have been calculated in multiple twin and family studies. However, results of these studies are inconsistent and no overview of the available evidence exists. Therefore, the objective of this paper is to summarize these genetic correlation coefficients in order to identify those MetS features that share much genetic variation with another MetS feature or with a MetS related biomarker.

Methods

An electronic literature search was conducted using PubMed. The search terms "(metabolic syndrome OR insulin resistance syndrome) and (heritability OR genetic correlation OR pleiotropy)" were used. Furthermore, we reviewed the reference lists of retrieved articles to identify other relevant publications.
Studies included were: 1) published before 7th July 2011; 2) family or twin studies; and 3) studies which presented genetic correlation coefficients between the different MetS features or studies which presented genetic correlation coefficients of adipose tissue, pro-inflammatory and pro-thrombotic biomarkers with MetS and its individual features. No further in or exclusion criteria were used. For each article the following information was extracted if applicable: genetic correlation coefficients of MetS features, study design (family study or twin study), health status of the study population, number of monozygotic twins, number of dizygotic twins, number of families, ethnicity, average age and average BMI.
As correlation coefficients are not normally distributed by definition, we summarized genetic correlations coefficients between MetS or MetS related features, by median genetic correlation coefficients. As a measure of variation we used the range of genetic correlation coefficients. By visual inspection we evaluated whether age, family history, ethnicity, and health status of the population influenced the genetic correlation coefficients. If we suspected a population characteristic to influence the genetic correlation coefficient and if the population characteristic was present in ≥ 3 studies, we conducted a sensitivity analysis.

Results

Our literature search through PubMed yielded 444 articles. In 21 of these articles genetic correlation coefficients with MetS features were described. Through review of references we identified 7 additional articles. Finally, we included 9 twin [7-15] and 19 family studies [3, 15-33], describing 239 genetic correlations. The number of subjects included ranged from 132 to 3234 in the twin studies and from 375 to 5376 in the fam-

Table 1 | Median genetic correlation coefficients of MetS features with each other and with adiponectin

	WC		**FPG**		**HOMA-IR**	
FPG	0.16 (0.01; 0.51)	(n=6)	-		-	
HOMA-IR	0.59 (0.41; 0.79)	(n=7)	-		-	
TG	0.32 (-0.61; 0.61)	(n=8)	0.19 (-0.32; 0.30)	(n=9)	0.37 (-0.12; 0.57)	(n=7)
HDL	-0.22 (-0.50; 0.03)	(n=7)	-0.07 (-0.26; 0.08)	(n=7)	-0.30 (-0.50; -0.09)	(n=6)
SBP	0.22 (0.00; 1.00)	(n=9)	0.07 (-0.15; 0.58)	(n=9)	0.23 (0.06; 0.59)	(n=6)
DBP	0.16 (0.08; 0.61)	(n=7)	0.10 (-0.08; 0.27)	(n=6)	0.33 (0.23; 0.43)	(n=6)
Adiponectin	-0.23 (-0.20; -0.29)	(n=3)	-0.10 (-0.40; 0.01)	(n=4)	-0.26 (-0.10; -0.60)	(n=4)

Data are presented as median genetic correlation coefficients (lowest genetic correlation coefficient – highest genetic correlation coefficient)
n, number of studies in which the genetic correlations has been presented; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride levels; HDL, HDL-cholesterol levels; FPG, fasting plasma glucose; INS fasting insulin; HOMA-IR, homeostasis model assessment insulin resistance; MetS, metabolic syndrome

ily studies. Thirteen studies were conducted in Caucasian populations [3, 10-12, 14-17, 22, 23, 29-32], eight in Hispanic populations [18, 21, 24, 26-28, 30, 33] and seven in Asian populations [7-9, 13, 19, 20, 25] (Supplementary table 1, Supplementary table 2).
In table 1, median values of genetic correlation coefficients between MetS features across all studies are presented. Genetic correlation coefficients were strongest for waist circumference and HOMA-IR (r^2: 0.41 to 0.79, median: 0.59) (figure 1), and for HDL cholesterol and triglycerides (r^2: 0.07 to –0.59, median –0.46) (figure 2). The genetic correlation coefficients for blood pressure and HDL cholesterol (r^2: –0.23 to 0.06, median; –0.09), blood pressure and triglycerides (r^2: –0.63 to 0.22, median; 0.10), blood pressure and fasting glucose (r^2: –0.15 to 0.58, median; 0.13) and for fasting glucose and HDL cholesterol (r^2: –0.26 to 0.08, median –0.07) were lowest. Functioning of glucose metabolism was measured with different indices. The genetic correlations with HOMA-IR were generally higher than the genetic correlations with fasting glucose.
In Supplementary table 2, genetic correlations of MetS and its features with related biomarkers are presented. Genetic correlations have been published for 9 biomarkers. Most studies have been published on adiponectin, whereas only a few studies have been published on other biomarkers. Total and high molecular weight adiponectin were most strongly genetically correlated with MetS (r^2: –0.32 to –0.43, median –0.37), HDL cholesterol (r^2: 0.22 to 0.51; median 0.27) and HOMA-IR (r^2: –0.10 to –0.60; median –0.26). In the study of Menzaghi et. al [31], the genetic correlations were stronger with total and high molecular weight adiponectin than with medium and low molecular weight adiponectin.
Having evaluated the individual estimates, the strength of the genetic correlation coefficients did not seem to depend on study type, ethnicity, age or BMI of the population (Supplementary table 1, Supplementary table 2). In a study population with a high family history of obesity, genetic correlation coefficients with waist circumference were higher than in other studies [3]. Furthermore, we suspected that the health status of the study population might influence genetic correlation coefficients. However, the median genetic correlation coefficients calculated from studies conducted among generally healthy populations, were very similar to the median genetic correlation coefficients calculated from all studies (e.g. HOMA-IR and waist circumference r^2=0.54; HDL cholesterol and triglycerides r^2=–0.46)

TG		**HDL**		**MetS**	
-		-		-	
-		-		-	
-		-		-	
-0.46 (-0.59; 0.07)	(n=12)	-		-	
0.08 (-0.42; 0.22)	(n=9)	-0.04 (-0.23; 0.07)	(n=10)	-	
0.09 (-0.63; 0.19)	(n=6)	-0.12 (-0.16; 0.03)	(n=6)	-	
-0.19 (-0.12; -0.35)	(n=4)	0.22 (0.27; 0.51)	(n=4)	-0.32 (-0.43; -0.37)	(n=2)

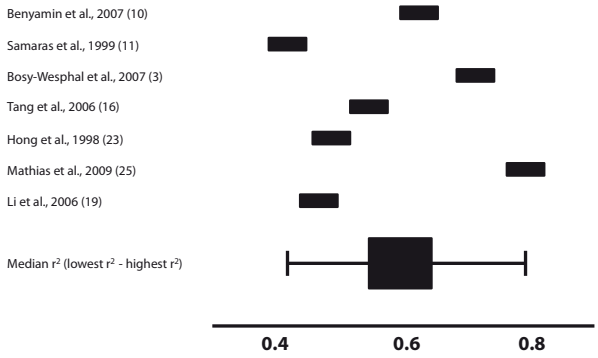

Figure 1 | Summary of genetic correlation coefficients between insulin resistance and waist circumference in 7 studies

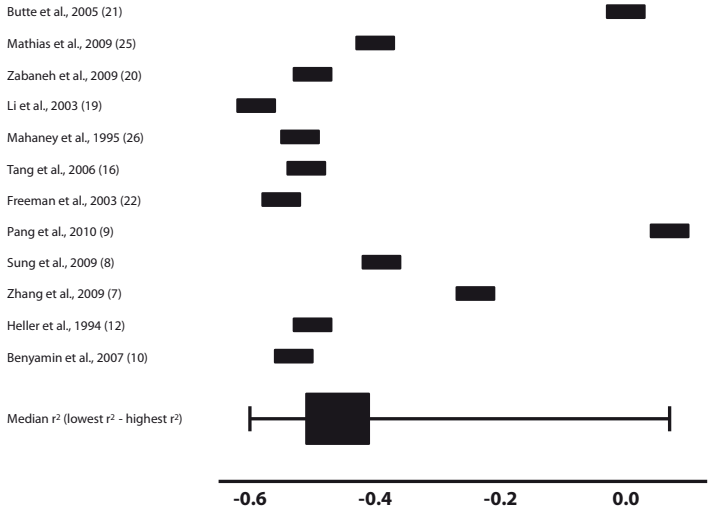

Figure 2 | Summary of genetic correlation coefficients between triglycerides and HDL cholesterol in 12 studies

Discussion

In this paper we have summarized the results of 28 family and twin studies that presented genetic correlation coefficients between the different MetS features, as well as genetic correlations between MetS features and MetS-related biomarkers. Genetic correlations were strongest between waist circumference and insulin resistance, HDL cholesterol and triglycerides, and adiponectin and MetS. The MetS features most strongly genetically correlated with adiponectin were insulin and HDL cholesterol.

Generally, the genetic correlations varied widely across studies. Differences in genetic architecture between study populations, such as different allele frequencies, could possibly explain this variation. In one study, for example, only families originating from overweight or obese probands were included [3]. Therefore this study population is enriched for obesogenic genes. Consequently, the percentage of insulin resistant cases caused by obesogenic genes may be relatively high explaining the higher genetic correlation between HOMA-IR and waist circumferences in this study, as compared to other studies. Furthermore, as all studies were conducted among different populations in different environments, gene-environment interactions could explain some of the differences between studies [6]. Finally, random error could also be an issue, especially in the smaller study populations [6].

Overall, genetic correlations were higher between MetS features and HOMA-IR, than between MetS features and fasting glucose, which increases secondary to insulin resistance [34]. As insulin resistance is one of the mechanisms proposed to initiate MetS development, it is not surprising that the genetic correlations are highest for those markers which most closely reflect insulin resistance, e.g. HOMA-IR [1]. Until now most studies on genetic variants have focused on fasting glucose instead of HOMA-IR. More research on genetic variants associated with insulin resistance may therefore help to identify genetic variants that influence multiple MetS features.

Currently, only a few genetic variants have been identified which are associated with more than one MetS feature. These identified genetic variants are all associated with MetS features that are clearly genetically correlated. For example, the high genetic correlation between triglycerides and HDL cholesterol may be partly explained by genetic variants in the *LPL* gene and genetic variants in the *APOA1/ APOC3/ APOA4/ APOA5* gene cluster. These genetic variants are associated with both HDL cholesterol and triglycerides [35]. Furthermore, genetic variants in the *FTO* gene are both associated with waist circumference and insulin resistance [36]. Therefore these genetic variants may partly explain the relatively high genetic correlation coefficient between abdominal obesity and insulin resistance.

Genetic correlations of adipose tissue biomarkers, pro-inflammatory biomarkers and pro-thrombotic biomarkers, such as adiponectin and CRP, with Mets and its features have been calculated in just a few studies [15, 16, 22, 28-33]. Only for adiponectin enough studies were conducted to draw meaningful conclusions [28-31]. Adiponectin is an adipose tissue secreted hormone, which is decreased in obese and diabetic patients [37]. Adiponectin was genetically most strongly correlated with HOMA-IR, fasting insulin, HDL cholesterol and MetS itself. The shared genetic variance of adiponectin with MetS and its individual features may be partly explained by the rs4311394 SNP, located in the *ADP-ribosylation factor-like 15* (*ARL15*) gene, a gene with unknown function [38]. In a recent genome wide association study, *ARL15* rs4311394 was not only associated with adiponectin, but also with insulin, T2D and CHD [38]. Furthermore, rs4311394 was modestly but not significantly associated with BMI [38]. To the best of our knowledge

the association between rs43111394 and HDL cholesterol or MetS has not been investigated yet. Besides the *ARL15* rs43111394 SNP, genetic variance at the 3q27 locus, may also partly explain the genetic correlation between adiponectin and MetS. The 3q27 locus, were the gene encoding adiponectin is located, has been associated with MetS and T2D in genome wide linkage studies [37].

In this paper we have pointed out the pairs of MetS features that have much genetic variation in common, by describing genetic correlation coefficients. A genetic correlation coefficient between two MetS features determines together with the heritabilities and environmental correlation coefficient of these features, the phenotypic correlation coefficient [6]. If the correlated characters have a low heritability the phenotypic correlation coefficient is mainly determined by the environmental correlation coefficient. Vice versa if both traits have a high heritability the phenotypic correlation coefficient is mainly determined by the genetic correlation coefficient. With the exception of blood pressure, MetS features have an average heritability of around 0.50 [29, 39-41]. Therefore, one can assume that, except for correlations with blood pressure, genetic correlation coefficients between two MetS features determine roughly 50% of the phenotypic correlation coefficients between those features.

The amount of genetic variation shared between two MetS features is determined both by the number of genetic variants and by the effect size of these variants. It is not possible to derive whether the shared genetic variation is caused by a small number of genetic variants with a large effect, or by a large number of genetic variants with a small effect. Furthermore, genetic correlations only estimate the amount of genetic variation shared between two MetS features and do no not give any hint on how this shared variation is realized. The shared genetic variation between two MetS features can be caused by two independent genetic effects, or the genetic effect on one MetS feature may be completely mediated by the effect of another MetS feature [6]. Lastly, one should realize that genetic correlation coefficients may be overestimated due to shared environmental factors [42]. To get further insight in how the shared genetic variation between two traits is realized, it is import to investigate which genetic variants are actually responsible for the shared genetic variation.

In conclusion, heritability studies suggest that genetic pleiotropy exist between HDL cholesterol and triglycerides and between waist circumference and insulin resistance. Furthermore, genetic pleiotropy seemed to be present between adiponectin and MetS. Further research, on actual genetic variants responsible for the pleiotropy of these MetS features, could provide more insight into the etiology of MetS. We suggest to start with the analysis of available GWAS for the associations between SNP's and those combinations of MetS features, which are highly genetically correlated.

Conflict of interest

The authors declare no conflict of interest

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Supplementary Table 1 | Genetic correlation coefficient between the features of the metabolic syndrome obtained from twin- and family studies.

Ref	Study Design	Healthy	Total (N)	MZ (N)	DZ (N)	Family (N)	Race	Age (y)	BMI (kg/m^2)
[7]	Twin	Y	3234	2232	1002		A	32.3	21.9
[8]	Twin	Y	944	732	212		A	42.4	-
[16]	Family	Y[p]	1940			445	C	51.5	27.2
[18]	Family	Y	375			21	H	41.6	27.4
[19]	Family	N[l]	913			179	A	41.5	24.8
[21]	Family	N[k]	1030			319	H	-	-
[10]	Twin	Y	1250	622	628		C	38	24.4
[11]	Twin	Y[p]	220	118	102		C	52.2	25.3
[3]	Family	N[n]	492			90	C	39.6	25.3
[16]	Family	Y[p]	1940			445	C	51.5	27.2
[23]	Family	Y[r]	512			98	C	-	25.9
[25]	Family	N[o]	524			26	A	-	-
[19]	Family	N[l]	913			179	A	41.5	24.8
[10]	Twin	Y	1250	622	628		C	38	24.4
[7]	Twin	Y	3234	2232	1002		A	32.3	21.9
[8]	Twin	Y	944	732	212		A	42.4	-
[9]	Twin	Y	1390	810	580		A	37	-
[22]	Family	Y	537			89	C	43.2	25.9
[16]	Family	Y[p]	1940			445	C	51.5	27.2
[3]	Family	N[n]	492			90	C	39.6	25.3
[18]	Family	Y	375			21	H	41.6	27.4
[24]	Family	Y	1510			41	H	-	30.3
[19]	Family	N[l]	913			179	A	41.5	24.8
[20]	Family	N[m]	1635			181	A	39.4	25.8
[25]	Family	N[o]	524			26	A	-	-
[21]	Family	N[k]	1030			319	H	-	-
[10]	Twin	Y	1250	622	628		C	38	24.4
[12]	Twin	Y	236 368				C	-	-
[7]	Twin	Y	3234	2232	1002		A	32.3	21.9
[8]	Twin	Y	944	732	212		A	42.4	-
[9]	Twin	Y	1390	810	580		A	37	-
[13]	Twin	Y	654	-	-		A	40	23.6
[22]	Family	Y	537			89	C	43.2	25.9
[16]	Family	Y[p]	1940			445	C	51.5	27.2
[3]	Family	N[n]	492			90	C	39.6	25.3
[26]	Family	Y	569			25	H	39.4	-
[27]	Family	Y[p]	1202			42	H	36.4	28.6
[24]	Family	Y	1510			41	H	-	30.3
[19]	Family	N[l]	913			179	A	41.5	24.8
[20]	Family	N[m]	1635			181	A	39.4	25.8
[25]	Family	N[o]	524			26	A	-	-
[21]	Family	N[k]	1030			319	H	-	-
[10]	Twin	Y	1250	622	628		C	38	24.4
[7]	Twin	Y	3234	2232	1002		A	32.3	21.9
[8]	Twin	Y	944	732	212		A	42.4	-
[13]	Twin	Y	654	-	-		A	40	23.6
[22]	Family	Y	537			89	C	43.2	25.9
[16]	Family	Y[p]	1940			445	C	51.5	27.2
[17]	Family	Y	5376			2184[j]	C	-	25.2
[3]	Family	N[n]	492			90	C	39.6	25.3
[18]	Family	Y	375			21	H	41.6	27.4
[24]	Family	Y	o10			41	H	-	30.3

ρ_genetic	WC	FPG	HOMA IR	TG	HDL
FPG	0.01	x	x	x	x
FPG	0.18	x	x	x	x
FPG	0.14	x	x	x	x
FPG	0.46	x	x	x	x
FPG	0.51	x	x	x	x
FPG	0.14	x	x	x	x
HOMA IR	0.64 [a]	x	x	x	x
HOMA IR	0.41 [h]	x	x	x	x
HOMA IR	0.73	x	x	x	x
HOMA IR	0.54 [d]	x	x	x	x
HOMA IR	0.48 [g,d]	x	x	x	x
HOMA IR	0.79 [d]	x	x	x	x
HOMA IR	0.46	x	x	x	x
TG	0.03	-	0.47 [a]	x	x
TG	0.40	-0.08		x	x
TG	0.36	0.19	-	x	x
TG	-	0.23	-	x	x
TG	-	-	0.31 [d]	x	x
TG	0.28	0.05	0.37 [d]	x	x
TG	0.61	-	0.57	x	x
TG	-0.61	-0.32	-	x	x
TG	-	-	0.30 [d]	x	x
TG	0.60	0.27	0.38	x	x
TG		0.22	-0.12	x	x
TG	-	0.3	-	x	x
TG	0.17	0.18	-	x	x
HDL	0.03	-	-0.16 [a]	-0.53	x
HDL	-	-	-	-0.48 [b]	x
				-0.43 [c]	
HDL	-0.13	0.08		-0.24	x
HDL	-0.25	0.06	-	-0.39	x
HDL	-	-0.22	-	0.07	x
HDL	-	-	-	-	x
HDL	-	-	-0.09 [d]	-0.55	x
HDL	-0.22	-0.09	-0.27 [d]	-0.51	x
HDL	-0.40	-	-0.50	-	x
HDL	-	-	-	-0.52	x
HDL	-	-	-0.33 [d]	-	x
HDL	-	-	-0.36 [d]	-	x
HDL	-0.50	-0.26	-0.38	-0.59	x
HDL	-	-0.07	-0.19	-0.50	x
HDL	-	-	-	-0.4	x
HDL	-0.21	0.04	-	-0.05	x
SBP	0.20	-	0.27 [a]	0.21	-0.01
SBP	0.00	0.05		0.22	-0.06
SBP	0.08 [i]	0.13 [i]	-	0.07 [i]	-0.05 [i]
SBP	-	-	-	-	-0.09
SBP	0.63	-	0.28 [d]	0.01	-0.10
SBP	0.16	0.18	0.18 [d]	0.17	0.03
SBP		-0.08 [i]			-0.10 [i]
SBP	1.00	-	0.59 [e]	-	-
SBP	0.00	-0.15	-	-0.42	-
SBP	-	-	0.06 [d]	-	-

Supplementary Table 1 | Continued

Ref	Study Design	Healthy	Total (N)	MZ (N)	DZ (N)	Family (N)	Race	Age (y)	BMI (kg/m^2)
[19]	Family	N [l]	913			179	A	41.5	24.8
[20]	Family	N [m]	1635			181	A	39.4	25.8
[25]	Family	N [o]	524			26	A	-	-
[21]	Family	N [k]	1030			319	H	-	-
[10]	Twin	Y	1250	622	628		C	38	24.4
[7]	Twin	Y	3234	2232	1002		A	32.3	21.9
[8]	Twin	Y	944	732	212		A	42.4	-
[16]	Family	Y [p]	1940			445	C	51.5	27.2
[17]	Family	Y	5376			2184 [j]	C	-	25.2
[3]	Family	N [n]	492			90	C	39.6	25.3
[18]	Family	Y	375			21	H	41.6	27.4
[24]	Family	Y	1510			41	H	-	30.3
[19]	Family	N [l]	913			179	A	41.5	24.8
[25]	Family	N [o]	524			26	A	-	-

Twin, Twin study; Family, Family study; A, Asian; C, Caucasian; H, Hispanci; Total (N), total number of people in the study population; MZ(N) number of study subjects which are monozygotic twins; DZ(N) number of study subjects which are dyzogotic twins; Family (N) number of families in the study; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride levels; HDL, HDL-cholesterol levels; FPG, fasting plasma glucose; INS fasting insulin; HOMA-IR, homeostasis model assessment insulin resistance

[a] BIGTT-SI, a marker for insulin sensitivity is measured. To estimate the effect on insulin resistance correlation coefficients have been multiplied by -1

[b] <=65.4 years

[c] >65.4 years

[d] Insulin is used

[e] male

[f] female

[g] abdominal fat measured by computed tomography scan

[h] Central body fat measured with DXA female

ρ_genetic	WC	FPG	HOMA IR	TG	HDL
SBP	0.27	0.07	0.07	-0.01	-0.23
SBP		-0.09		0.12	-0.01
SBP	-	0.58	-	-	-
SBP	0.33	0.44	-	0.07	0.08
DBP	0.27	-	0.23 [a]	0.10	-0.13
DBP	0.13	0.08		0.19	-0.16
DBP	0.08 [i]	0.13 [i]	-	0.07 [i]	-0.05 [i]
DBP	0.14	0.11	0.25 [d]	0.12	0.03
DBP		-0.08 [i]			-0.10 [i]
DBP	0.61	-	0.35 [e]	-	-
DBP	0.19	-0.06	-	-0.63	-
DBP	-	-	0.30 [d]	x	x
DBP	0.16	0.27	0.43	0.02	-0.14
DBP	-	-	0.42 [d]	-	-

[i] mean blood pressure is used

[j] households instead of families are used

[k] Probands are obese or overweight children (4-19y)

[l] All probands were diabetic

[m] All probands had premature coronary heart disease

[n] All included families had at least one overweight or obese family member

[o] Probands were either participants in the Chennai Urban Rural Epidemiology study, or paricipants of the Chennai Urban Population study, or diabetic subjects from an outpatient clinic

[p] Diabetic patients are excluded

[q] CVD cases, pregnant and breasfeading women, and people taking weight reducing medication were excluded

[r] People with a BMI>40, with conditions life-threatening with cycle exercise, with lipid lowering or hypertensive medication were excluded

Supplementary Table 2 | Genetic correlation coefficient of MetS features with inflammatory, thrombotic and adipose tissue biomarkers across twin and family studies

Ref	Study Design	Healthy	Total (N)	MZ (N)	DZ (N)	Family (N)	Race	Age (y)	BMI (kg/m^2)
[28]	Family	Y	898			-	H	41.2	29.2
[29]	Family	Y	2256			22	C	28.6	26.9
[30]	Family	N [b]	805			-	H	-	-
[31]	Family	Y [h]	640			235	C	40.3	26.3
[31]	Family	Y [h]	640			235	C	40.3	26.3
[31]	Family	Y [h]	640			235	C	40.3	26.3
[14]	Twin	Y	1250	622	628		C	37.2	24.4
[32]	Family	Y	737			264	C	40.5	26.2
[15]	Twin	Y	216	152	64		C	40.7	24.8
[16]	Family	Y [h]	1940			445	C	51.5	27.2
[16]	Family	Y [h]	1940			445	C	51.5	27.2
[22]	Family	Y	537			89	C	43.2	25.9
[22]	Family	Y	537			89	C	43.2	25.9
[22]	Family	Y	537			89	C	43.2	25.9
[22]	Family	Y	537			89	C	43.2	25.9
[33]	Family	Y	428			20	H	38.5	28.8

Twin, Twin study; Family, Family study; C, Caucasian; H, Hispanci; Total (N), total number of people in the study population; Family (N) number of families in the study; Adip, adiponectin; WC, waist circumference; FPG, fasting; INS, insulin; HOMA-IR, homeostasis model assessment insulin resistance; TG, triglyceride levels; HDL, HDL-cholesterol levels; SBP, systolic blood pressure; DBP, diastolic blood pressure; MetS, Metabolic Syndrome; HMW, high molecular weight; MMW, medium molecular weight; LMW, low molecular weight; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator; FVII, Factor VII; CRP, C-reactive protein; ICAM-1, intracellular adhesion molecule-1

[a] 2h-glucose used

[b] Probands are obese or overweight children (4-19y)

ρ_genetic	WC	FPG	HOMA IR	TG	HDL	SBP	DBP	MetS
Adipose tissue biomarkers								
Adip	-0.29	-0.40 [a]	-0.60 [d]	-0.35	0.51			
Adip	-0.20	-0.03	-0.19	-0.12	0.25	-0.10 [e]	-0.10 [e]	-0.43
Adip		0.01	-0.10	-0.21	0.32			
HMW adip	-0.23	-0.15	-0.32	-0.17	0.22	-0.01	-0.24	-0.32 [c]
MMW adip	-0.05	-0.10	-0.08	-0.02	0.14	-0.21	-0.02	-0.13 [c]
LMW adip	-0.05	-0.10	-0.08	-0.02	0.14	-0.21	-0.02	-0.13 [c]
Leptin	0.65 [f]							
	0.64 [g]							
Pro-inflammatory biomarkers								
Resistin	0.32	0.26	0.28	0.10	-0.08			0.35 [c]
CRP				0.45		0.28	0.29	
CRP	0.57	-0.05	0.50 [d]	0.26	-0.20	0.39	0.24	
Thrombotic biomarkers								
PAI-1	0.62	0.07	0.56 [d]	0.54	-0.37	0.01	0.21	
PAI-1			0.17 [d]	0.59	-0.04	0.61		
t-PA			0.02 [d]	0.23	0.04	0.70		
Fibrinogen			0.42 [d]	0.36	-0.35	0.13		
FVII			0.30 [d]	0.36	0.02	0.29		
ICAM-1	0.26	0.01	0.22	0.06	-0.24	0.05	0.04	

[c] MetS score is used

[d] Insulin is used

[e] mean blood pressure is used

[f] in male

[g] in female

[h] in non-diabetic subjects

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Genetic variants and the metabolic syndrome: a systematic review

Supplementary tables
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Abstract

Several candidate gene studies on the metabolic syndrome (MetS) have been conducted. However, for most single nucleotide polymorphisms (SNP's) no systematic review on their association with MetS exists. A systematic electronic literature search was conducted until the 2nd of June 2010, using HuGE Navigator. English language articles were selected. Only genes of which at least one SNP – MetS association was studied in an accumulative total population ≥4000 subjects were included. Meta-analyses were conducted on SNP's with 3 or more studies available in a generally healthy population. In total 88 studies on 25 genes were reviewed. Additionally, for nine SNP's in seven genes *(GNB3, PPARG, TCF7L2, APOA5, APOC3, APOE, CETP)* a meta-analysis was conducted. The minor allele of rs9939609 *(FTO)*, rs7903146 *(TCF7L2)*, *C56G (APOA5)*, *T1131C (APOA5)*, *C482T (APOC3)*, *C455T (APOC3)* and *174G>C (IL6)* were more prevalent in subjects with MetS, whereas the minor allele of Taq-1B *(CETP)* was less prevalent in subjects with the MetS. After having systematically reviewed the most studied SNP-MetS associations, we found evidence for an association with the MetS for 8 SNP's, mostly located in genes involved in lipid metabolism.

Introduction

The metabolic syndrome (MetS) is a common multi-component condition including abdominal obesity, dyslipidemia, hypertension, and hyperglycemia. It is associated with an increased risk of coronary heart disease (CHD) and type 2 diabetes (T2D). The prevalence of MetS, which is currently around 30%, is rising worldwide [88]. Heritability estimates for MetS range from 10-30% [89-91], indicating that MetS is partly heritable. Knowledge of the exact genetic factors underlying MetS development may help to explain why the features of MetS frequently co-occur within one individual. In order to detect genes underlying MetS development several candidate gene studies, have been performed with inconsistent results. However, no systematic review has been conducted to date, and thus no clear overview of the available evidence on the genetics of the MetS exists. Therefore, the objective of this paper is to systematically review the studies on single nucleotide polymorphisms (SNP's) and MetS, and where possible to summarize the results using meta-analyses.

Methods

Search strategy

An electronic literature search was conducted using HuGE Navigator. HuGE Navigator is a database of published population-based epidemiologic studies of human genes extracted and curated from PubMed since 2001 [92]. Previous validations on selected gene-disease associations showed that HuGE Navigator was equally sensitive, but more specific than PubMed [93].

For the Huge Navigator search, the search term "metabolic syndrome x [Text MesH]" was used. This search retrieved articles on the association between MetS and any genetic variant. The latest search was undertaken on 2nd of June 2010. As HuGE Navigator only retrieves articles published since 2001, an additional PubMed search was done. For the PubMed search, the search term "metabolic syndrome x [Text + MesH] with limits on publication date from 1990/1/1 to 2001/12/31" was used.

Eligibility criteria

Articles were included when they contained MetS as outcome and were: published in English; original research articles; conducted in humans; and testing for SNP main effects. All existing definitions of MetS (supplementary table 1) were eligible as study outcome.

Genes were included if two or more articles were retrieved on the same gene, and at least for one of the SNP's in this gene the accumulative total study population was ≥4000 subjects. A study with 4000 subjects has a power of 80% to detect an OR ≤0.8 or an OR≥1.2, assuming a significance level of 0.05, a MetS prevalence of 30% and a minor allele frequency (MAF) of 0.25. An exception was made for the *ADIPOQ* gene, which has been related to MetS in linkage studies (7). The *ADIPOQ* G276T (rs1501299) polymorphism was studied in an accumulative total population of 3865 subjects only. However, because the MAF of this SNP was 0.30 instead of 0.25, the power to detect an association was 90%. For other SNP's investigated in 3000-3999 subjects either the MAF was too low to obtain sufficient power, or the prior evidence substantiating an association with MetS was weak.

Included studies were eligible for inclusion in the meta-analyses if they had a cross-sectional or case-control design, and if the crude genotype distribution according to MetS status was available. If the genotype distribution could not be extracted from the original research article, investigators were contacted via email. Meta-analyses were carried out for SNP's with both 3 or more eligible studies available in a generally healthy population and with inconsistent study outcomes.

Data extraction

Data extraction was conducted by one author (CMP). For quality control, data were extracted by two of the other authors (JMAB, ER) for ten percent of the entered papers. Only minor discrepancies were found. For each article the following information was extracted: authors, publication year, sample size, number of MetS cases, ethnicity, health status of the population (e.g. CHD or T2D patients), study design, mean age, percentage men, crude genotype distribution by MetS status, odds ratio and the reported measure of variance. For the selected genes all SNP – MetS associations published, independent of sample size, were extracted. If results were given for multiple MetS definitions, results for the definition of the NCEP ATPIII, which is the most common definition, were extracted. If results were presented separately for men and women an aggregate effect measure was calculated where possible.

Data analyses

For SNP's located in the same gene we checked the correlation coefficients according to HapMap. If SNP's had a correlation ≥0.8 we mentioned this in the results.

For SNP's included in the meta-analyses, OR's of individual studies were recalculated from the available genotype distributions according to an allelic model. Afterwards, combined OR's were calculated using random effect models and forest plots were drawn. Heterogeneity was investigated by the I^2 statistic. Roughly, I^2 values of 25%, 50% and 75% can be regarded as low, moderate, and high heterogeneity [94]. The following sources of heterogeneity were explored by meta-regression: health status of the population (e.g. CHD or T2D patients), gender, age, MetS definition, study design and ethnicity. In some cases too few studies were available to conduct meta-regression with STATA. In those cases sensitivity analyses were performed. Funnel plots, Egger's and BEGG's test were used to check for publication bias. STATA 11 (StataCorp LP, Collega Station, TX, USA) was used to perform all analyses.

Results

Our literature search yielded 104 articles identified through PubMed and 465 articles identified through Huge Navigator (figure 1). None of the studies identified through PubMed were eligible, while 186 identified through Huge Navigator were. Of the eligible papers, 51 were excluded because <2 articles were published on the same gene, and 48 were excluded because all SNP's in the gene described in the article were studied in <4000 subjects. Finally, 87 articles on 25 genes were included in this review. In these 87 articles 88 studies were described.

The majority of the studies were cross-sectional studies (*n*=73; 83%). Of the remaining studies, 11 were case-control studies, 3 were family studies and 1 was a prospective study. Most studies were either conducted in subjects of Caucasian (*n*=56; 64%) or Asian origin (*n*=21; 24%). The average prevalence of MetS across all studies was 30%. In 75% (*n*=66) of the studies MetS was defined according to the criteria of the NCEP ATP III. Meta-analyses were carried out for those SNP's with 3 or more eligible studies available in a generally healthy population, which included 37 studies [1-13, 15, 17-29, 33, 36-43] on 9 SNP's located in 7 genes *(GNB3, PPARG, TCF7L2, APOA5, APOC3, APOE, CETP)*. In none of the meta-analyses the Egger's test, the Begg's test, or the funnel plots could indicate the presence of publication bias.

First we will describe the association between MetS and those genes with sufficient data for meta-analyses. Secondly, we will describe the remaining SNP-MetS associations in a narrative review. In table 1, an overview is provided of all genes studied, the pathways they are involved in and the results of the meta-analyses. Detailed information on all studies is available in supplementary tables 2a-2y.

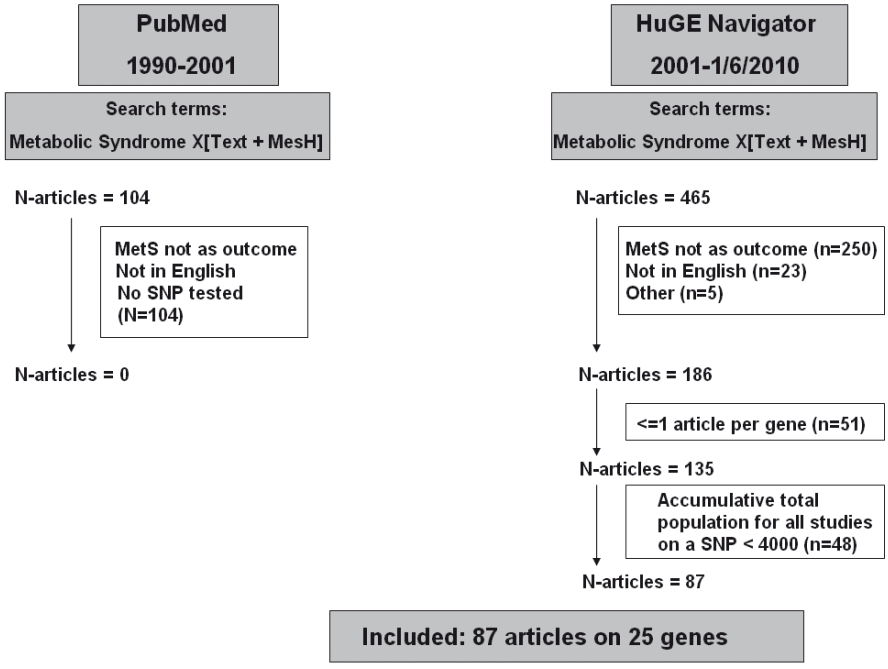

Figure 1 | Literature search results. SNP, single nucleotide polymorphism.

Results of the Meta-analyses

PPARG

PPARG is a nuclear receptor involved in glucose and fatty acid metabolism [17]. The *Pro12Ala* (rs1801282) polymorphism of the *PPARG* gene has been consistently associated with T2D [95, 96]. However, of the 16 studies investigating the association between *Pro12Ala* (rs1801282) and MetS [3-11, 13-17], most showed no effect [1, 3-17]. This is confirmed by our meta-analysis among 13 studies[1, 3-13, 17] (pooled OR of Ala vs. Pro 1.08; 95% 0.93- 1.24, I^2=48.3%) (figure 2). Meta regression revealed that population characteristics such as ethnicity and health status could not explain the moderate heterogeneity present in this meta-analysis (supplementary table 3). Interestingly, although the 12Pro allele is associated with increased risk of T2D and insulin resistance independent of BMI [95], from the meta-analysis it can be concluded that if any effect on MetS exists, *12Ala* is the risk allele. As the *12Ala* genotype has been associated with BMI in a meta-analysis among Caucasian subjects [95], this effect could possibly be mediated by BMI.

The association between *C1431T* (rs3856806), another well-known *PPARG* polymorphism, and MetS, has been investigated in 6 cross-sectional studies (17-19, 21, 23, 25) and 1 family study [16]. In the family study, conducted among 423 Chinese subjects, the prevalence of the *1431T* allele was lowest in subjects with MetS. However, in our meta-analysis of the 6 cross-sectional studies (17-19, 21, 23, 25) there was no association between *C1431T* (rs3856806) and MetS (pooled OR of T v C 0.97, 95%CI 0.86-1.11, I^2=0%) (figure 3).

Table 1 | Summary of the reviewed SNP's in relation to metabolic syndrome

Gene	- SNP's	Pathways involved			
		Weight regulation	Glucose metabolism	Lipid metabolism	Inflammation
Meta-analyses					
PPARG	- *Pro12Ala* (rs1801281) - *C134T* (rs3856806)	x	x	x	
TCF7L2	- rs7903146		x		
APOA5	- *T113C* (rs662799) - *C56G* (rs3135506)			x	
APOC3	- *C482T* (rs2854117) - *C455T* (rs2854116)			x	
APOE	- *ε2/ε3/ε4 (ε2/- v ε3/ ε3)* - *ε2/ε3/ε4 (ε4/- v ε3/ ε3)*			x	
CETP	- *Taq-1B* (rs708272)			x	
GNB3	-*C825T* (rs5433)	x		x	
FTO	-rs9939609	x			

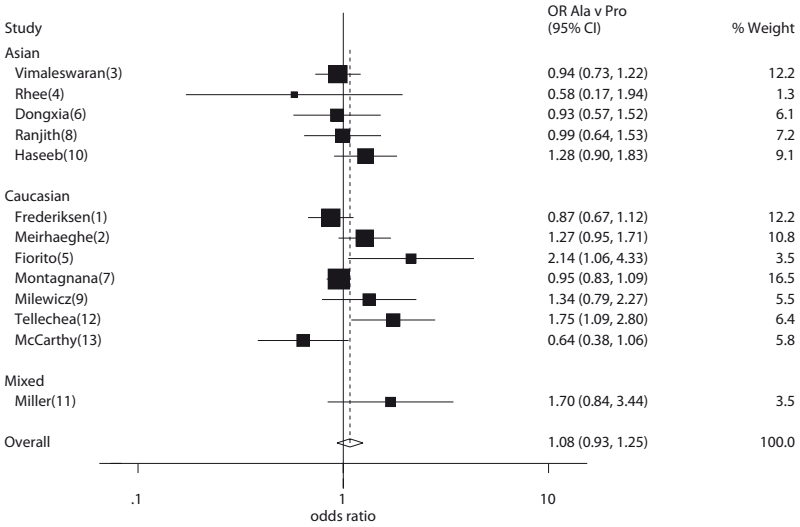

Figure 2 | Meta-analysis on the association between the *PPARG Pro12Ala* (rs1801282) polymorphism and the metabolic syndrome; heterogeneity $I^2 = 48.3\%$; MAF Caucasian 0.06–0.17; MAF Asian 0.05–0.13; MAF mixed population 0.09; MAF, minor allele frequency; OR, odds ratio

	Results	
Blood pressure	**Pooled OR**	**I^2**
	1.08 (95%CI: 0.93-1.24)	48.3%
	0.97 (95%CI: 0.86-1.11)	0%
	1.18 (95%CI 1.04-1.34)	25.6%
	1.24 (95%CI 1.10-1.41)	47.7%
	1.26 (95%CI 1.09-1.47)	0%
	1.57 (95%CI 1.00-2.48)	90.5%
	NA	
	0.91 (95%CI 0.70-1.18)	7.5%
	1.61 (95%CI 0.87-2.97)	88.3%
	0.93 (95%CI 0.80-1.90)	59.8%
	0.89 (95%CI 0.80-0.97) a	4.4%
x	1.03 (95%CI 0.94-1.12)	0%
	1.17 (95%CI 1.10-1.25)45	0%

Table 1 | Continued

Gene	- SNP's	Pathways involved			
		Weight regulation	Glucose metabolism	Lipid metabolism	Inflammation
Narrative review					
UCP1		x			
UCP2		x			
LEPR		x	x	x	
ADIPOQ	*-G276T* (rs1501299)		x	x	x
IL6	*-174G>C* (rs1800795)				x
RETN	*-420C>G* (rs1862513)	x			x
LMNA	*-H566H* (rs4641)	x	x	x	
ADRB2	*-Arg16Gly* (rs1042713) *-Gln27Gln* (rs1042714)		x	x	
ADRB3	*-Trp64Arg* (rs4994)		x	x	
PPARD	*-87T>C* (rs2016520)	x	x		
PPARGC1A	*-Gly482Ser* (rs8192678) *-Thr394Thr* (rs3755863)		x	x	
FABP2	*-Ala54Thr* (rs179883)		x	x	
CAPN10	*-UCSNP43* (rs3792267)		x		
IRS1	*-Gly927Arg* (rs1801278)		x		
ENPP1	*-K21Q* (rs1044498)		x		
GCK	*-30G>A* (rs1799884)		x		
KCNJ11	*-E23K* (rs5219)		x		

NA, not available; (+), sufficient evidence for an association based on the narrative review; (-), insufficient evidence for an association based on the narrative review;
a Results of a sensitivity analysis in non-patients

Interestingly, although both the 12Ala and the 1431C allele did not seem to increase MetS risk significantly in our meta-analyses, a haplotype containing the same alleles was associated with an increased prevalence of MetS in a cross-sectional study among 1115 French subjects [17]. Other SNP's in the *PPARG* gene have not been associated with MetS (22, 34).

Blood pressure	Results Evidence level
	(-)
	(-)
	(-)
	(-)
	(+)
	(-)
	(-)
x	(-)
	(-)
x	(-)
	(-)
	(-)
	(-)
	(-)
	(-)
	(-)
	(-)
	(-)
	(-)

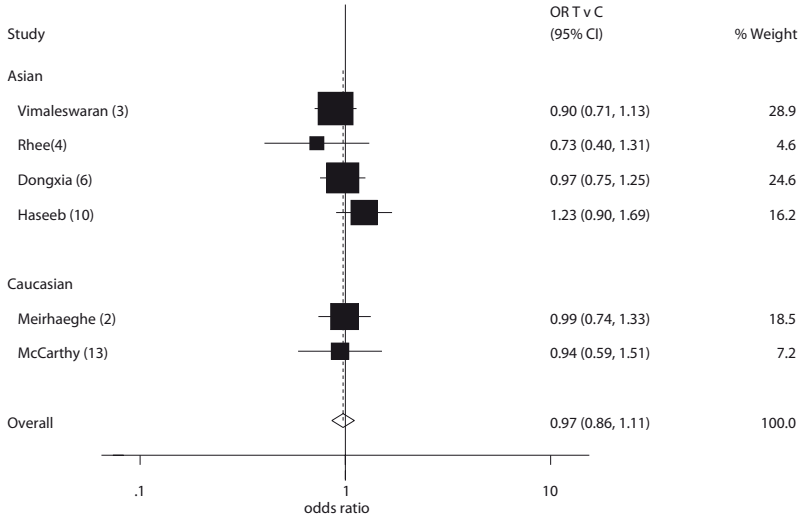

Figure 3 | Meta-analysis on the association between the *PPARG C1431T* (rs3856806) polymorphism and the metabolic syndrome; heterogeneity $I^2 = 0\%$; MAF Asian 0.14–0.30; Caucasian 0.08–0.15; MAF, minor allele frequency; OR, odds ratio

TCF7L2

The *TCF7L2* gene is involved in Wnt signaling and insulin secretion [97]. The T-allele of the rs7903146 polymorphism in the *TCF7L2* gene increases the risk of T2D [98]. The T allele also increased MetS risk in our meta-analysis of 5 studies (pooled OR 1.18, 95%CI 1.04-1.34) (figure 4) [18-22]. The heterogeneity between studies was low (I^2=25.6%), and decreased to 0% in a sensitivity analysis among generally healthy subjects [19-22]. The pooled OR increased to 1.29 (95%CI 1.10-1.36).

Although both Begg's (P=0.01) and Egger's test (P=0.008) were significant, no publication bias was present, as the largest studies had the largest effect. One expects that in case of publication bias, the smallest studies would show the highest OR's [99, 100].

A prospective study among 16143 Swedes confirmed the results of our meta-analysis. In this prospective study the OR for developing MetS in 23 years was 1.10 (95%CI 1.04-1.17) [14].

As expected results for the rs12255372 polymorphism [19-21] were similar as those of the completely correlated rs7903146 polymorphism (r^2=1 HapMap CEU).

Furthermore, in one study among obese hypertensive patients the *TCF7L2* copy number variation, *DG10S478X*, was associated with MetS [18].

APOA5

APOA5 reduces plasma triglyceride levels by stimulating the hydrolysis of triglycerides through the activation of lipoprotein lipase (LPL) and by inhibiting very low density lipoproteins (VLDL) production [101]. The C allele of the *T1131C* (rs662799) polymorphism in the APOA5 gene is associated with higher triglycerides and reduces HDL-cholesterol levels [30-32]. The *T1131C* (rs662799) polymorphism, or genetic variants highly correlated with the *T1131C* (rs662799) polymorphism, were significantly associated with MetS in all [15, 23-32], but 3 studies [23, 26, 31]. Accordingly, in our meta-analysis among 9 of these studies [15, 23-29] the C-allele of the *T1131C* (rs662799) polymorphism increased MetS risks (pooled OR 1.24, 95%CI 1.10-1.41) (figure 5). Meta-regres-

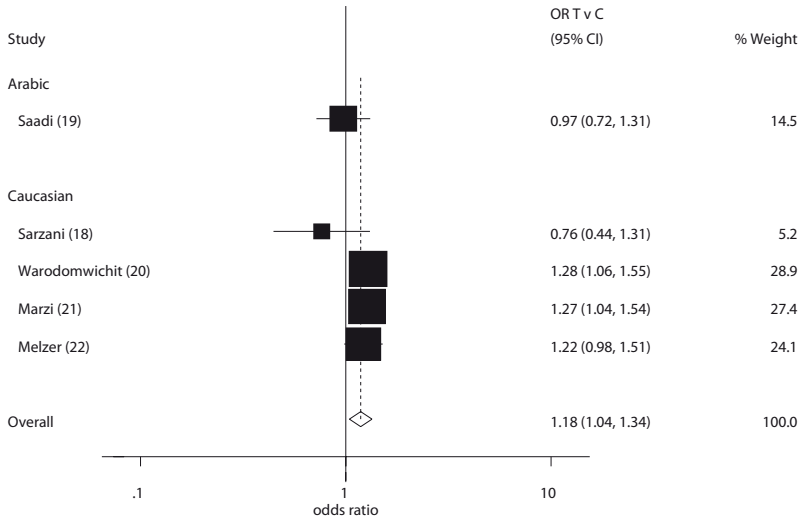

Figure 4 Meta-analysis on the association between the *TCF7L2* rs7903146 polymorphism and the metabolic syndrome; heterogeneity $I^2 = 25.6\%$; MAF Caucasian 0.28–0.35; MAF Arabic 0.39; MAF, minor allele frequency; OR, odds ratio

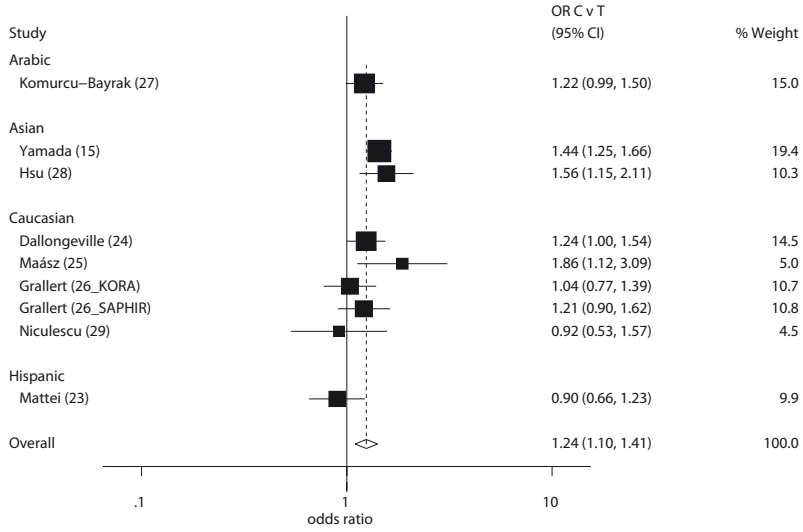

Figure 5 Meta-analysis on the association between the *APOA5 T1131C* (rs662799) polymorphism and the metabolic syndrome; heterogeneity $I^2 = 47.7\%$; MAF Arabic 0.13; MAF Asian 0.30–0.31; MAF Caucasian 0.06–0.08; MAF Hispanic 0.14; MAF, minor allele frequency; OR, odds ratio

sion analysis revealed that the moderate heterogeneity (I^2 = 47.7%) present could be explained by population characteristics such as sex and ethnicity (supplementary table 4). Therefore, we performed a sensitivity analysis in Caucasian subjects only. The OR in this sensitivity analysis was somewhat lower (pooled OR C versus T 1.20, 95%CI 1.02-1.41, I^2 = 19.0%).

Another *APOA5* polymorphism which has been frequently investigated in relation to MetS is the *C56G* (rs3135506) polymorphism. The meta-analysis included 5 studies [23, 26, 27, 29] and showed that the G allele of the *C56G* (rs3135506) polymorphism increased MetS risk (pooled OR 1.26, 95% CI 1.09-1.47, I^2 =0%) (figure 6). However, the *C56G* (rs3135506) polymorphism was not associated with MetS in a study among 2417 Japanese, which could not be included in the meta-analysis, because the genotype distribution could not be obtained [30].
Three other *APOA5* polymorphisms, all not correlated with one of the polymorphisms discussed above, have also been investigated in relation to MetS [24, 26, 30]. Two of these polymorphisms, *12,238T>C* (rs625524) [24] and *Gly185Cys* (rs2075291) [30] were associated with MetS, in one single study.

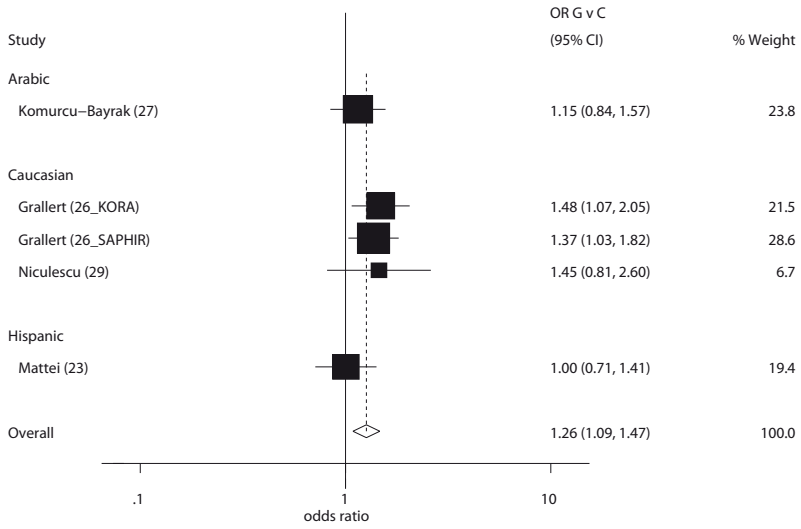

Figure 6 Meta-analysis on the association between the *APOA5 C56G* (rs3135506) polymorphism and the metabolic syndrome; heterogeneity I^2 = 0%; MAF Arabic 0.05; MAF Caucasian 0.06–0.09; MAF Hispanic 0.10; MAF, minor allele frequency; OR, odds ratio

APOC3

APOC3 increases plasma triglycerides levels, by the inhibition of LPL activity and by the interference with ApoE mediated uptake of triglycerides [34, 102]. The minor *482T* allele of the *APOC3 C482T* (rs2854117) polymorphism is associated with increased triglyceride levels [103]. The same allele also increased MetS risk in 4 [11, 29, 33, 34] out of 5 studies [11, 24, 29, 33, 34]. Our meta-analysis among the 4 studies with genotype distributions available [11, 24, 29, 33] confirmed that the 482T allele increased MetS risk (pooled OR 1.57, 95%CI 1.00-2.48) (figure 7). However, although the direction of the effect was the same for most studies, the heterogeneity between studies was high (I^2=90.5%). Both the heterogeneity and the OR were slightly lower among cross-sectional studies (OR 1.24, 95%CI 0.90-2.01, I^2=78.2%) [24, 33] and studies in Caucasian subjects (OR 1.16, 95%CI 0.79-1.70, I^2=84.9%) [24, 29, 33].

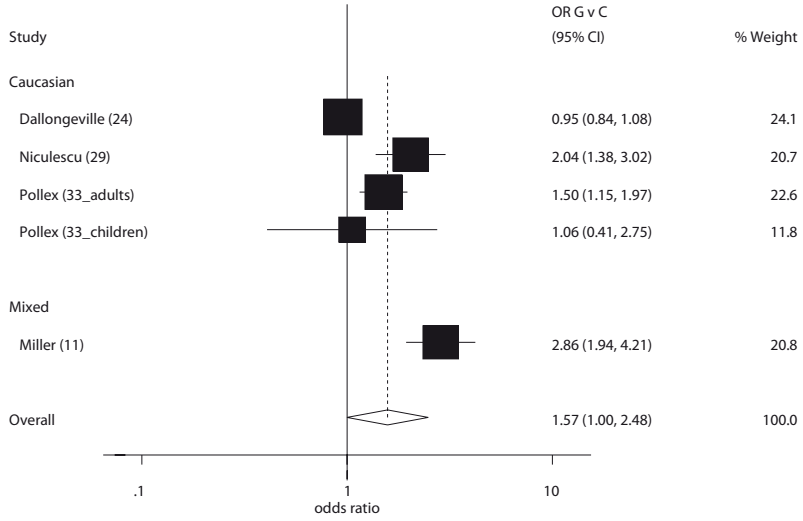

Figure 7 Meta-analysis on the association between the *APOC3 C482T* (rs2854117) polymorphism and the metabolic syndrome; heterogeneity I^2 = 90.5%; MAF mixed population 0.24; MAF Caucasian (excluding Oji Cree aboriginals33) 0.21–0.27; MAF Oji Cree aboriginals33 0.43–0.44; MAF, minor allele frequency; OR, odds ratio

As expected results for the *c455t* (rs2854116) polymorphism [11, 29, 33-35] were similar to those of the highly correlated *c482t* (rs2854117) polymorphism (r^2=0.97 HapMaP CEU) [33]. On the contrary, for *APOC3 1100C>T*[15] and *APOC3 SstI* [13, 24] no association with MetS could be detected.

APOE

Apolipoprotein-E (APOE) has an important function in the clearance of chylomicron remnants and VLDL from plasma. Three APOE isoforms encoded by the *ε2/ε3/ε4* haplotype exist. The *ε3* isoform is the most prevalent isoform. In comparison with the *ε3* isoform, the *ε2* isoform decreases cholesterol levels and increases triglyceride levels, whereas the *ε4* isoform increases both cholesterol and triglyceride levels [104]. In our meta-analysis among 5 studies [11, 36-39] the *ε2/-* genotype (*ε2/ε3* + *ε2/ε2*) none significantly decreased MetS risk (pooled OR *ε2/- v ε3/ε3* 0.91; 95%CI 0.70-1.18, I^2=7.5%) whereas the *ε4/-* genotype (*ε4/ε4* + *ε4/ε3*) tended to increase MetS risk (pooled OR *ε4/- v ε3/ε3* 1.61, 95%CI 0.87-2.97, I^2=88.3%) (figure 8a, figure 8b). The fact that 4 out of 5 studies were conducted in subjects of different ethnicity, may explain the high heterogeneity (I^2=88.3%) observed for the *ε4/-* genotype.

In two studies the effect of individual SNP's in the APOE gene instead of the effect of the *ε2/ε3/ε4* haplotype was investigated. In a study among 1788 Japanese [15], in which 3 SNP's of the APOE gene had been genotyped, the Arg158Cys (rs7412) polymorphisms, which is part of the *ε2/ε3/ε4* haplotype, was associated with MetS. However, this association could not be replicated in 305 Caucasian CAD patients [13]

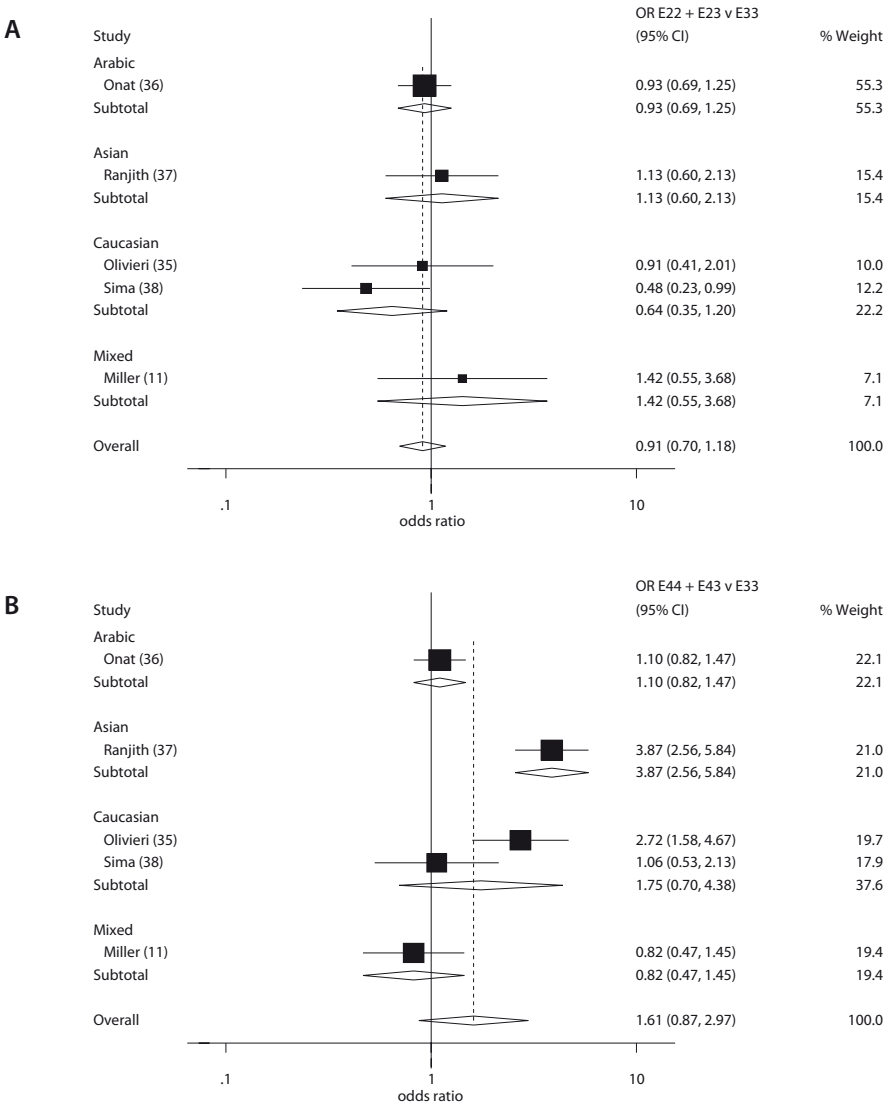

Figure 8 A Meta-analysis on the association between the *APOE ε2/ε3/ε4* haplotype and the metabolic syndrome; heterogeneity $I^2 = 7.5\%$; frequency *ε2* Arabic 0.12; frequency *ε2* Asian 0.05; frequency *ε2* Caucasian 0.06–0.09; frequency *ε2* mixed 0.05; OR, odds ratio;
B Meta-analysis on the association between the *APOE ε2/ε3/ε4* haplotype and the metabolic syndrome; heterogeneity $I^2 = 88.3\%$; frequency ε4 Arabic 0.07; frequency *ε4* Asian 0.09; frequency *ε4* Caucasian 0.08–0.09; frequency *ε4* mixed 0.20; OR, odds ratio

CETP

The cholesteryl ester transfer protein (CETP) plays an import role in reverse cholesterol transport. The *B2* allele of the *CETP Taq-1B* (rs708272) polymorphism increases HDL cholesterol levels and decreases triglyceride levels and CETP activity [105]. In our meta-analysis including 4 studies [37, 40-42], the *B2* allele tended to decrease MetS risk (pooled OR 0.93, 95% 0.80- 1.09, I^2 = 59.8%.) (figure 9). When we excluded the study of Ranjith et all.[37] among 592 patients with acute MI from our meta-analysis the het-

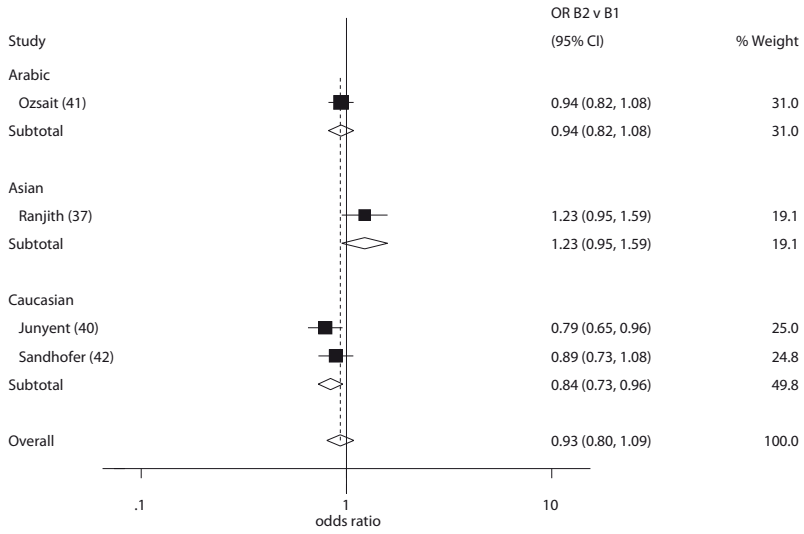

Figure 9 | Meta-analysis on the association between the *CETP Taq-1B* (rs708272) polymorphism and the metabolic syndrome; heterogeneity I^2 = 59.8%; MAF Arabic 0.43; MAF Asian 0.48; MAF Caucasian 0.41–0.43; MAF, minor allele frequency; OR, odds ratio

erogeneity decreased (I^2 = 4.4%), and the OR became significant (pooled OR B2 v B1 0.89, 95%CI 0.80-0.97). The study among 1788 Japanese, which could not be included in the meta-analysis showed no association between the Taq-1B (rs708272) polymorphism and MetS [30]. Furthermore, in studies on other polymorphisms in the *CETP* gene, no associations with MetS were observed [13, 15, 30].

FTO

Studies in humans and rodents suggest that FTO regulates food intake and effects the lypolytic activity in adipose tissue [106]. The A-allele of the rs9939609 polymorphism in the FTO gene has been associated with increased BMI and T2D risk in multiple genome wide association studies (GWAS) [45]. The A-allele of the rs9939609 also increased MetS prevalence in a large meta-analysis among 12555 European subjects (OR per A allele 1.17 ; 95%CI 1.10- 1.25, P=3.0 * 10^{-6}) [45] and in a smaller meta-analysis among 2112 subjects of mixed ethnicity (AA + AT vs. TT OR 1.26; 95%CI 1.02-1.57) [44]. OR's of the individual studies included in the meta-analyses ranged from 1.10 to 1.44. In line with these results, the OR per A-allele for developing MetS in 23 years was 1.08 (95%CI 1.02-1.14) in a large prospective study among 16143 non-diabetic Swedes [14]. Furthermore, the rs1421085 polymorphism, which is highly correlated with the rs9939609 polymorphism (r^2=0.93), was associated with MetS in 2 independent studies [47, 48]. On the contrary, rs9939609 and 2 other highly correlated polymorphisms were not associated with MetS in a study among 1488 Japanese [46].

GNB3

The *GNB3* gene is involved in G-protein signal transduction. The *C825T* (rs5433) polymorphism in the *GNB3* gene has been associated with obesity, hypertension, dyslipidemia and T2D, which are all features of MetS [2, 43]. However, although in one study the *C825T* (rs5433) polymorphism was associated with MetS in Oji Cree women [33], other studies could not replicate these results [2, 11, 15, 43]. Also, our meta-analysis of 4 studies [2, 11, 33, 43] (figure 10) could not demonstrate an association between the *C825T* (rs5433) polymorphism and MetS (pooled OR of 825T v C 1.03, 95%CI 0.94-1.12, I^2=0). In one study, among 2417 Japanese, the association with the *GNB3* 1429C>T (rs5446) polymorphism was investigated [30]. Also this polymorphism was not associated with MetS.

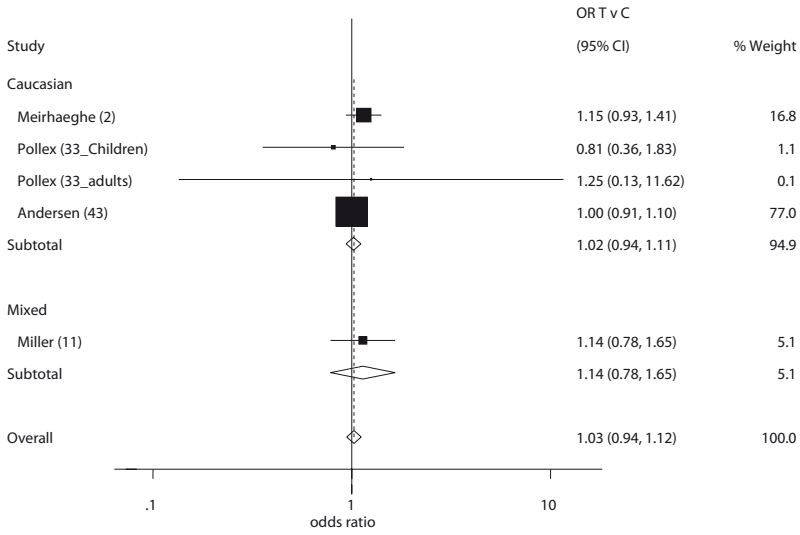

Figure 10 Meta-analysis on the association between the *GNB3 C825T* (rs5433) and the metabolic syndrome; heterogeneity I^2 = 0%; MAF mixed 0.34; MAF Caucasian (excluding Oji Cree aboriginals33) 0.31–0.34; MAF Oji Cree aboriginals33 0.46–0.49; MAF, minor allele frequency; OR, odds ratio

Narative review of associations with MetS for SNP's not elegible for meta-analysis

In this narrative review we describe SNP's which were not elegible for meta-analysis because they have been studied in too few studies with generally healthy subjects. Detailed information about these SNP's can be found in the online supplementary tables 2i-2y.

Of all SNP's, the strongest evidence for an association with MetS was found for the *IL6 174G>C* (rs1800795) promoter polymorphism. IL-6 is a cytokine with a broad range of effects, e.g. it is the primary determinant of hepatic CRP secretion [63]. Elevated plasma IL-6 levels are associated with T2D and CHD, both end stages of MetS [63]. The association between the *IL6 174G>C* (rs1800795) promoter polymorphism and MetS was significant in 3 [13, 62, 63] out of 4 [13, 61-63] studies. In three studies the *174C* allele increased MetS risk [61-63], while in a fourth study the direction of the association was not reported [13]. In most studies on inflammatory SNP's other than *IL6 174G>C* (rs1800795), such as SNP's in *RETN*[15, 64, 65] and *ADIPOQ* [54-58], no association with MetS was found. Especially for *ADIPOQ* this was remarkable. The *ADIPOQ* gene encodes for adiponectin. Lower plasma adiponectin concentrations have been associated with several features of MetS including insulin resistance [58]. Furthermore, in a linkage study the *ADIPOQ* locus, 3q27, was associated with MetS [107]. However, in most studies the *ADIPOQ G276T* (rs1501299) polymorphism was not associated with MetS [54, 55, 57-60] . Furthermore, in the single study in which an effect was shown for *ADIPOQ G276T* (rs1501299) [56], this effect was opposite to the effect expected based on the association of *ADIPOQ G276T* (rs1501299) with adiponectin and insulin sensitivity [108]. The *ADIPOQ G276T* (rs1501299) polymorphism was not the only SNP in which, despite strong prior evidence for possible involvement of the gene in MetS development, an association with MetS seemed absent. Also, no association with MetS seemed to exist for SNP's in the *LMNA* [66-69] gene, while the *LMNA* gene is associated with lipodystrophy, a syndrome which shares many features with MetS [66]. Involvement of a gene in multiple MetS pathways, did not guarantee an association for SNP's in this gene with MetS. The evidence for an association with MetS was weak for SNP's in the *ADRB2* [13-15, 70] and *ADRB3* [14, 15, 71-73] gene, genes involved in glucose metabolism, lipid metabolism, and blood pressure regulation [70], SNP's in the *LEPR* gene[30, 52, 53], which is involved in body weight regulation, fatty acid oxidation and glucose metabolism [109]; SNP's in the *PPARD* gene [15, 74, 75], which regulates both glucose and energy metabolism [75]; and SNP's in the *PPARGC1A* gene [3, 14, 15, 76], which is involved in lipid and glucose metabolism [76]. However, for the *Ala54Thr* (rs1799883) SNP [11, 33, 34, 77-79] in the *FABP2* gene, which is involved in both fatty acid and glucose metabolism [77, 78], some evidence for an association with MetS exists. In the majority of studies [11, 33, 77-79], most of which were conducted in patient populations [34, 77-79], the *Thr54* allele increased MetS risk, although in most studies the association was not statistically significant [11, 33, 77, 79]. For all other SNP's reviewed, either located in genes involved in energy metabolism (*UCP1* and *UCP2* [3, 14, 15, 30, 49-51]) or in genes involved in glucose metabolism (*CAPN10* [14, 15, 80-83], *IRS1* [8, 14, 15], *ENPP1* [14, 15, 84-86], *GCK*[15, 87] and *KCNJ11*[8, 14, 15]) the evidence for an association with MetS was not substantial.

Discussion

In this systematic review we described the most studied SNP's in relation to MetS. The overall results suggest an association with MetS for SNP's in the *FTO*, *TCFL72*, *IL6*, *APOA5*, *APOC3* and *CETP* genes.

The *FTO* rs9939609 and the *TCF7L2* rs7903146 polymorphism are the top hits of GWAS on respectively BMI [110] and T2D [111]. The *TCF7L2* rs7903146 polymorphism influences insulin secretion, and to a lesser extent this SNP also affects insulin resistance[112]. The *174C* allele of the *IL6 174G>C* (rs1800795) polymorphism increased MetS risk in 3 [13, 62, 63] out of 4 studies [13, 61-63]. In line with the effect on MetS the *174CC* genotype tended to increase BMI and IL6 levels[113], both MetS-associated features, in meta-analysis on 15 and 17 studies, respectively. Accordingly, in another meta-analysis on 7 studies the 174CC genotype also tended to increase CHD risk, an end stage of MetS [114]. However, contrary to the effect on MetS, the *174CC* genotype significantly decreased glucose levels in a meta-analysis on 7 studies [113]. The other SNP's which were associated with MetS, the *T1131C APOA5* (rs662799) [26], the *C56G APOA5* (rs3135506) [26], the *C455T* (rs2854116) *APOC3*, the *C482T* (rs2854117) *APOC3* [103] and the *Taq-1B* (rs708272) *CETP* [105] polymorphisms, are all associated with hypertriglyceridemia. Furthermore, the *C482T* (rs2854117) polymorphism, which is located in the insulin response element of the *APOC3* gene promoter, has also been associated with insulin and glucose levels [102, 115].

Focussing on combined phenotypes, like MetS, may lead to the discovery of new SNP's that would not have been found when studying the phenotypes separately. The fact that the study of combined phenotypes may lead to the discovery of new risk loci is nicely illustrated by a recent GWAS on Crohn's and Celiac disease, where the focus on risk loci shared between Crohn's and Celiac disease leads to the discovery of six new risk loci [116]. All SNP's included in this review which were associated with MetS were also strongly associated with an individual feature of MetS. Up till now no SNP has been found, which has only a minor effect on individual MetS features, but which does affect the clustering of the different features. Nevertheless, such a SNP may still be discovered. Interestingly, we observed that although all SNP's associated with MetS were associated with an individual MetS feature the reverse is not always true. For example, both *PPARG Pro12Ala* (rs1801282) and *TCF7L2* rs7903146 are associated with hyperglycemia. However, only *TCF7L2* rs7903146 and not *PPARG Pro12Ala* (rs1801282) seemed to be associated with MetS. This subdivision of on the one hand SNP's which are associated with one MetS feature only, and on the other hand SNP's which are associated with multiple MetS features, and thus also associated with MetS, may facilitate the discovery of pathways responsible for the clustering of MetS features.

Interestingly, although disturbances in glucose metabolism [88], weight regulation [88] and inflammation [117] all three have been proposed to initiate MetS, most SNP's associated with MetS are located in genes involved in lipid metabolism. The associations of these SNP's in the *CETP*, *APOC3* and *APOA5* genes with the MetS may be mediated by hypertriglyceridemia. Accumulation of triglycerides in the muscles may stimulate the development of insulin resistance [118]. Furthermore, dysfunctioning of the *APOA5* and *APOC3* gene increases free fatty acid levels [102, 119], which in turn may stimulate development of MetS features, such as dyslipidemia, overweight, insulin resistance, hypertension or inflammation [118]. Alternatively, the overrepresentation of SNP's in lipid metabolism, may be caused by the stress put on lipid metabolism in MetS definition. In the most common MetS definitions, the NCEP ATP III and the IDF definition, a disturbed lipid metabolism is characterized by 2 MetS features i.e. low HDL cholesterol

levels and increased triglyceride levels, whereas disturbances in the other mechanisms such as weight regulation, are all only characterized by 1 MetS feature.
In this review we have focussed on SNP-MetS associations which have been investigated in at least two studies. Consequently, significant SNP-MetS associations which have not been researched yet or which have only been researched in one study were not described. One of the best ways to test a large number of not investigated SNP - MetS associations is to conduct a GWAS. Unfortunately, to the best of our knowledge, such a GWAS has not been conducted yet.
Strength of this review is the unbiased way in which we have summarized results of the available studies on SNP – MetS associations. For all genes described, at least one SNP – MetS association was investigated in an accumulative total population across all published studies ≥4000 subjects. The number of 4000 subjects allowed us to detect SNP – MetS associations of moderate effect size (OR ≤0.8 or an OR ≥1.2). Therefore, we may have missed associations of smaller effect size. E.g. the pooled OR of 0.90 for the APOE *ε2ε3ε4* haplotype was not statistically significant in our meta-analysis. Population characteristics, such as ethnicity and health status of the study population, differed between the studies included in this review. Despite these differences, study outcomes were homogeneous for some SNP's, e.g. the *GNB3 C825T* (rs5433) and *PPARG C1431T* (rs3856806) polymorphism. However, for other SNP's these differences could explain the observed heterogeneity in study outcomes. For example, ethnicity explained nearly all heterogeneity present in the meta-analysis on *APOA5 T1131C* (rs662799). Furthermore, heterogeneity decreased and the OR increased, if studies in patient populations were excluded from the meta-analyses on the *TCF7L2* rs7903146 and the *CETP Taq-1B* (rs708272) polymorphisms. In two meta-analyses, on the *APOC3 C482T* (rs2854117) polymorphism and *APOE ε2ε3ε4* haplotype, a high unexplained heterogeneity was present. Especially for these genetic variants it will be valuable to conduct an updated meta-analysis stratified for several subgroups, if more studies become available. The Egger's and Begg's test did not indicate that in any of the meta-analyses publication bias was present. However, both tests have a low power unless a large number of studies ($n \geq 25$) are analyzed [99, 100]. As our meta-analyses were conducted among a smaller number of studies, we can not rule out the possibility that publication bias is present anyway.
In conclusion, we found evidence for an association with MetS for 8 SNP's. All of these SNP's were also associated with an individual MetS feature, most of them with dyslipidemia. This suggests that lipid metabolism plays a central role in MetS development.

Conflict of interest

The authors declare no conflict of interest

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SNP's involved in insulin resistance, weight regulation, lipid metabolism and inflammation in relation to metabolic syndrome

Submitted

Abstract

Introduction

Mechanisms involved in metabolic syndrome (MetS) development include insulin resistance, weight regulation, inflammation and lipid metabolism. Aim of this study is to investigate the association of single nucleotide polymorphisms (SNP's) involved in these mechanisms with MetS.

Methods

In a random sample of the EPIC-NL study (*n*=1886), 38 SNP's associated with waist circumference, insulin resistance, triglycerides, HDL cholesterol and inflammation in genome wide association studies (GWAS) were selected from the 50K IBC array and one additional SNP was measured with KASPar chemistry. The five groups of SNP's, each belonging to one of the metabolic endpoints mentioned above, were associated with MetS and MetS-score using Goeman's global test. For groups of SNP's significantly associated with the presence of MetS or MetS-score, further analyses were conducted.

Results

The group of waist circumference SNP's was associated with waist circumference (P=0.03) and presence of MetS (P=0.03). Furthermore, the group of SNP's related to insulin resistance was associated with MetS score (P<0.01), HDL cholesterol (P<0.01), triglycerides (P<0.01) and HbA1C (P=0.04). Subsequent analyses showed that *MC4R* rs17782312, involved in weight regulation, and *IRS1* rs2943634, related to insulin resistance were associated with MetS (OR 1.16, 95%CI 1.02-1.32 and OR 0.88, 95% CI 0.79; 0.97, respectively). The groups of inflammation and lipid SNP's were neither associated with presence of MetS nor with MetS score.

Conclusion

In this study we found support for the hypothesis that weight regulation and insulin metabolism are involved in MetS development. *MC4R* rs17782312 and *IRS1* rs2943634 may explain part of the genetic variation in MetS.

Introduction

Methods

EPIC-NL: Study design

In the EPIC-NL cohort the two Dutch contributions to the European Investigation into Cancer and Nutrition (EPIC) project are combined: the Prospect-EPIC and the MORGEN-EPIC (Monitoring Project on Risk Factors for Chronic Diseases) cohorts. Both cohorts were initiated in 1993.The study design of the combined cohort is described in detail elsewhere [8]. In brief, Prospect is a prospective cohort study among 17 357 women aged 49-70 who participated in a breast cancer screening program between 1993 and 1997. The MORGEN-project consists of 22 654 men and women aged 20-59 years recruited from three Dutch towns (Amsterdam, Doetinchem, and Maastricht). From 1993 to 1997, each year a new random sample of approximately 5000 individuals were examined for the MORGEN-project.

A 6.5% random sample of the EPIC-NL study was selected and blood and buffy coat was retrieved for these subjects (*n*=2604). We used this random sample for our analyses. After exclusion of participants with missing blood samples (*n*=157), missing values for haemoglobin A1c (HbA1C), waist circumference, high-density lipoprotein(HDL) cholesterol, systolic blood pressure, diastolic blood pressure, triglycerides or C-reactive protein (*n*=128), or with missing SNP data (*n*=438) the study population consisted of 1886 participants. All participants signed informed consent before study inclusion. Both studies complied with the Declaration of Helsinki. The Prospect-EPIC study was approved by the Institutional Review Board of the University Medical Center Utrecht and the MORGEN project was approved by the Medical Ethical Committee of TNO, The Netherlands.

Baseline measurements

At baseline, a physical examination was performed and non-fasting blood samples were drawn. During the physical examination, systolic and diastolic blood pressure measurements were performed twice in the supine position on the right arm using a BosoOscillomat (Bosch & Son, Jungingen, Germany) (Prospect) or on the left arm using a random zero sphygmomanometer (MORGEN). The mean of both measurements was taken. Waist circumference and height were measured to the nearest 0.5 cm. Body weight was measured with light indoor clothing without shoes on, to the nearest 100 gr.

Biomarker measurements

Levels of the biochemical parameters were measured in EDTA or citrate plasma. HbA1c was measured with a homogeneous assay with enzymatic endpoint. Trigycerides were measured using enzymatic methods, whereas high sensitve C-reactive protein (hsCRP) was measured with a turbidimetric method [8]. MetS was defined according to an adapted version of the AHA/NHLBI MetS definition as having at least 3 of the following 5 MetS features [9]: abdominal obesity (waist circumference ♂ ≥102 cm; ♀ ≥88 cm); low HDL cholesterol (♂ <1.0; ♀ <1.3 mmol/L); hypertriglyceridemia (≥1.7 mmol/L); hypertension (≥130/85 mm Hg or hypertensive medicine); hyperglycemia (HbA1C ≥5.7% or glucose lowering medication) [10, 11]. MetS-score was calculated by summing the number of MetS features present in each participant.

Genotyping

Genomic DNA was extracted in different batches using standard methods, such as salting out, QIAamp® Blood Kit (Qiagen Inc., Valencia, CA, USA). We were able to isolate DNA from 2398 participants in the random sample. For 206 participants in the random sample this was not possible due to missing buffy coats or blood samples. The participants were genotyped using a gene-centric 50K iSelect chip array, previously described as the IBC CVD chip [12]. The design and coverage of the IBC array compared to conventional genome-wide genotyping arrays has been described in detail elsewhere [12]. Additionally, the *MC4R* rs17700633 SNP was available for 853 women of the random sample. For this SNP genomic DNA was extracted with an in-house developed extraction method at Kbiosciences (Hoddesdon Herts, UK). (http://www.kbioscience.co.uk/lab%20services/DNA%20extraction/Ext_services_intro.html) *MC4R* rs17700633 was genotyped with the KASPar chemistry, an allele-specific PCR SNP genotyping that uses FRET quencher cassette oligos [13].

From the available SNP's, we selected those significantly associated ($P \leq 1.0*10^{-5}$) with waist circumference, inflammatory markers, triglycerides, HDL cholesterol or HOMA-IR ($p<0.05$). As only a few GWAS on HOMA-IR are conducted, SNP's both associated with a glucose related traits in GWAS ($P \leq 1.0*10^{-5}$) and with HOMA-IR ($P \leq 0.05$) were also included. Highly correlated SNP's were included in case the original SNP from the GWAS was not available on the IBC array ($r^2 \geq 0.80$). If SNP's were only found in a GWAS, without a replication sample, they were excluded. In total we included 39 SNP's: 2 SNP's associated with waist circumference, 5 SNP's associated with insulin resistance, 6 SNP's associated with inflammation, 16 SNP's associated with triglycerides and 16 SNP's associated with HDL cholesterol (table 1).

Statistics

Distributions of genotypes were tested for deviation from HWE by chi-square analyses. Triglycerides and hsCRP were log-transformed to improve normality. Participants on blood pressure medication were excluded from the analyses on blood pressure, participants on glucose lowering medication from the analysis on HbA1C, and participants with acute inflammation (hsCRP>10 mmol/L) from the analyses on hsCRP.

SNP's were divided into 5 groups (table 1) according to the known associations in GWAS. These groups of SNP's were associated with the corresponding phenotype using the linear regression model of Goeman's global test [7]. For example, we tested if the group of SNP's associated with waist circumference in GWAS was also associated with waist circumference in our data. Subsequently, for each group of SNP's the association with MetS was analysed using the log-linear model of Goeman's global test, and the association with MetS-score using the linear regression model of Goeman's global test. If one of these associations was significant, we conducted additional data-analyses. First, to see whether the association with the group of SNP's was mediated by the corresponding phenotype from GWAS, we adjusted the association between MetS and the group of SNP's for this phenotype. Second, we tested if the group of SNP's was also associated with the individual MetS features or hsCRP using the linear regression model of Goeman's global test. Third, we analysed the association of the individual SNP's in this group with MetS using log-linear models and with MetS-score using linear regression. For the individual SNP's which were significantly associated with MetS or MetS-score, we analysed associations with the individual MetS features and hsCRP using linear regression. See figure 1 for an overview of our data-analyses scheme. All analyses were corrected for age, sex and cohort. Significance was defined as a 2-sided P-value <0.05.

Table 1 | SNPs included in the analyses of random sample of EPIC-NL (*n*=1886)

Gene	SNP (literature)	SNP (dataset)	MAF (dataset)	ref	r^2 SNPs
Insulin resistance					
PPARG	rs1801282	rs1801282	G: 0.13	[25]	-
IRS1	rs2943634	rs2943634	A: 0.35	[21]	-
GCKR	rs780094	rs780094	T: 0.37	[25]	-
IGF1	rs35767	rs35767	A: 0.16	[25]	-
GCK	rs4607517	rs1799884	T: 0.18	[25]	1
Abdominal obesity					
FTO	rs1421085	rs1421085	C: 0.40	[32]	-
MC4R [a]	rs17782313	rs17782313	C: 0.25	[32]	-
Inflammation					
IL6R	rs4537545	rs4537545	T: 0.39	[33]	-
LEPR	rs6700896	rs1805096	A: 0.38	[33]	0.89
CRP	rs7553007	rs1341665	A: 0.32	[33]	1
ADIPOQ	rs1648707	rs182052	A: 0.34	[34]	1
IL18	rs1834481	rs5744256	G: 0.26	[35]	1
GCKR	rs780094	rs780094	T: 0.37	[36]	-
Triglycerides					
AFF1	rs442177	rs3775214	G: 0.43	[37]	0.96
APOB	rs673548	rs673548	A: 0.22	[38]	-
APOB	rs693	rs693	G: 0.50	[39]	-
APOA5-A4-C3-A1	rs12286037	rs12286037	T: 0.08	[40]	-
APOA5	rs6589566	rs2075290	C: 0.06	[41]	1
FADS1	rs174548	rs174548	G: 0.29	[37]	
FADS1-2-3	rs174547	rs174577	A: 0.35	[42]	1
GALNT2	rs4846914	rs4846914	G: 0.41	[39]	-
LPL	rs328	rs328	G: 0.10	[39]	-
MLXIPL	rs17145738	rs17145750	T: 0.16	[40]	0.86
PLTP	rs7679	rs6073952	A: 0.20	[42]	0.82
TRIB1	rs2954029	rs2954029	T: 0.47	[42]	-
CLIP2	rs16996148	rs16996148	T: 0.10	[40]	-
GCKR	rs780094	rs780094	T: 0.37	[27]	-
ANGPTL3-DOCK7	rs1748195	rs1748197	A: 0.35	[40]	1
ANGPTL3-DOCK7	rs12130333	rs12130333	T: 0.24	[39]	-
HDL cholesterol					
ABCA1	rs1883025	rs1883025	T: 0.24	[42]	-
ABCA1	rs3890182	rs3890182	A: 0.10	[37]	-
APOB	rs11902417	rs11902417	A: 0.24	[37]	-
CETP	rs1800775	rs1800775	A: 0.46	[43]	-
CETP	rs3764261	rs3764261	A: 0.31	[44]	-
FADS1	rs174548	rs174548	G: 0.29	[37]	-
FADS1-2-3	rs174547	rs174577	A: 0.35	[42]	-
GALNT2	rs4846914	rs4846914	G: 0.41	[39]	-
LCAT	rs255052	rs255052	A: 0.17	[40]	-
LCAT	rs12449157	rs1109166	C: 0.18	[37]	0.94
LIPC	rs1800588	rs1800588	T: 0.22	[39]	-
LIPG	rs2156552	rs2156552	A: 0.16	[37]	-
LPL	rs328	rs328	G: 0.10	[39]	-
PLTP	rs7679	rs6073952	A: 0.20	[42]	0.82
MMAB MVK	rs2338104	rs10774708	A: 0.47	[42]	1
HNF4A	rs1800961	rs1800961	T: 0.04	[42]	-

[a] Data available in 853 women

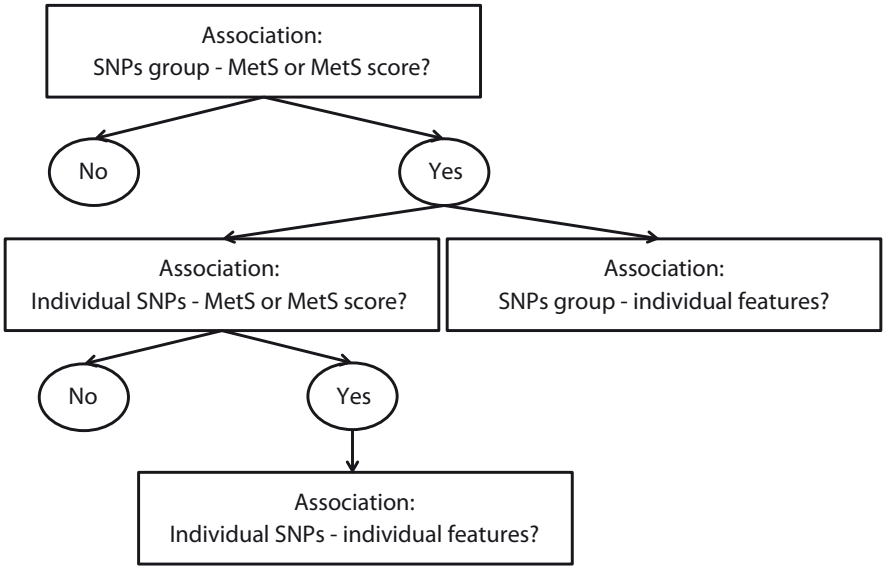

Figure 1 | Flow diagram of analyses

The global test was calculated in R version 2.12.1 (R Foundation for Statistical Computing; www.r-project.org). The analyses for individual SNP's were performed with SAS version 9.2 (SAS Institute, INC., Cary, North Carolina).

Results

All SNP's were in HWE (P>0.05). Minor allele frequency of the SNP's ranged from 0.04-0.47 (table 1). The random sample of EPIC-NL consisted of 465 men and 1421 women (table 2). The mean age was 50.1 (SD=11.7) and 30.3% of all participants had MetS. Mean waist circumference was 85.5 cm (SD 11.6), mean HbA1C was 5.46% (SD 0.69), median triglycerides 1.32 mmol/L (P25-P75 0.91-1.98), mean HDL cholesterol 1.27 mmol/L (SD 0.35), mean systolic blood pressure 129.9 mm/Hg (SD 18.5) and mean diastolic blood pressure 78.2 mmHg (SD 10.4).The group of abdominal obesity SNP's was significantly associated with waist circumference (P=0.01), the group of insulin resistance SNP with HbA1C (P=0.04) and the group of inflammation SNP's with hsCRP ($P=7.3*10^{-6}$). In contrast, the group of triglyceride SNP's and the group of HDL cholesterol SNP's were not significantly associated with triglycerides (P=0.08) and HDL cholesterol (P=0.32), respectively.

P-values for the association of all groups of SNP's with MetS or MetS-score are shown in table 3. The group of SNP's, known for their association with insulin resistance, was borderline significantly associated with MetS (P=0.06) and statistical significantly associated with MetS-score (P=0.003). This group of SNP's was also significantly associated with HbA1C, triglycerides and HDL cholesterol (table 4). The associations of this group of SNP's with MetS-score and MetS features weakened slightly after adjustment for HbA1C (table 4). Of the five insulin resistance SNP's included in the group *IRS1* rs2943634 was the only SNP individually associated with MetS or MetS-score (table 5). These associations remained after adjustment for HbA1C (data not shown). *IRS1*

Table 2 | Characteristics of 1886 subjects of the random sample of EPIC-NL

	Total (*n*=1886)	Men (*n*=465)	Women (*n*=1421)
Sex (% men)	24.6 (465)		
Age (yr)	50.1 (11.7)	43.9 (11.1)	52.2 (11.2)
Waist circumference (cm)	85.5 (11.6)	92.0 (11.4)	82.9 (10.5)
Abdominal obesity(%) [a]	27.7 (522)	21.9 (102)	29.6 (420)
HbA1C (%)	5.46 (0.69)	5.27 (0.61)	5.53 (0.71)
Hyperglycemia [a]	28.3 (534)	17.8 (83)	31.7 (451)
Diabetic medication(%)	1.2 (22)	0.2 (1)	1.5 (21)
HDL-cholesterol (mmol/L)	1.27 (0.35)	1.14 (0.28)	1.31 (0.36)
Low HDL-cholesterol(%) [a]	47.8 (902)	29.2 (136)	53.9 (766)
Triglyceride (mmol/L) [b,c]	1.32 (0.91-1.98)	1.72 (1.15-2.40)	1.22 (0.85-1.80)
Hypertriglyceridemia [a,b]	33.8 (637)	50.8 (236)	28.2 (401)
Systolic blood pressure (mm Hg)	126.9 (18.5)	127.1 (14.8)	126.8 (19.5)
Diastolic blood pressure (mm Hg)	78.2 (10.4)	80.3 (10.1)	77.5 (10.4)
Hypertension(%) [a]	45.6 (860)	47.1 (219)	45.1 (641)
Blood pressure lowering medication (%)	10.7 (202)	5.6 (26)	12.4 (176)
High sensitive CRP (mmol/L)	1.41 (0.62-3.39)	1.20 (0.53-2.82)	1.49 (0.66-3.70)
MetS-score (number of features)	1.8 (1.4)	1.7 (1.3)	1.9 (1.4)
MetS prevalence(%) [a]	30.3 (572)	25.2 (117)	32.0 (455)

Data are presented as means (standard deviation), median with inter-quartile range or % (*n*); MetS, metabolic syndrome

[a] Abdominal obesity, low HDL, hypertension, hypertriglyceridemia and MetS are defined according to the criteria of AHA-NHLBI (2005). Hyperglycemia is defined according to the criteria of the American Diabetes Association (2010). Abdominal obesity: ♂ ≥102 cm; ♀≥ 88 cm; Low HDL: ♂ <1.0; ♀<1.3 mmol/L; Hypertriglyceridemia: ≥1.7 mmol/L ;Hypertension: ≥130/85 mm Hg or hypertensive medicine; Hyperglycemia: HbA1C in National GlycohemoglobinStandarization Program (NGSP) units ≥ 5.7% or glucose lowering medication; MetS is defined as having at least 3 MetS features

[b] Non-fasting values

[c] No information on lipid lowering medication is available

Table 3 | P-values for Goeman's global test, testing the association of inflammation, waist circumference, insulin resistance and lipid SNPs with on MetS and MetS-score

Group of SNPs	MetS (*n*=1886)	MetS score (*n*=1886)
Inflammation	P=0.37	P=0.15
Waist circumference [a]	P=0.03	P=0.08
Insulinresistance	P=0.06	P=0.003
Triglycerides + HDL cholesterol	P=0.72	P=0.73
Triglycerides	P=0.45	P=0.62
HDLcholesterol	P=0.97	P=0.87

All analyses are adjusted for age, sex and cohort

[a] Data available in 853 women

rs2943634 was also associated with HbA1C (per allele difference -0.034, 95%CI -0.070; 0.002), triglycerides (per allele difference -0.051, 95%CI -0.085; -0.017) and HDL cholesterol (per allele difference 0.029, 95%CI 0.008; 0.052).

The group of SNP's, known for their association with waist circumference, was statistical significantly associated with MetS (P=0.03) and tended to be associated with MetS-score (P=0.08) (table 3). The association with MetS and the suggested associa-

Table 4 | P-values for Goeman's global test, testing the association of waist circumference and insulin resistance SNPs with metabolic syndrome and related features

Group of SNPs	MetS	MetS-score	WC (cm)	HbA1C (%)	Log (TG) (mmol/L)
n	*1886*	*1886*	*1886*	*1864* [a]	*1886*
WC [d]	P=0.03	P=0.08	P=0.01	P=0.73	P=0.81
Adj WC	P=0.16	P=0.80	-	P=0.47	P=0.55
IR	P=0.06	P=0.003	P=0.45	P=0.04	P=0.0003
Adj HbA1C	P=0.12	P=0.01	P=0.70	-	P=0.0005

All analyses are adjusted for age, sex and cohort; Mets, Metabolic syndrome; WC, waist circumference; TG, triglycerides; HDL, HDL-cholesterol; HbA1C, haemoglobin A1c; SBP systolic blood pressure; DBP diastolic blood pressure; Adj, adjusted; IR, insulin resistance

[a] Subjects which are using glucose lowering medication are excluded

[b] Subjects with CRP > 10 mmol/L are excluded

[c] Subjects with blood pressure lowering medication are excluded

[d] Data available in 853 women

Table 5 | Individual SNPs associated with waist circumference or insulin resistance in GWAS in relation to MetS and MetS-score

		MetS (*n*=1886)	MetS-score (*n*=1886)
Waist circumference			
FTO	rs1421085	1.02 (0.93; 1.12)	0.05 (-0.03; 0.14)
MC4R	rs17782313 [a]	1.16 (1.02; 1.32)	0.10 (-0.05; 0.24)
Insulin resistance			
PPARG	rs1801282	1.04 (0.91; 1.19)	0.10 (-0.02; 023)
IRS1	rs2943634	0.88 (0.79; 0.97)	-0.14 (-0.23;-0.06)
GCKR	rs780094	0.99 (0.89; 1.09)	0.05 (-0.04; 0.13)
IGF1	rs35767	1.03 (0.91; 1.16)	0.03 (-0.08; 0.14)
GCK	rs1799884	1.07 (0.95; 1.20)	0.08 (-0.03; 0.22)

Data are presented as PR per minor allele for MetS and as minor allele change for MetS Score; MetS, Metabolic syndrome;

All analyses are adjusted for age, sex and cohort

[a] Data available in 853 women

tion with MetS-score disappeared after adjustment for waist circumference (table 4). Furthermore, no association was found with any individual MetS feature except for waist circumference (table 4). Of the 2 abdominal obesity SNP's only *MC4R* rs17782313 was individually associated with MetS (table 5). This association remained after adjustment for waist circumference.

MC4R rs17782313 was not associated with any individual MetS feature, including waist circumference itself (data not shown).

The groups of SNP's linked in GWAS with inflammation, triglycerides or HDL cholesterol were neither associated with MetS nor with MetS-score (table 3). Therefore no further data-analyses were done for these groups of SNP's.

HDL (mmol/L)	SBP (mm HG)	DBP (mm HG)	Log (CRP) (mmol/L)
1886	*1684*[c]	*1684*[c]	*1683*[b]
P=0.36	P=0.29	P=0.11	P=0.22
P=0.09	P=0.36	P=0.34	P=0.68
P=0.0005	P=0.07	P=0.16	P=0.16
P=0.0008	P=0.10	P=0.22	P=0.17

Discussion

In this population based study of 465 men and 1421 women, we studied the relation between MetS and groups of SNP's associated in GWAS with waist circumference, insulin resistance, inflammation, triglycerides or HDL cholesterol. Only the group of waist circumference SNP's and the group of insulin resistance SNP's were associated with MetS or MetS-score.

In our study the group of SNP's which were associated with waist circumference in GWAS (*MC4R* rs17782313 and *FTO* rs1421085) was associated with waist circumference, as well as with MetS. However, the association with MetS disappeared after adjustment for waist circumference. This result is in line with the theory that visceral obesity induces the development of MetS and related features, such as insulin resistance, dyslipidemia and hypertension [1, 3, 14]. The association with MetS was mainly driven by *MC4R* rs17782313. In the KORA study among 7888 adults, an association between *MC4R* rs2229616 (r^2=1 with rs17782313) and MetS was found [15], supporting our findings. Unfortunately, in our study, data on MC4R rs17782313 were available for women only. However, as in the KORA study [15] the association between *MC4R* rs2229616 and MetS was not dependent on sex, we expect that this did not influence our findings. Although we found an association between *MC4R* rs17782313 and MetS, we found no association between *MC4R* rs17782313 and any individual MetS feature, including waist circumference. Furthermore, the association between *MC4R* rs17782313 and MetS, did remain after adjustment for waist circumference. This suggests that the association between *MC4R* rs17782313 and MetS, is at least in part, independent of body weight. Both human and animal studies found that *MC4R* rs17782313 has an effect on insulin resistance, independent of body weight [16, 17]. Therefore, the association between *MC4R* rs17782313 and MetS is probably partly mediated by insulin resistance. Contrary to a meta-analysis among 12555 Europeans, in which *FTO* rs9939609 (r^2=1 with rs1421085) was significantly associated with MetS (OR 1.17 ; 95%CI 1.10- 1.25) [18], we did not observe an association between *FTO* rs1421085 and MetS. This discrepancy may be explained by the weak association between *FTO* rs1421085 and waist circumference in our study. In our study the regression coefficient between *FTO* rs1421085 and waist

circumference was 0.03 per SD, whereas in other studies it ranged from 0.07 per SD to 0.14 per SD [18].

We found an association between insulin resistance SNP's and MetS and MetS-score that remained after adjustment for HbA1C. This suggests that this association is not mediated by HbA1C. However, as HbA1C is not an optimal marker of insulin resistance (r^2 between HOMA-IR – HbA1C ≈0.50 [19]), we do not exclude the possibility that insulin resistance mediates the association between insulin resistance SNP's and MetS. Out of the group of five insulin resistance SNP's, *IRS1* rs2943634 was the only SNP significantly associated with MetS and MetS score. It was also associated with HbA1C, triglycerides and HDL cholesterol. Associations of *IRS1* rs2943634 with glucose related [20] and lipid traits [21] have been observed by others. In contrast, in a study among 1126 non-Hispanic whites, 898 non-Hispanic blacks and 906 Mexican Americans, the IRS1 rs7578326 (r^2 with rs2943634=0.82) was not associated with MetS, neither in the overall population, nor in specific ethnic groups [22]. However, as the number of Caucasian participants and MetS prevalence were lower in the previous than in our study, the power to detect an association with MetS in Caucasian was also lower in the previous than in our study. *IRS1* rs2943634 was not associated with waist circumference, both in our study and in a much larger study among 39576 individuals [21]. In accordance with the human data described above, an IRS1 knock-out mouse model displayed a non-obese MetS like phenotype with insulin resistance, increased blood pressure, increased triglycerides, decreased HDL cholesterol and decreased LPL activity [23]. Besides *IRS1* rs2943634 the group of insulin resistance SNP's consisted of *PPARG* rs1801282, *GCKR* rs780094, *GCK* rs1799884, and *IGF1* rs35767. In line with other studies, none of these SNP's were associated with MetS in our data [4, 22, 24]. This may in part be explained by the relatively relatively weak effect of *PPARG* rs1801282, *GCK* rs1799884, and *IGF1* rs35767 on HOMA-IR compared to *IRS1* rs2943634 [20, 25]. Furthermore, pleiotropic effects may explain the lack of an association of *PPARG* rs1801282 and *GCKR* rs780094 with MetS. The *12Pro* allele of *PPARG* rs1801282 has opposite effects on insulin resistance and BMI in Caucasian subjects [26], whereas *GCKR* rs780094 has opposite effects on insulin resistance and lipid levels [27]. These opposite effects may result in a zero association with MetS. In summary, it seems that the association between the groups of insulin resistance SNP's and MetS, was mainly driven by *IRS1* rs2943634.

We did not observe an association between groups of SNP's known for their association with triglycerides or HDL cholesterol and MetS. On the contrary, in a GWAS [5] and a systematic review of genetic association studies[4], the majority of SNP's associated with MetS was involved in lipid metabolism. Possibly, lack of an association between lipid SNP's and MetS in our study can be explained by the weak association of lipid SNP's with lipid levels in EPIC-NL. Subgroup analyses revealed that the weak associations we observed between lipid SNP's and lipid levels could not be explained by medication use, sex or a difference between the MORGEN and Prospect study. Furthermore, it is unlikely that the non-fasting state of our samples gives an explanation, as in a GWAS, the association with lipid levels was independent of the fasting state for most SNP's [28].

We found no significant association between a group of inflammation SNP's and MetS. This zero association is in line with a study among 4286 British women, in which a *CRP* haplotype was not associated with the individual features of MetS [29]. Furthermore, Rafiq *et al.* could not detect an association between type 2 diabetes, an endpoint of MetS and 8 SNP's known to alter circulating levels of inflammatory proteins, which were located in the *IL-18, IL1RN, IL6R, MIF, PAI1* and *CRP* genes [30]. Overall, this evi-

dence may suggest that inflammation does not play a causal role in MetS development. However, for several reasons it can not be ruled out that SNP's in inflammatory pathways exist that are causally related to MetS. First, for not all inflammatory proteins a SNP-MetS association has been investigated. Second, as the global test gives a combined result for all SNP's, the global test may be not significant, despite the presence of an association between one of the single SNP's and MetS.

In this study we have explored the biomarkers involved in MetS development, by studying SNP's related to these biomarkers. Advantage of this approach is that, according to the principles of Mendelian randomization the associations we investigated are neither affected by reverse causality nor by socioeconomic and behavioural confounders [31]. Furthermore, as all participants were Caucasian, it is unlikely that our study results have been affected by population stratification. Sixteen SNP's which were associated in GWAS with waist circumference, inflammatory biomarkers and lipid levels were not on the IBC CVD array (appendix I). Inclusion of the three waist circumference SNP's, which were not on the array, might have increased the possibility to find associations with several MetS features. As the global test of inflammation SNP's on hsCRP was already highly significant ($P=7.3*10^{-6}$), inclusion of additional SNP's, which were absent on the IBC CVD array, would not have changed our results for the global test, but may have revealed additional individual SNP's. The IBC CVD array covered all lipid genes detected in GWAS, but not for every gene each SNP was available. However, the total number of lipid SNP's in our study was relatively large and relatively few lipid SNP's were missing. Therefore we believe that inclusion of additional lipid SNP's, would not have changed our results considerably on the group level. The IBC CVD array covered all insulin resistance SNP's discovered in GWAS. However, up till now for only three SNP's a genome wide association with HOMA-IR has been found and replicated. To increase power we also included those SNP's associated with glucose related traits in GWAS, which were also associated with HOMA-IR ($P\leq0.05$). However, as the association between the group of insulin resistance SNP's and HbA1C was just significant, the power to detect associations with MetS and its features was still low.

In conclusion, we found that SNP's associated with waist circumference or insulin resistance in GWAS were also associated with MetS. These results are in line with the hypotheses that weight regulation and insulin metabolism are causative factors for MetS.

Individual SNP's for which we found an association with MetS were *MC4R* rs17782312 which is involved in weight regulation and *IRS1* rs2943634 which is involved in insulin resistance.

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Conflict of Interest

None declared

Appendix I

Loci related to waist circumference, insulin resistance, inflammatory biomarkers, triglycerides and HDL cholesterol in genome wide association studies till 01-01-2011 which are not on the IBC CVD array

Waist circumference

NRXN3	– rs10146997 [45]
TFAP2B	– rs987237 [32]
MSRA	– rs7826222 [32]

Insulin resistance

Inflammatory biomarkers

HNFIA	– *rs1183910* [36]
ARL15	– rs43111394 [34]
APOE, APOCI, APOCII	– rs4420638 [46]
CDHI3	– rs3865188 [47]

Triglycerides

LPL	– rs326 [48]
APOAI	– rs2075292 [48]
APOAI, APOC3, APOA4, APOA5	– rs10892151 [49]
APOA1, APOC3, APOA4, APOA5	– rs4938303[37]
CLIP2	– rs7557067 [42]

HDL cholesterol

CETP	– rs9989419 [37]
LIPC	– rs10468017 [42]
CLIP2	– rs2304130 [37]
MAB, MVK	– rs9943753 [37]

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Genetic variants in lipid metabolism are independently associated with multiple features of the metabolic syndrome

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Abstract

Background

Our objective was to find single nucleotide polymorphisms (SNP's), within transcriptional pathways of glucose and lipid metabolism, which are related to multiple features of the metabolic syndrome (MetS).

Methods

373 SNP's were measured in 3575 subjects of the Doetinchem cohort. Prevalence of MetS features, i.e. hyperglycemia, abdominal obesity, decreased HDL cholesterol levels and hypertension, were measured twice in 6 years. Associations between the SNP's and the individual MetS features were analyzed by log-linear models. For SNP's related to multiple MetS features (P<0.01), we investigated whether these associations were independent of each other.

Results

Two SNP's, *CETP Ile405Val* and *APOE Cys112Arg*, were associated with both the prevalence of low HDL cholesterol level (*Ile405Val* P=<.0001; *Cys112Arg* P= 0.001) and with the prevalence of abdominal obesity (*Ile405Val* P=0.007; *Cys112Arg* P=0.007). For both SNP's, the association with HDL cholesterol was partly independent of the association with abdominal obesity and vice versa.

Conclusion

Two SNP's, mainly known for their role in lipid metabolism, were associated with two MetS features i.e., low HDL cholesterol concentration, as well as, independent of this association, abdominal obesity. These SNP's may help to explain why low HDL cholesterol levels and abdominal obesity frequently co-occur.

Introduction

The metabolic syndrome (MetS) is a common multi-component condition including abdominal obesity, dyslipidemia, hypertension, and hyperglycemia. It is associated with an increased risk of cardiovascular disease and type 2 diabetes [1]. A central question in understanding the MetS is why these traits cluster together [1]. The clustering may be explained by a complex physiological cascade of events, in which the occurrence of one trait initiates the occurrence of a second. Alternatively, a causative factor common to several metabolic traits may explain the clustering. This factor could be either of genetic or environmental nature [2].

Family and twin studies indicate that the different features of the MetS share a common genetic component [2-5]. Twin studies show that the correlation between the features of the metabolic syndrome is higher in monozygotic compared to dizygotic twins [2, 6, 7]. Family studies also show significant genetic correlations between the different features of the metabolic syndrome [4]. Heritability estimates of the MetS itself range from 13-27% [3-5]. However, despite the evidence from these heritability studies, only a few single nucleotide polymorphisms (SNP's) have been linked to multiple features of the MetS[1].

Disturbances in lipid and glucose metabolism may lead to the development of one or more MetS features [8]. Therefore, genes involved in these pathways are potentially pleiotropic for multiple MetS features. In a population based cohort study, we studied 373 SNP's mainly selected from transcriptional pathways of glucose and lipid metabolism, and their association with multiple features of the MetS.

Methods

Study population

The Doetinchem Study is a population-based cohort study on lifestyle, biological risk factors and chronic diseases [9]. Between 1987 and 1991, 12404 subjects, aged 20-59, all inhabitants of Doetinchem, a town in a rural area in east of the Netherlands, were enrolled in the baseline cohort. A random sub-sample of this cohort (63%) was invited for a second measurement round (1993-1997; response 79%) and for a third measurement round (1998-2002; response 75%). Overall, the Doetinchem Cohort comprises 4662 persons with repeated measurements.

Pregnancy and alteration in smoking behavior are factors that influence body weight and therewith the MetS. Therefore, subjects of the Doetinchem Cohort who changed their smoking habits (n=750), who had missing data on smoking status (n=11) or who where pregnant at the time of measurement (n=122) were excluded from the current study. This resulted in a final study population of 3779 subjects. The second and third measurement rounds included glucose and waist circumference measurements and were used for the present study. All participants gave written informed consent and approval was obtained from local Medical–Ethical Committees.

Measurements

During each measurement round, a questionnaire on lifestyle factors was administered and anthropometric and biochemical variables were measured. For a more detailed description see [10].

During the second and third visit waist circumference was measured according to written instructions based on WHO criteria for waist measurement (1989). Waist circumference was determined to the nearest 0.5 cm, at midway between the lowest rib and the iliac crest, with subjects in standing position and after breathing out gently. Waist circumference was measured in duplicate and the mean of the two measurements was taken. Blood pressure (BP) was measured in each round, with the subject in sitting position using a random-zero sphygmomanometer. Systolic pressure was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic blood pressure was recorded at the disappearance of sounds (fifth-phase Korotkoff). BP measurement was repeated and values were averaged. During the physical examination, regular audits were performed to check adherence to the BP measuring protocol (e.g. resting time, adequate cuff size).

Non-fasting blood samples were taken by venapuncture for all subjects. Blood samples were fractionated into serum, buffy coat and erythrocytes and subsequently stored at -30°C until further use. Plasma glucose levels were measured as described by Tietz [11]. HDL cholesterol was measured in EDTA-plasma until 1998, and from 1998 onwards in serum, at the Lipid Reference Laboratory (LRL) of the university Hospital Dijkzigt in Rotterdam, using standardized enzymatic methods. Performance for enzymatic HDL cholesterol measurements fulfilled National Cholesterol Education Program (NCEP) recommendations throughout the entire study period.

Genomic DNA was extracted from the buffy coat fraction with a salting out method. A total of 139 subjects were not eligible for genotyping, mainly because of failure to extract DNA or unavailability of buffy coats. For 3640 subjects, 401 SNP across 270 candidate genes were genotyped. A set of 383 SNP's across 253 candidate genes, passed the Illumina design tool and were genotyped with the Illumina Golden Gate assay using the Sentrix Array Matrix platform (Illumina Inc, San Diego, California) [11]. 18 Additional SNP's were genotyped by KBioscience (Hoddesdon, Hertfordshire, UK) using the KASPar chemistry, which is a competitive allele specific PCR SNP genotyping system using FRET quencher cassette oligonucleotides http://www.kbioscience.co.uk). Two SNP's (rs7412 and rs429358 in *APOE*) that failed in the Illumina Golden Gate assay were successfully re-genotyped with Taqman assay.

A detailed description of the SNP selection procedure and a full SNP list have been published elsewhere [10]. In short, 270 candidate genes were selected by a pathway-driven approach, with emphasis on regulatory pathways that control fatty acid, glucose, cholesterol and bile salt homeostasis [10]. The selection procedure started from the master regulator genes encoding nuclear receptors (PPAR's, LXR, NRIH4) and transcription factors (SREBP's) and continued by selecting their co-activators, co-repressors and target genes. In addition, hormonal receptors (insulin receptor), their down-stream signaling proteins and genes involved in β-signaling were selected. For each gene out of these pathways, 1-7 SNP's most likely to carry functional properties were selected. For 26 SNP's genotyping was unsuccessful. In addition, 33 SNP's were not in Hardy Weinberg Equilibrium (HWE). Verification was carried out in a random sample (n=96) for the eight SNP's (24%) that deviated most strongly from HWE. All yielded the same results, except for 2 SNP's, which were therefore excluded [10]. After the exclusion of subjects with genotype failure or discordance on gender control (n=65), 3575 subjects were available for data analyses. Finally, data on 373 SNP's in 254 genes were available for 3575 subjects.

Statistical analyses

Abdominal obesity, low HDL cholesterol levels, hyperglycemia and hypertension were defined according to the criteria of the AHA/NHLBI (2005)[12].

All analyses were performed with SAS version 9.1 (SAS Institute, INC., Cary, North Carolina). Distributions of genotypes were tested for deviation from HWE by chi-square analyses (PROC ALLELE). Associations with individual MetS features and co-occurrence of MetS features were tested. To optimize precision, subjects who changed phenotype between the two rounds were excluded. This means that subjects being e.g. hypertensive in one round and normotensive in the other round or vice versa were excluded. All analyses were adjusted for age and sex.

In a first series of analyses, the association between individual MetS features and each SNP was analyzed by log-linear models. The prevalence ratios of change per allele were calculated with an additive genetic model. To avoid chance findings we only followed up those SNP's which were related to multiple MetS features with P≤0.01. We determined the expected number of SNP's related to 2 or more MetS features with P<0.01 by chance alone and under the assumption of independent random outcomes using the following formula: Chance (P≤0.01 for SNP_1 - MetS feature_1 association) * Chance (P≤0.01 for the SNP_2 - MetS feature_2 association) * Chance (association 1 and 2 in the same direction) * number of MetS feature combinations * number of SNP's. The expected number appeared to be 0.12 (0.01 * 0.01 * 0.5 * 6 * 373). Subsequently we tested whether the number of observed SNP's associated with 2 or more MetS features differed significantly from the expected 0.12 SNP's.

In our study abdominal obesity and decreased HDL cholesterol appeared to be the MetS features both associated with the same SNP's. In a second series of analyses, it was

Table 1 | Characteristics of 3575 subjects of the Doetinchem Cohort in round 2 and 3

	Round 2 1993-1997	Round 3 1998-2002
Age (yr)	46.5 (9.7)	51.5 (9.7)
Sex (% men)	47.8	47.8
Waist circumference (cm)	90.2 (11.1)	92.9 (11.4)
Increased waist circumference (%) [a]	31.3	40.3
Glucose levels (mmol/L) [b]	5.3 (1.3)	5.4 (1.5)
Diabetic medication (%)	0.8	2.3
Hyperglycemia (%) [a]	28.8	33.6
HDL-cholesterol (mmol/L)	1.38 (0.38)	1.37 (0.39)
Low HDL-cholesterol (%) [a]	25.4	29.0
Diastolic Blood Pressure (mm Hg)	79.9 (10.6)	81.4 (10.7)
Systolic Blood Pressure (mm Hg)	125.1 (16.4)	129.3 (18.01)
Hypertension (%) [a]	50.8	58.5
Blood Pressure lowering medication (%)	6.5	11.0
MetS-score (number of features)	1.34 (1.1)	1.61 (1.1)
Metabolic syndrome prevalence (%)	14.9	22.7

Data are presented as means (standard deviation) or %

[a] Abdominal obesity, hyperglycemia, low HDL, hypertension and MetS are defined according to the criteria of AHA-NHLBI (2005). Abdominal obesity: ♂ ≥ 102 cm; ♀ ≥ 88 cm; Low HDL: ♂ < 1.0; ♀ < 1.3 mmol/L; hypertension: ≥130/85 mm Hg or hypertensive medicine; Hyperglycemia ≥ 5.6 (mmol/L) or glucose lowering medication; MetS is defined as having 3 MetS features measured in Doetinchem Cohort

[b] Non-fasting values

tested if the association between these SNP's and HDL cholesterol was independent of the association with abdominal obesity, and vice versa. This was done both by adjustment and by stratification. The HDL cholesterol analyses were adjusted for abdominal obesity and vice versa. For stratified analyses, the association with abdominal obesity was analyzed in subjects with high HDL cholesterol levels. Low HDL cholesterol was analyzed in subjects without abdominal obesity.

Results

Baseline characteristics among the 3575 subjects of the Doetinchem cohort are presented in table 1. Hypertension was the most prevalent MetS feature (41.6% of the subjects were stable hypertensive and 32.3% of the subjects were stable normotensive). The least prevalent MetS feature was low HDL cholesterol (18.7% were stable for low HDL and 64.1% were stable for high HDL). The most frequent combination of co-occurring MetS features was hypertension and abdominal obesity (14.5% were stable positive,

Table 2 | SNP's associated (P<0.01) with stable MetS features among subjects of the Doetinchem Cohort over 2 surveys (1993-1997; 1998-2002)

SNP	MAF	Gene	PR / allele [a] (95%CI)	P-Value
Hyperglycemia (n=2280)				
rs1137101	0.46	*LEPR*	0.84 (0.76; 0.93)	0.001
rs3842748	0.21	*INS-IGF2*	1.20 (1.07; 1.35)	0.002
rs6795441	0.45	*RAF1*	0.86 (0.77; 0.95)	0.003
rs7903146	0.29	*TCF7L2*	1.17 (1.05; 1.30)	0.005
rs1143634	0.24	*IL1B*	1.17 (1.05; 1.31)	0.005
Abdominal obesity (n=2931)				
rs35724	0.38	*NR1H4*	0.91 (0.85; 0.97)	0.005
rs10860603	0.14	*NR1H4*	0.86 (0.78; 0.96)	0.006
rs1800796	0.04	*IL6*	0.77 (0.64;0.93)	0.007
rs5882	0.31	*CETP*	0.90 (0.83;0.97)	0.007
rs429358	0.16	*APOE*	1.12 (1.03;1.23)	0.007
Hypertension (n=2643)				
rs130005	0.10	*CREBBP*	0.89 (0.82; 0.97)	0.006
rs3759324	0.25	*SCCN1A*	1.07 (1.02; 1.12)	0.009
Low HDL-Cholesterol (n=2959)				
rs1800777	0.03	*CETP*	1.60 (1.56;2.32)	3.3^{E-12}
rs3208305	0.30	*LPL*	0.70 (0.63;0.79)	9.3^{E-10}
rs328	0.11	*LPL*	0.60 (0.49; 0.72)	1.2^{E-7}
rs5882	0.31	*CETP*	0.76 (0.69;0.86)	2.1^{E-6}
rs429358	0.16	*APOE*	1.21 (1.07;1.37)	0.001
rs174546	0.33	*FADS1*	1.18 (1.07;1.30)	0.001
rs780094	0.36	*GCKR*	1.17 (1.06; 1.29)	0.002
rs268	0.02	*LPL*	1.45 (1.12; 1.86)	0.004
rs5275	0.31	*PTGS2*	1.15 (1.04; 1.27)	0.006

MAF = Minor allele frequency; PR = Prevalence Ratio;

[a] Prevalence ratios are expressed per minor allele assuming an additive genetic model

21.2% were stable negative). The least frequent combination was decreased HDL cholesterol levels and hyperglycemia (3.5% were stable positive, 32.6 % were stable negative). 19 SNP's were related to at least one of the stable MetS features with P<0.01 (table 2). Two of them, *Ile405Val (rs5882)* in the *Cholesteryl Ester Transfer Protein (CETP)* gene and *Cys112Arg (rs429358)* in the *Apolipoprotein E (APOE)* gene were related to 2 MetS features each with P<0.01. This number differs significantly from the expected 0.12 SNP's to be associated with two features or more by chance alone (p<0.005 chi-square with Yates correction). Both SNP's were in HWE (*Ile405Val* P=0.21; *Cys112Arg* P=0.48). The minor *Val* allele of *Ile405Val* in the *CETP* gene was associated with both a decreased prevalence of low HDL cholesterol levels (PR/allele 0.76, 95%CI 0.69; 0.86) and a decreased prevalence of abdominal obesity (PR/allele 0.90, 95%CI 0.83; 0.97) (table 3). The minor *Arg* allele of the *Cys112Arg* in the *APOE* gene was associated with an increased prevalence of low HDL cholesterol levels (PR/allele 1.21, 95% CI 1.07; 1.37) and an increased prevalence of abdominal obesity (PR/allele 1.12, 95% CI 1.03; 1.23) (table 4). Results for both SNP's remained significant after adjusting the abdominal obesity analyses for HDL cholesterol and vice versa. Further analyses showed that both SNP's were associated with the simultaneous occurrence of abdominal obesity and low HDL cholesterol levels, with decreased HDL cholesterol levels in a subgroup of people without abdominal obesity,

Table 3 | Association of *Ile405Val* (rs5882) in the *CETP* gene with abdominal obesity and low HDL-cholesterol levels

Outcome [a]	***Ile / Ile***	***Ile / Val***	***Val / Val***	**PR / allele** [b] **(95%CI)**	**P-trend**
Prevalence of low HDL [c]					
Overall (*n*=669, total *n*=2959)	26.0%	20.6%	14.4%	0.76 (0.69;0.86)	<.0001
Adjusted for abdominal obesity (*n*=669, total *n*=2959)	24.7%	21.3%	16.0%	0.83 (0.74;0.93)	0.002
Among subjects without abdominal obesity (*n*=252, total *n*=1684)	18.4%	13.0%	7.8%	0.68 (0.56;0.82)	<0.001
Prevalence of abdominal obesity [d]					
Overall (*n*=958, total *n*=2931)	34.7%	31.9%	26.4%	0.90 (0.83;0.97)	0.0072
Adjusted for low HDL (*n*=958, total *n*=2931)	33.1%	30.4%	27.0%	0.92 (0.83;1)	0.05
Among subjects with high HDL levels (*n*=470, total *n*=1902)	27.9%	22.8%	18.7%	0.82 (0.73;0.93)	0.0014
Prevalence of both low HDL and abdominal obesity [e]					
Overall (*n*=298, total *n*=1730)	19.5%	16.3%	11.2%	0.81 (0.69;0.94)	0.0076

PR = Prevalence Ratio

[a] All analyses are adjusted for age and sex

[b] Prevalence ratios are expressed per minor VAL allele assuming an additive genetic model

[c] Subjects with low HDL-cholesterol in round 2, but not in round 3 or vice versa, were excluded

[d] Subjects with abdominal obesity in round 2, but not round 3 or vice versa, were excluded

[e] Only subjects with either both abdominal obesity and low HDL-cholesterol levels in round 2 and 3 or with both no abdominal obesity and high HDL-cholesterol levels were included. Subjects without abdominal obesity and with high HDL-cholesterol levels in round 2 and 3 were used as the reference category

Table 4 | Association of *Cys112Arg* (rs429358) in the *APOE* gene with abdominal obesity and low HDL-cholesterol levels

Outcome [a]	*Cys/Cys*	*Cys/Arg*	*Arg/Arg*	PR / allele [b] (95%CI)	P-trend
Prevalence of low HDL [c]					
Overall (*n*=669, total *n*=2959)	21.2%	25.5%	32.3%	1.21 (1.07;1.37)	0.0013
Adjusted for abdominal obesity (*n*=669, total *n*=2959)	21.0%	25.5%	30.2%	1.20 (1.06;1.36)	0.005
Among subjects without abdominal obesity (*n*=252, total *n*=1684)	13.5%	18.0%	26.0%	1.35 (1.11; 1.65)	0.0031
Prevalence of abdominal obesity [d]					
Overall (*n*=958, total *n*=2931)	31.2%	35.8%	39.6%	1.12 (1.03;1.23)	0.0074
Adjusted for low HDL (*n*=958, total *n*=2931)	30.1%	33.1%	38.9%	1.12 (1.0l;1.25)	0.04
Among subjects with high HDL levels (*n*=470, total *n*=1902)	23.5%	27.3%	34.5%	1.16 (1.01;1.33)	0.03
Prevalence of both low HDL and abdominal obesity [e]					
Overall (*n*=298, total *n*=1730)	15.7%	20.7%	27.8%	1.29 (1.08;1.54)	0.0045

PR = Prevalence Ratio

[a] All analyses are adjusted for age and sex

[b] Prevalence ratios are expressed per minor *ARG* allele assuming an additive genetic model

[c] Subjects with low HDL-cholesterol in round 2, but not in round 3 or vice versa, were excluded

[d] Subjects with abdominal obesity in round 2, but not round 3 or vice versa, were excluded

[e] Only subjects with either both abdominal obesity and low HDL-cholesterol levels in round 2 and 3 or with both no abdominal obesity and high HDL-cholesterol levels were included. Subjects without abdominal obesity and with high HDL-cholesterol levels in round 2 and 3 were used as the reference category

and with abdominal obesity in a subgroup of people with normal HDL cholesterol levels (table 3; table 4).

The *Cys112Arg* genotype of the *APOE* gene is part of the *ε2, ε3, ε4* haplotype. Results of the *ε2ε3ε4* haplotype analyses were similar to the results of the *Cys112Arg* analyses. Compared to the *ε3/ε3* isoform, the ε4/- isoforms (*ε3/ε4* and *ε4/ε4*) were associated with an increased prevalence of low HDL cholesterol levels (PR 1.24, 95%CI 1.07; 1.44) and an increased prevalence of abdominal obesity (PR 1.13, 95%CI 1.01; 1.26). No associations were found with the *ε2/-* isoforms (*ε3/ε2* and *ε2/ε2*).

Discussion

In this explorative study of 373 SNP's, mainly located in pathways related to lipid and glucose metabolism, we found a significant association between the *Ile405Val* genotype in the *CETP* gene and the *Cys112Arg* genotype in the *APOE* gene, with multiple features of the metabolic syndrome, i.e. the prevalence of abdominal obesity and prevalence of low HDL cholesterol. For both SNP's, the association with abdominal obesity was partly independent of the association with HDL cholesterol, and vice versa. No, association was found between SNP's in genes involved in glucose metabolism or blood pressure regulation and multiple MetS features.

In humans, CETP and ApoE are expressed in the liver and in peripheral tissues, such as adipose tissue [13, 14]. Both genes are involved in plasma lipid homeostasis. CETP stimulates the clearance of HDL cholesterol from plasma [14]. Furthermore, CETP increases the formation of small dense LDL particles and triglycerides [15]. ApoE removes atherogenic lipoproteins, such as VLDL, from the circulation [16]. This results in lower cholesterol and triglyceride levels. Besides having a role in lipid homeostasis, a few studies indicate that CETP and ApoE may be involved in other metabolic processes such as weight regulation. For example, APOE plays a role in the deposition of dietary fat in adipose tissue [17]. As CETP is synthesized in the adipose tissue, CETP may affect adipose tissue characteristics [18].

The *Ile405Val* polymorphism in the CETP gene induces a change in amino acid sequence. Therefore it is likely to be a functional SNP. In our study, the Val allele of the *Ile405Val* genotype was associated with a lower prevalence of abdominal obesity and a lower prevalence of low HDL cholesterol levels. The stratified and adjusted analyses in our study suggested that the association with prevalence of abdominal obesity and prevalence of low HDL cholesterol levels was partly independent of each other. This suggests that CETP regulates weight and HDL cholesterol via independent pathways.

In line with our results, a meta-analysis of 29 studies, showed that *Val* allele carriers had higher HDL levels [19]. Furthermore, a Chinese case-control study in 934 obesity cases and 924 controls showed a decreased obesity risk for *Val/Val* homozygotes, which persisted after adjustment for HDL cholesterol levels [20]. In previous studies, the *405Val* allele has been associated with lower CETP mass and lower CETP activity [19]. Lower CETP plasma levels are correlated with a lower obesity risk [21]. The *405Val* allele has also been associated with other positive health outcomes such as, increased HDL and LDL particle size [15], decreased coronary heart disease risk [22], and increased longevity [15], all of which are related to the MetS. In summary, cumulative evidence indicates that *Ile405Val* is involved in several metabolic processes, including lipid level control and weight regulation.

The *Cys112Arg* genotype of the APOE gene is a non-synonymous genotype. Together with *Arg158Cys* (rs7412), the *Cys112Arg* forms the *ε2ε3ε4* haplotype. The *ε2*, *ε3* and *ε4* ApoE isoforms differ markedly on the structural and functional level [16]. In our study the *Arg* allele of the *Cys112Arg* genotype was associated with an increased prevalence of low HDL cholesterol levels and an increased prevalence of abdominal obesity. Again the stratified and adjusted analyses suggested that the associations with the prevalence of abdominal obesity and prevalence of low HDL cholesterol levels were partly independent of each other. The *ε4* isoform showed a similar, though less pronounced, pattern of associations. No associations were observed with *Arg158Cys* or *ε2* isoform, of the *ε2ε3ε4* haplotype.

Previous studies generally focused on the *ε2*, *ε3* and *ε4* haplotype and did not take associations with the individual *Arg158Cys* and *Cys112Arg* into account. In line with our study, the ε4 isoform was associated with a more detrimental metabolic profile in most studies. A meta-analysis of 19 studies in 9751 subjects, showed that *ε3/ε4* carriers had lower HDL cholesterol levels than *ε3/ε3* carriers [23] . Most studies showed either a positive [24-27] or no [28-31] association between the ε4 isoform and body weight. However, some showed a negative association (31, 32). Arbones Mainar *et al.* [28] showed that compared to ApoE3 mice, ApoE4 mice fed a western diet were more prone to the development of several MetS features, such as increased insulin resistance, decreased fat tolerance and increased fat cell size. However, they gained less body weight. This suggests that the positive association between the *ε4* isoform and abdominal obesity

may be driven by the development of other MetS features, such as insulin resistance [28]. Furthermore, these results suggest that the *ε4* isoform may be associated with MetS. This has indeed been shown in other epidemiological studies [25, 26, 29].

Strength of our pathway driven candidate gene study was the relatively large sample size. Contrast and precision were increased by exclusively including people with consistent MetS phenotype, i.e. classified as healthy or not healthy for a particular metabolic phenotype over two measurement rounds. Furthermore we tried to keep the probability of chance findings low by including only those SNPS that were related to two or more MetS features with P<0.01 into the second round of data-analysis. We found 2 SNP's, which differed significantly from the expected 0.12 SNP's (p<0.005 chi-square with Yates correction). However, the 0.12 expected SNP's were obtained assuming independent random outcomes. As HDL cholesterol and abdominal obesity are not completely independent, this assumption is partly violated. However, the associations with abdominal obesity and HDL cholesterol remained significant in our stratified and adjusted analyses. A weakness of our study may be that blood samples were taken from non-fasting subjects. This may have randomly affected the glucose measurements. Another weakness is that triglycerides levels were not measured in our study. Therefore, we may have missed SNP's which were related to hypertriglyceridemia and one or two other MetS feature. For example, the *CETP Ile405Val* mutation has been associated with triglycerides in previous studies [19]. We therefore expect that in our study population this SNP will not only be associated with HDL cholesterol and abdominal obesity, but also with triglyceride levels.

In this explorative study of 373 SNP's among 3575 subjects, we emphasized on the intricate links between several MetS features. We have showed that two SNP's, mainly known for their role in lipid metabolism, influenced both abdominal obesity and low HDL cholesterol levels, partly independent of each phenotype. If the pleiotropic effects of these genes are further confirmed by others it might be possible to develop medication which increases HDL cholesterol leves and reduces waist circumference, and so affects the development of MetS

Conflict of interest

The authors declare no conflict of interest

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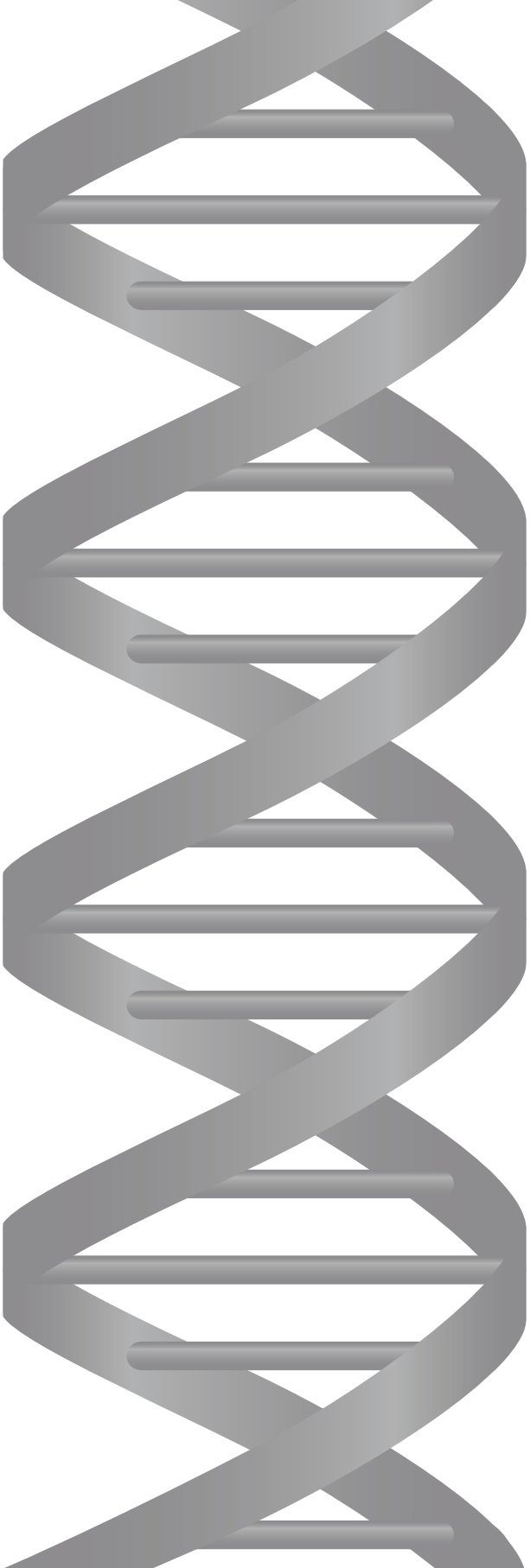

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Glucose levels and genetic variants across transcriptional pathways: interaction effects with BMI

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Abstract

Objective

Much of the genetic variation in glucose levels remains to be discovered. Especially, research on gene-environment interactions is scarce. Overweight is one of the main risk factors for hyperglycemia. As transcriptional regulation is important for both weight maintenance and glucose control, we analyzed 353 single nucleotide polymorphisms (SNP's), occurring in transcriptional pathways of glucose and lipid metabolism in interaction with body mass index (BMI) on glucose levels.

Research design and methods

SNP's were measured in 3244 participants of the Doetichem cohort. Non-fasting glucose levels and BMI were measured twice in 6 years. SNP x BMI interactions were analyzed by mixed models and adjusted for age, sex, time since last meal and follow-up time. False Discovery Rate (FDR) <0.2, was used to adjust for multiple testing.

Results

Two SNP's in the *PPARGC1A* gene, (rs8192678 FDR=0.07; rs3755863 FDR=0.17), showed a significant interaction with BMI. The rare allele of both SNP's was associated with significantly lower glucose levels in subjects with a BMI ≤ 25 kg/m^2 (rs8192678 P=0.02; rs3755863 P=0.03). An inverse association was suggested in subjects with a BMI > 28 kg/m^2. A small intervention study (*n*=120) showed similar, though non-significant, results.

Conclusions

Using a pathway-based approach we found that BMI significantly modified the association between two SNP's in the *PPARGC1A* gene and glucose levels. The association between glucose and *PPARGC1A* was only present in lean subjects. This suggests that the effect of the *PPARGC1A* gene, which is involved both in fatty acid oxidation and glucose metabolism, is modified by BMI.

Introduction

Elevated plasma glucose levels are a risk factor for type 2 diabetes mellitus. Even within a non-diabetic population there is a substantial variation in glucose levels [1]. This variation arises from both genetic and environmental factors. Identification of factors that influence glucose levels may improve our understanding of the pathogenesis of diabetes.

The heritability of glucose levels, as estimated by family studies, is approximately 30% [2-4]. However, as the established polymorphisms so far explain a limited amount of genetic variation in glucose levels [1], many more polymorphisms remain to be discovered. Most studies analyzed the individual association between polymorphisms and glucose levels without taking gene-environment interaction effects into account. Therefore, exploration of interaction effects may unravel new polymorphims. Furthermore, interaction effects may explain discrepancies between studies.

Overweight is one of the main risk factors for elevated plasma glucose levels and diabetes [5]. The association between plasma glucose levels or diabetes and several polymorphisms (e.g. in *PPARG*) is modulated by obesity status [6, 7]. As transcriptional regulation is an important factor for maintaining glucose and energy homeostasis, polymorphisms occurring in transcriptional pathways related to glucose and lipid metabolism may be involved both in weight regulation and development of hyperglycemia [8]. Surveying genetic variants across these pathways in interaction with body weight could provide new insights into genetic determinants of glucose levels.

In this study, we studied the interaction of 353 single nucleotide polymorphisms (SNP's), occurring in transcriptional pathways of glucose and lipid metabolism, with body mass index (BMI) in relation to repeated measures of glucose levels in a population based cohort study.

Research, Design and Methods

Study population

The Doetinchem Study is a population-based cohort study on lifestyle, biological risk factors and chronic diseases [9]. Between 1987 and 1991, 12404 subjects, aged 20-59, all inhabitants of Doetinchem, a town in a rural area in east of the Netherlands, were enrolled in the baseline cohort. A random sub-sample of this cohort (63%) was invited for a second measurement round between 1993 and 1997 (response 79%) and for a third measurement in the period 1998-2002 (response 75%). Overall, the Doetinchem cohort comprises 4662 persons with repeated measurements.

Pregnancy and alteration in smoking behavior are factors that influence body weight, whereas diabetic medication affects glucose levels. Therefore, participants of the Doetinchem Cohort who changed their smoking habits (n=750), who had missing data on smoking status (n=11), who where pregnant at the time of measurement (n=122) or who took diabetic medication (n=76) were excluded from the current study. The second and third measurement rounds included glucose measurements and were used for the present study. However, glucose levels were not measured in the beginning of round 2 from January till April 1993, and thus another 255 subjects were excluded. This resulted in a final study population of 3448 participants.

General questionnaire and anthropometric measurements

During each measurement round, anthropometric data and data on lifestyle factors were collected. Data on pregnancy , status (non-, ex-, current smoker) and physical activity were obtained by a standardized questionnaire [10]. The Cambridge Phyiscal Activity Index (CPAI), as described by Wareham *et al.*,[11] was calculated by combining occupational physical activity with time spent on cycling and sporting in summer and winter. Subjects were divided into four physical activity categories (inactive, moderately inactive, moderately active and active). No information on the CPAI was available for the beginning of round 2 (1993). Body weight was measured on subjects wearing light indoor clothing with emptied pockets and without shoes. To adjust for light indoor clothing, 1 kilogram was subtracted from the measured body weight. Body weight (kg) was measured to the nearest 0.5 kg and height (cm) to the nearest 0.5 cm. BMI was calculated as weight (kg) divided by the square of height (m^2).

Assessment of plasma glucose and biochemical measurements

Non-fasting blood samples were taken by venapuncture for all participants. Time of venapuncture and time of last meal were recorded. Time between venapuncture and last meal was calculated. Blood samples were fractionated into serum, buffy coat, and erythrocytes and subsequently stored at -30°C until further use. Plasma glucose levels were measured as described by Tietz [12].

Selection of candidate genes and SNP's

A total of 253 candidate genes were selected by a pathway-driven approach, with emphasis on regulatory pathways that control fatty acid, glucose, cholesterol and bile salt homeostasis [10, 13]. In short, the selection procedure started from the master regulator genes encoding nuclear receptors (PPAR's, LXR, NRIH4) and transcription factors (SREBP's) and continued by selecting their co-activators, co-repressors and target genes. In addition, hormonal receptors (insulin receptor) and their down-stream signaling proteins were selected. For each gene out of these pathways one to seven SNP's were selected using the procedure described in detail elsewhere [10]. Finally a set of 383 SNP's across 253 candidate genes, passed the Illumina Assay Design Tool and were included.

Genotyping

Genomic DNA was extracted from the buffy coat fraction with a salting out method [14]. A total of 139 subjects were not eligible for genotyping, mainly because of failure to extract DNA or unavailability of buffy coats. For 3309 subjects high throughput SNP genotyping was performed with the Illumina Golden Gate assay using the Sentrix Array Matrix platform (Illumina Inc, San Diego, California) [15]; Sixty-five subjects were excluded due to genotype failure or discordance on gender control and 28 SNP's were excluded because genotyping was unsuccessful. In addition, 33 SNP's were not in Hardy Weinberg Equilibrium (HWE). Verification was carried out in a random sample (n=96) for the eight SNP's (24%) that deviated most strongly from HWE. All yielded the same results, except for 2 SNP's, which were therefore excluded[16]. Thus, data on 353 SNP's in 239 genes were available for 3244 participants.

Study on Lifestyle intervention and Impaired glucose tolerance Maastricht (SLIM)

All SNP's which were measured in the Doetinchem study were also measured in the SLIM study, using the same Illumina array. Study design, inclusion and exclusion criteria of SLIM, an intervention study, have earlier been described in detail [17]. In short, BMI and

fasting plasma glucose levels were measured yearly from 1999 to 2005. Data of 120 participants from year 1999 to 2003, with both anthropometric and genotype information available, were used for replication analyses. Data from year 2004 and 2005 were not used, because in these years information was only available for 68 participants.

Statistical analyses

All analyses were performed with SAS version 9.1 (SAS Institute, INC., Cary, North Carolina). Distributions of genotypes were tested for deviation from HWE by chi-square analyses (PROC ALLELE).

As the glucose distribution was skewed, glucose values were log-transformed. Repeated measurements of log-glucose and BMI were used in multilevel random coefficient models (PROC MIXED) to study interaction effects of SNP's x BMI on log-glucose levels. This model accounts for correlation between repeated measures within subjects. In order to account for individual differences in glucose levels, a random intercept was used. Analyses were adjusted for age, sex, time since last meal (continuous), time since last meal (<=2 hours; >2 hours) and follow up time. After a meal, glucose levels peak shortly, and they return to near normal levels in approximately 2 hours. After 2 hours glucose levels decrease slowly [18].

The false discovery rate (FDR), which is a commonly accepted method in high-throughput genomic studies, was used to adjust for multiple testing [19, 20]. Significance was defined as FDR ≤0.20 [21]. When a significant interaction was found, the association between the SNP and log-glucose, stratified by BMI (≤25 kg/m^2, >25 kg/m^2 and ≤28 kg/m^2, >28 kg/m^2) was analyzed. The cut off points of 25 kg/m^2 and 28 kg/m^2 equal, respectively, the 50th and 75th percentile of the study population rounded to the nearest whole number. The model used was similar to that of the interaction analyses. For the stratified and replication analyses, significance was defined as a P-value ≤0.05.

Results

The Doetinchem Cohort

Among the 3244 subjects of the Doetinchem cohort median non-fasting glucose levels increased from 5.1 mmol/L (P25-P75: 4.7-5.6) in round 2 to 5.2 mmol/L (P25-P75: 4.8-5.8) in round 3 (table 1). The number of participants with elevated non-fasting glucose levels (≥ 11.1 mmol/L) was small, n=2 and n=17 in round 2 and round 3 respectively. BMI also increased slightly from round 2 till round 3. The overall correlation coefficient between log-glucose and BMI was 0.22 (P<0.001).

Table 1 | Baseline characteristics of 3244 subjects of the Doetinchem Cohort

	Round 2 1993-1997	**Round 3 1998-2002**
Age (yr)	46.5 (9.7)	51.5 (9.7)
Sex (% men)	47.8 % (*n*=1552)	47.8 % (*n*=1552)
BMI (kg/m^2)	25.3 (3.5)	26.0 (3.8)
Glucose levels (mmol/L) [a]	5.1 (4.7-5.6)	5.2 (4.8-5.8)
Time since last meal (h)	3.2 (3.6)	3.1 (3.3)

Data are presented as means (standard deviation) or median with inter-quartile range

[a] Non-fasting values

Two out of the 353 SNP's, rs8192678 (P-value=0.0002; FDR=0.07) and rs3755863 (P=0.0012; FDR=0.17), showed a significant interaction with BMI after adjustment for multiple testing. Results were similar after additional adjustment for physical activity in a subgroup of 2844 people with data available (rs8192678*BMI - P<0.0001; rs3755863*BMI - P=0.0005). The rs8192678 and rs3755863 SNP of the *PPARGC1A* gene, better know as *Gly482Ser* and *Thr528Thr*, were highly correlated (r^2=0.88; P<0.001). Both SNP's were in HWE (rs8192678 P=0.14; rs3755863 P=0.25). In subjects with a BMI ≤25 kg/m², the *A/A* genotype of rs8192678 (P=0.02) and rs3755863 (P=0.03) was significantly associated with lower log-glucose levels (table 2). In contrast, in subjects with a BMI>28 kg/m² an opposite trend was found, the A/A genotype of both SNP's were associated with slightly higher log-glucose values (rs8192678 P=0.12; rs3755863 P=0.14). No association was found in subjects with a BMI between 25 and 28 kg/m². Taking alternative cut-off points between 25 and 28 kg/m² did not change the results. According to our data, both SNP's seem to be inherited in a recessive mode. Single SNP and haplotype analysis under a recessive model showed similar results.

Some other SNP's in the *PPARGC1A* pathway showed an interaction with BMI under a significance level of P<0.05. These SNP's were located in *GSK3B* (rs334558: P-value=0.004, FDR=0.29), *UCP2* (rs659366: P-value=0.005, FDR=0.29; rs660339: P-value=0.01, FDR=0.35), *PCK2* (rs2759409: P-value=0.007, FDR=0.29), *LIPC* (rs1800588: P-value=0.02, FDR=0.43), *EP300* (rs4822012: P-value=0.02, FDR=0.43), *PPARG* (rs3856806: P-value=0.04, FDR=0.56), and *AKT2* (rs748236: P-value=0.04, FDR=0.56) (Supplementary table 1).

Table 2 | Log-glucose levels according to genotype stratified by BMI

rs8192678 - *Gly482Ser*	***A/A*** ***Ser/Ser*** **(*n*=415)**	***A/G*** ***Ser/Gly*** **(*n*=1545)**	***G/G*** ***Gly/Gly*** **(*n*=1609)**	**P-value**
BMI ≤ 25 (kg/m²)	1.59 (0.01)	1.63 (0.01)	1.61 (0.01)	0.02
BMI > 25 and ≤ 28 (kg/m²)	1.67 (0.01)	1.66 (0.01)	1.67 (0.01)	0.62
BMI > 28 (kg/m²)	1.73 (0.02)	1.70 (0.01)	1.71 (0.01)	0.12
rs3755863 - *Thr528Thr*	***A/A*** ***Thr/Thr*** **(*n*=503)**	***A/G*** ***Thr/Thr*** **(*n*=1514)**	***G/G*** ***Thr/Thr*** **(*n*=1224)**	**P-value**
BMI ≤ 25 (kg/m²)	1.60 (0.01)	1.62 (0.01)	1.62 (0.01)	0.02
BMI > 25 and ≤ 28 (kg/m²)	1.67 (0.01)	1.67 (0.01)	1.67 (0.01)	0.90
BMI > 28 (kg/m²)	1.72 (0.01)	1.70 (0.01)	1.71 (0.01)	0.14

Replication in the SLIM study

Among the 120 SLIM participants, mean fasting glucose levels ranged from 6.0 (0.78) mmol/L in round 1 to 6.3 (0.92) mmol/L in round 5. Mean BMI was 29.5 (3.5) kg/m^2 in round 1, decreased somewhat in round 2 and returned to 29.5 kg/m^2 in round 5. For rs3755863 genotype calling did not succeed due to overlap between genotype clusters. The interaction effect of rs8192678*BMI on fasting log-glucose levels was similar to the interaction in the Doetinchem cohort, though not statistically significant (P=0.15). In subjects with a BMI >28 kg/m^2, the AA genotype of rs8192678 showed somewhat higher log-glucose values (AA, AG, GG: 1.89 (0.04), 1.82 (0.02), 1.81 (0.02); P= 0.20). However, in subjects with a BMI ≤28 kg/m^2, no association was found (AA, AG, GG: 1.75 (0.05), 1.76 (0.02), 1.77 (0.02); P= 0.87). These results remained the same after further correction for intervention status.

Discussion

In this explorative study of 353 SNP's, mainly located in pathways related to lipid and glucose metabolism, we found a significant interaction between 2 SNP's in the *PPARGC1A* gene (rs8192678; rs3755863) and BMI on glucose levels. The rare allele of both SNP's was associated with lower glucose levels in subjects with a BMI ≤25 kg/m^2, but with higher glucose levels in subjects with a BMI >28 kg/m. A small cohort study of 120 mainly overweight or obese subjects suggested a similar association.

PPARGC1A is a transcriptional co-activator that interacts with a broad range of transcription factors involved in a wide variety of biological processes, including glucose metabolism in the liver and muscle, mitochondrial biogenesis, lipid oxidation, and adipocyte differentiation [22]. One of the transcription factors regulated by *PPARGC1A* is *PPARG*. In line with our results, Wei *et al.* [7] found a significant interaction effect of 2 SNP's in the *PPARG* gene (rs4135304; rs4135247) and BMI on fasting plasma glucose levels in African Americans. For both polymorphisms allelic effects on glucose levels were reversed among individuals with lower BMI and individuals with higher BMI. However, neither Wei *et al.* [7] noir we observed an interaction effect between BMI and the well-studied rs1801282 (*Pro12Ala*) polymorphism of *PPARG*. Interestingly, we found an interaction effect with another SNP in the *PPARG* gene, the rs3856806 polymorphism (P= 0.04). However, this interaction was no longer statistically significant after adjustment for multiple testing. Besides, we found interactions with BMI for SNP's in other genes of the *PPARGC1A* pathway (*GSK3B*, *UCP2*, *PCK2*, *EP300*, *LIPC* and *AKT2*) under a significance level of P<0.05. These associations were no longer significant after adjustment for multiple testing. Furthermore, to the best of our knowledge, there are no other human studies showing an interaction between BMI and SNP's in these genes on glucose levels.

Owing to the high correlation between rs8192678 and rs3755863, both SNP's showed similar associations with serum glucose levels. Of these SNP's, rs8192678 or *Gly482Ser*, induces a change in amino acid sequences, whereas rs3755863 or *Thr528Thr* does not. Although the amino acid change induced by the *Gly482Ser* polymorphism has not been shown to affect a regulatory regions within *PPARGC1A* [23], Ling *et al.* [24, 25] showed that the *Gly482Ser* polymorphism changed *PPARGC1A* mRNA expression.

The Ser allele of the *Gly482Ser* mutation has been related to diabetes [26] and obesity [27, 28]. In a meta-analysis of eight studies, the *Ser* allele of the *Gly482Ser* polymorphism was associated with modest increase of type 2 diabetes risk [26]. However, in the

same meta-analysis, no association was found with fasting glucose levels or fasting insulin levels. This may be due to between–study heterogeneity, which could result from differences in genetic or environmental factors. Indeed, several environmental factors, such as age [25, 28, 29], physical activity [28, 30] and acarbose treatment [31], have been shown to interact with the *Gly482Ser* polymorphism. Furthermore, Goyenchea *et all*. [32] showed in an 8 week weight loss trial, that insulin resistance decreased significantly more in *Ser/Ser* subjects compared to *Gly/Ser* and *Gly/Gly* subjects, whereas the decrease in body weight and adiposity were similar among the different genotypes. These results support our finding that body weight modifies the association between the *Gly482Ser* polymorphism and glucose.

The *Ser* allele of the *Gly482Ser* polymorphism has been shown to correlate with decreased *PPARGC1A* mRNA expression in muscle [25] and insulin islets [24].We speculate that the *Ser* allele may also down-regulate *PPARGC1A* expression in other tissues. Down-regulation of *PPARGC1A* in liver and muscle has opposite effects on glucose levels. In liver, *PPARGC1A* down-regulation decreases gluconeogenesis [22], which has a blood glucose lowering effect. In contrast, *in vitro* studies suggest that down-regulation of *PPARGC1A* in muscle increases insulin resistance, by down-regulating GLUT4 transporters [22] and by stimulating muscle lipid accumulation [33]. This, in turn, increases blood glucose levels. Our results suggest that body weight affects the balance between the glucose lowering and glucose increasing processes. We hypothesize that in lean people, the *Ser* allele may decrease glucose levels through decreased gluconeogenesis, whereas in obese people, who are generally more insulin resistant, the balance may shift towards increased insulin resistance properties of the *Ser* allele, leading to increased glucose levels.

Strengths of this pathway driven candidate gene study were the relatively large sample size and the availability of repeated anthropometric measurements, which improve study power and precision. Furthermore, the SLIM study showed a similar interaction with a larger effect size. Owing to the smaller sample size, these results were, however, not statistically significant. Our blood samples were taken from non-fasting subjects. To account for this, we corrected our analysis for time since last meal. Moreover, the error induced by the non-fasting state of the subjects is of random rather than of systematic nature. As mentioned above, our findings are in line with evidence from other studies and might be biologically plausible. Nevertheless, it can not be excluded that our results are based on chance alone.

In this explorative study among 3244 subjects investigating 353 SNP's in 253 candidate genes, we emphasized on the intricate links between glucose and lipid metabolism through common transcriptional factors. We found that BMI significantly modifies the association between two SNP's in the *PPARGC1A* gene (rs8192678; rs3755863) and glucose levels. Although the *Ser/Ser* genotype of *PPARGC1A* was associated with lower glucose levels in lean subjects, it may be inversely associated in obese people. This suggests that the effect of the polymorphisms in the *PPARGC1A* gene, which is involved both in fatty acid and glucose metabolism, is modified by BMI. Based on this study, it can be speculated that *Ser/Ser* subjects are more susceptible to develop hyperglycemia in an obesogenic environment, but less susceptible to develop hyperglycemia in a non-obesogenic environment.

Conflict of interest

The authors declare no conflict of interest

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Supplementary Table 1 | SNP * BMI interaction on log-glucose

Gene	BMI*SNP	Minor Allele	P-value	FDR
PPARGC1A	BMI*rs8192678	A	0.000235	0.065189
PPARGC1A	BMI*rs3755863	A	0.001205	0.166837
GSK3B	BMI*rs334558	G	0.004309	0.29138
UCP2	BMI*rs659366	T	0.005321	0.29138
INSR	BMI*rs2963	T	0.006259	0.29138
NR1H3	BMI*rs1449627	G	0.007226	0.29138
PCK2	BMI*rs2759409	G	0.007363	0.29138
PTGS2	BMI*rs5277	C	0.00919	0.318204
UCP2	BMI*rs660339	T	0.011213	0.345111
NCOA3	BMI*rs2230782	C	0.014025	0.38849
EP300	BMI*rs4822012	T	0.018023	0.428762
PCK1	BMI*rs707555	C	0.020475	0.428762
LIPC	BMI*rs1800588	T	0.020912	0.428762
SCARB1	BMI*rs5888	C	0.022145	0.428762
CYP51A1	BMI*rs7797834	G	0.023605	0.428762
ACACA	BMI*rs9330250	C	0.024766	0.428762
CYP4A11	BMI*rs3890011	C	0.029296	0.477345
CD5L	BMI*rs2261295	G	0.032276	0.496698
PPARG	BMI*rs3856806	T	0.040955	0.568366
GPAM	BMI*rs2254537	A	0.042365	0.568366
AKT2	BMI*rs748236	T	0.043089	0.568366
ACLY	BMI*rs2304497	G	0.047107	0.586427
SCCN1A	BMI*rs3759324	C	0.051463	0.586427
MMP9	BMI*rs2664538	G	0.054448	0.586427
IL1B	BMI*rs1143634	T	0.059483	0.586427
FABP2	BMI*rs1799883	A	0.059538	0.586427
MTP	BMI*rs1800591	T	0.064304	0.586427
MTP	BMI*rs3816873	C	0.064849	0.586427
GNB3	BMI*rs5443	T	0.065076	0.586427
PRKCA	BMI*rs7210446	G	0.066813	0.586427
APOB	BMI*rs1367117	A	0.067978	0.586427
EIF4EBP1	BMI*rs6605631	G	0.069138	0.586427
HRAS	BMI*rs4963176	C	0.070197	0.586427
PCAF	BMI*rs3021408	T	0.073325	0.586427
LIPC	BMI*rs690	G	0.074182	0.586427
SHC1	BMI*rs4845401	G	0.076214	0.586427
LDLR	BMI*rs688	T	0.080163	0.600136
PDK1	BMI*rs1005273	T	0.084385	0.607966
CETP	BMI*rs1800775	A	0.085598	0.607966
APOA2	BMI*rs3813628	C	0.088619	0.613685
CYP4B1	BMI*rs2297809	A	0.098437	0.66505
ABCB4	BMI*rs31653	A	0.104653	0.665498
LPL	BMI*rs3208305	T	0.105892	0.665498
PLTP	BMI*rs553359	C	0.110713	0.665498
ABCA1	BMI*rs2230806	A	0.112913	0.665498
IFNG	BMI*rs2069718	T	0.116511	0.665498
FASN	BMI*rs2228309	T	0.119636	0.665498
PPARA	BMI*rs1055659	T	0.123904	0.665498
PPARA	BMI*rs4253655	A	0.124946	0.665498
INSR	BMI*rs2059806	A	0.128458	0.665498
RXRG	BMI*rs3818569	A	0.128999	0.665498
PPARGC1A	BMI*rs2932976	A	0.135899	0.665498
PIK3CA	BMI*rs7614305	A	0.136569	0.665498
DEFA6	BMI*rs1803898	A	0.137127	0.665498

Supplementary Table 1 | Continued

Gene	BMI*SNP	Minor Allele	P-value	FDR
ADIPOR2	BMI*rs1044471	A	0.138786	0.665498
LDLR	BMI*rs5925	T	0.139671	0.665498
CYP7A1	BMI*rs3808607	C	0.141088	0.665498
AGTR1	BMI*rs5186	G	0.141449	0.665498
PPARA	BMI*rs1800206	C	0.141749	0.665498
NFKB1	BMI*rs170731	G	0.156387	0.721985
MVD	BMI*rs8854	A	0.162712	0.738873
ACOX2	BMI*rs1137582	C	0.167246	0.746601
UE2I	BMI*rs8063	A	0.173655	0.746601
LPL	BMI*rs328	G	0.176861	0.746601
SNX26	BMI*rs231228	T	0.177197	0.746601
PMVK	BMI*rs1891805	C	0.178659	0.746601
LPL	BMI*rs1059507	T	0.180586	0.746601
SREBF1	BMI*rs8066560	A	0.18441	0.746974
PPARG	BMI*rs1801282	G	0.186069	0.746974
PPARA	BMI*rs135539	G	0.19087	0.755302
CPT1B	BMI*rs470117	A	0.209639	0.7846
CCND3	BMI*rs3218108	A	0.211231	0.7846
RARA	BMI*rs7217852	G	0.213328	0.7846
PPARG	BMI*rs709158	G	0.218222	0.7846
ABCG1	BMI*rs2839482	G	0.221404	0.7846
STAT1	BMI*rs2066802	C	0.224112	0.7846
KLF5	BMI*rs4885062	G	0.224744	0.7846
FADS1	BMI*rs482548	T	0.225762	0.7846
PCK1	BMI*rs2071023	G	0.232153	0.7846
PPARA	BMI*rs6008259	A	0.233414	0.7846
ABCB11	BMI*rs2287622	T	0.235132	0.7846
NOS3	BMI*rs1799983	T	0.235183	0.7846
MSR1	BMI*rs3747531	C	0.236436	0.7846
PPARA	BMI*rs4253778	C	0.242028	0.7846
RHOQ	BMI*rs1451152	A	0.246498	0.7846
SOS1	BMI*rs1059310	A	0.247673	0.7846
CETP	BMI*rs1800777	A	0.250015	0.7846
RXRA	BMI*rs1805352	C	0.25022	0.7846
ACAT2	BMI*rs3464	T	0.252092	0.7846
HPG2	BMI*rs8752	G	0.255425	0.786142
ABCG8	BMI*rs4148211	G	0.259303	0.789308
NCOA6	BMI*rs6088618	A	0.265591	0.795784
SREBF1	BMI*rs4925119	A	0.267177	0.795784
ABCB4	BMI*rs8187799	G	0.270892	0.798266
EDNRA	BMI*rs5333	C	0.274637	0.800783
ACACA	BMI*rs8071315	C	0.278398	0.803294
APOC2	BMI*rs1132899	C	0.282353	0.806307
IRS1	BMI*rs1801278	A	0.289563	0.815104
CREBBP	BMI*rs130005	G	0.292575	0.815104
AGT	BMI*rs5050	G	0.299766	0.815104
CEBPB	BMI*rs6020348	A	0.299796	0.815104
RAF1	BMI*rs6795441	G	0.305584	0.815104
PPARGCA1	BMI*rs2970870	C	0.307915	0.815104
AACS	BMI*rs900410	C	0.310908	0.815104
IL6	BMI*rs1800795	C	0.316768	0.815104
PTPN1	BMI*rs718630	C	0.318295	0.815104
ESRRA	BMI*rs2276014	T	0.320217	0.815104
LEPR	BMI*rs1137101	G	0.32484	0.815104

Supplementary Table 1 | Continued

Gene	BMI*SNP	Minor Allele	P-value	FDR
CCL2	BMI*rs4586	C	0.325476	0.815104
PPARGC1B	BMI*rs1076064	C	0.329592	0.815104
PGA4	BMI*rs6800271	T	0.329688	0.815104
MEF2D	BMI*rs1925950	G	0.335332	0.815104
MEF2C	BMI*rs2457979	A	0.337239	0.815104
NCOA6	BMI*rs3787220	A	0.340094	0.815104
SIRT1	BMI*rs7069102	C	0.340364	0.815104
THRB	BMI*rs1394761	T	0.341343	0.815104
GSK3B	BMI*rs3755557	A	0.344853	0.81606
PIK3R2	BMI*rs613339	A	0.347987	0.81606
CDKN2A	BMI*rs3731249	A	0.356533	0.81606
NCOA2	BMI*rs3088092	T	0.358346	0.81606
PTGS2	BMI*rs5275	T	0.358641	0.81606
EDNRB	BMI*rs5352	A	0.361263	0.81606
CETP	BMI*rs5882	G	0.365077	0.81606
ACE	BMI*rs4343	A	0.365312	0.81606
AKT1	BMI*rs3001371	T	0.378395	0.838524
HMGCS1	BMI*rs1548097	G	0.383518	0.843131
PDK4	BMI*rs6931	A	0.390197	0.85106
RETN	BMI*rs1862513	C	0.39346	0.851472
PIAS1	BMI*rs1489599	G	0.400735	0.859872
ADIPOR2	BMI*rs1029629	C	0.40355	0.859872
AGER	BMI*rs1800624	A	0.410775	0.868584
ADAMTS4	BMI*rs4233367	T	0.416011	0.871879
FRAP1	BMI*rs2076657	C	0.419551	0.871879
AGT	BMI*rs699	C	0.421775	0.871879
PTGS2	BMI*rs20417	C	0.426112	0.874319
FOXC2	BMI*S04_FOXC2	C	0.434449	0.879361
PP3CA	BMI*rs3804404	G	0.438029	0.879361
UCP3	BMI*rs1800849	T	0.438406	0.879361
CPT1B	BMI*rs140515	C	0.443753	0.879361
PPARD	BMI*S14_PPARD	T	0.45037	0.879361
IDI1	BMI*rs7075141	G	0.45406	0.879361
SC5DL	BMI*rs1061332	A	0.459678	0.879361
NR1H4	BMI*rs35724	A	0.470885	0.879361
NRIP1	BMI*rs2229741	A	0.474247	0.879361
APOB	BMI*rs1042031	A	0.478923	0.879361
NCOA3	BMI*rs2076546	C	0.483282	0.879361
EDN1	BMI*rs1794849	T	0.487208	0.879361
NCOR1	BMI*rs178802	A	0.488972	0.879361
SIRT1	BMI*rs2273773	C	0.489899	0.879361
ABCA1	BMI*rs2275543	C	0.496515	0.879361
FBXW7	BMI*rs2676330	À	0.504131	0.879361
AGER	BMI*rs1062070	G	0.506254	0.879361
FABP1	BMI*rs2241883	C	0.508115	0.879361
NFKB1	BMI*rs1801	C	0.508204	0.879361
USF1	BMI*rs3737787	T	0.508342	0.879361
CAMK4	BMI*rs1469442	G	0.508854	0.879361
ACADVL	BMI*rs507506	A	0.509834	0.879361
ACADL	BMI*rs2286963	G	0.511423	0.879361
ABCG8	BMI*rs4148217	A	0.513685	0.879361
DHCR7	BMI*rs1044482	G	0.517608	0.879361
PLA2G7	BMI*rs1051931	A	0.520037	0.879361
PTRN2	BMI*rs11700	C	0.523228	0.879361

Supplementary Table 1 | Continued

Gene	BMI*SNP	Minor Allele	P-value	FDR
FDFT1	BMI*rs1047643	C	0.524581	0.879361
MVK	BMI*rs7957619	A	0.525217	0.879361
CD36	BMI*rs1049654	A	0.525997	0.879361
PLTP	BMI*rs394643	A	0.531151	0.879361
PPARGC1B	BMI*rs7732671	C	0.534657	0.879361
ELOVL5	BMI*rs209512	G	0.53687	0.879361
RAPGEF1	BMI*rs7854489	C	0.537473	0.879361
IRS2	BMI*rs4773092	A	0.54179	0.879361
MAPK14	BMI*rs6457878	T	0.542855	0.879361
RXRB	BMI*rs1547387	C	0.550786	0.884504
SAH	BMI*rs5716	T	0.553847	0.884504
GABPA	BMI*rs2829887	A	0.555609	0.884504
ELOVL2	BMI*rs4532436	G	0.563535	0.88698
NCOR2	BMI*rs2229840	A	0.563568	0.88698
NRF1	BMI*rs1882094	C	0.579951	0.890693
PPARGC1A	BMI*rs2970869	A	0.583459	0.890693
CD36	BMI*rs1049673	G	0.585428	0.890693
GRB2	BMI*rs4788891	A	0.586453	0.890693
FDFT1	BMI*rs9205	C	0.587329	0.890693
IL6	BMI*rs1800796	C	0.590609	0.890693
NFKBIA	BMI*rs2233409	T	0.592353	0.890693
HNF4A	BMI*rs1800961	T	0.59753	0.890693
HNF4A	BMI*rs745975	A	0.598127	0.890693
MBTPS1	BMI*rs3759972	G	0.602233	0.890693
INSIG2	BMI*rs9308762	C	0.605574	0.890693
ME1	BMI*rs1144181	A	0.617659	0.890693
CRSP3	BMI*rs2781667	T	0.620369	0.890693
PTEN	BMI*rs2735343	C	0.626388	0.890693
MEF2C	BMI*rs244760	G	0.628438	0.890693
LEP	BMI*rs2167270	A	0.631762	0.890693
RELB	BMI*rs10856	T	0.636616	0.890693
LPL	BMI*rs268	G	0.64311	0.890693
INPPL1	BMI*rs2276048	C	0.643233	0.890693
ABCA1	BMI*rs1800977	T	0.643983	0.890693
CREBBP	BMI*rs1296720	G	0.64544	0.890693
ECE1	BMI*rs213045	T	0.651646	0.890693
HMGCR	BMI*S06_HMGCR	T	0.654892	0.890693
SQLE	BMI*rs966946	C	0.656351	0.890693
ECE1	BMI*rs213046	C	0.66267	0.890693
ABCD3	BMI*rs16946	A	0.662991	0.890693
NOS3	BMI*rs2070744	C	0.663683	0.890693
RETN	BMI*rs3745367	A	0.665386	0.890693
CYP8B1	BMI*rs735320	A	0.666844	0.890693
NR1H2	BMI*rs1405655	C	0.669296	0.890693
ADD1	BMI*rs4961	T	0.670869	0.890693
MYBBP1A	BMI*rs751670	A	0.675275	0.890693
ADIPOR1	BMI*rs2275737	A	0.676487	0.890693
STAT5A	BMI*rs2293158	G	0.677816	0.890693
NFKB2	BMI*rs1056890	T	0.679427	0.890693
ABCG1	BMI*rs915843	T	0.681686	0.890693
UCP1	BMI*rs1800592	G	0.688533	0.891824
CREB1	BMI*rs2551640	G	0.689587	0.891824
ELOVL6	BMI*rs4146696	A	0.692478	0.891824
FOXO1A	BMI*rs2721044	T	0.69543	0.891824

Supplementary Table 1 | Continued

Gene	BMI*SNP	Minor Allele	P-value	FDR
CDKN2A	BMI*rs3088440	A	0.716576	0.909838
TRIB3	BMI*rs6115830	T	0.720328	0.909838
ABCG5	BMI*rs6720173	C	0.720418	0.909838
TM7SF2	BMI*rs1546532	C	0.722615	0.909838
PPRC1	BMI*rs2815402	G	0.73382	0.919766
PPARG	BMI*rs880663	C	0.737424	0.920119
HMGCS2	BMI*rs536662	A	0.750738	0.928169
CREBBP	BMI*rs2239316	G	0.75161	0.928169
SOCS3	BMI*rs4969170	A	0.753928	0.928169
NPPB	BMI*rs198389	G	0.767064	0.932032
IGF1R	BMI*rs2229765	A	0.76909	0.932032
CBL	BMI*rs4938638	G	0.770973	0.932032
APOA5	BMI*rs3135506	C	0.778446	0.932032
CPT1B	BMI*rs131758	A	0.779092	0.932032
STAT5B	BMI*rs6503691	T	0.780444	0.932032
PPARD	BMI*rs2016520	G	0.781015	0.932032
LIPC	BMI*rs6082	G	0.78646	0.932032
EDNRA	BMI*rs1801708	A	0.789384	0.932032
APOA2	BMI*rs5082	C	0.790713	0.932032
NR3C1	BMI*rs6191	G	0.810333	0.938321
ESR1	BMI*rs2234693	C	0.81227	0.938321
LEPR	BMI*rs8179183	C	0.813497	0.938321
CYP11B2	BMI*rs1799998	C	0.814218	0.938321
SREBF2	BMI*rs4822063	C	0.822145	0.938321
INS	BMI*rs3842748	G	0.825159	0.938321
NCOA6IP	BMI*rs7823773	G	0.829573	0.938321
NR1H4	BMI*rs4764980	A	0.834018	0.938321
RPS6KB1	BMI*rs1051424	G	0.835287	0.938321
LIPE	BMI*rs1206034	T	0.83628	0.938321
SUMO1	BMI*rs6755690	G	0.841353	0.938321
UCP3	BMI*rs2075577	C	0.842162	0.938321
PRKACA	BMI*rs729372	A	0.84289	0.938321
PPARGC1A	BMI*rs2970847	T	0.845176	0.938321
FDP2	BMI*rs2297480	C	0.84686	0.938321
NPPA	BMI*rs5063	A	0.854276	0.941631
SCAND1	BMI*rs6060717	C	0.856646	0.941631
SREBF2	BMI*rs6002527	C	0.867366	0.949645
PPARBP	BMI*rs4795367	G	0.872848	0.951885
KCNMB1	BMI*rs827778	A	0.884768	0.959136
ABCB4	BMI*rs2109505	A	0.890739	0.959136
SREBF2	BMI*rs2269657	T	0.890762	0.959136
LEPR	BMI*rs1137100	G	0.893347	0.959136
PIK3R1	BMI*rs706713	T	0.899935	0.962478
RXRB	BMI*rs6531	C	0.903784	0.962878
NR3C1	BMI*rs6196	G	0.90762	0.96326
PPARBP	BMI*rs7501488	T	0.91113	0.963294
PPARGC1A	BMI*rs3796407	A	0.925507	0.967845
SPP1	BMI*rs1126616	T	0.927351	0.967845
UCP1	BMI*S18_UCP1	A	0.928637	0.967845
NCOR2	BMI*rs2272368	C	0.929411	0.967845
NOS2A	BMI*rs2297518	A	0.938649	0.970514
FBP1	BMI*rs1754435	A	0.938981	0.970514
FADS1	BMI*rs174546	T	0.944561	0.972652
PPARD	BMI*rs1053049	C	0.949636	0.974256

Supplementary Table 1 | Continued

Gene	BMI*SNP	Minor Allele	P-value	FDR
TNF	BMI*rs1800629	A	0.953944	0.975065
RXRA	BMI*rs3118571	G	0.981898	0.995623
ABCA1	BMI*rs2066716	A	0.984487	0.995623
RXRA	BMI*rs3132300	T	0.987806	0.995623
NOS2A	BMI*rs1060826	A	0.988434	0.995623
RXRA	BMI*rs1805348	A	0.99592	0.996683
INSR	BMI*rs2860172	A	0.996683	0.996683

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8

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General Discussion

General Discussion

Abdominal obesity, hyperglycemia, hypertriglyceridemia, low serum High Density Lipoprotein (HDL) cholesterol levels and hypertension frequently co-occur within individuals. The cluster of these features is referred to as metabolic syndrome (MetS). Currently, it is unclear how, i.e. by which endpoints, the clustering of MetS features can be best studied. Also the pathophysiology behind the clustering of MetS features is not fully understood. Therefore the research questions posed in this thesis were:

Which metabolic endpoints should be studied in order to explain the clustering of MetS features best?

Wich pathophysiology is underlying the clustering of MetS features?

All together the research described in this thesis aims to contribute to a better understanding of MetS. In chapter 2 and 3 we aimed to identify endpoints or combinations of endpoints, that should be studied in order to successfully elucidate factors responsible for the clustering of MetS features. In chapters 4 to 7 we investigated genetic variants in relation the clustering of MetS features, therewith aiming to give insight in the pathological pathways responsible for this clustering.
This final chapter starts with an overview of our main findings embedded within a discussion of the results of other studies. This section is followed by a section on methodological considerations, a section on the implications for public health, a section on recommendations for future research and by an overall conclusion.

Main findings

MetS is a very heterogeneous phenotype. Because of this heterogeneity critics of MetS doubt whether the traditional MetS features represent one entity, and consequently they question whether MetS exists [1]. If MetS is a clinical entity, characterized by a unifying pathophysiological factor, the symptoms characterizing the disease are most likely highly correlated and represent a statistical entity. In chapter 2 we observed, in line with other studies [2-8], that a one-factor confirmatory factor analysis(CFA) MetS model composed of traditional MetS features had an acceptable model fit. This indicates that it is possible to construct a single statistical entity out of the traditional MetS features. In order to increase the predictive ability of MetS for T2D and CVD, it has been proposed to add additional features to the definition of MetS. We have tested the model fit of one-factor CFA MetS models additionally including hsCRP, uric acid, albumin or liver enzymes. Of these models only the model additionally including hsCRP represented a single entity. Compared to a MetS model including traditional MetS features only, this model predicted T2D and CVD somewhat better. Therefore inclusion of hsCRP in future MetS definitions may be considered. Although the model fit of a one-factor MetS model including traditional MetS features was acceptable, it was not perfect. This implies that it is impossible to explain all covariance between the different MetS features with one MetS factor. Consequentially, the causes and mechanisms behind the clustering of MetS features will never be fully explained by studies focussing on one MetS factor.

To fully explain this clustering, the different combinations of MetS features should be studied. For those combinations of MetS features with the largest genetic pleiotropy, it will probably be easiest to detect common genetic variants. In chapter 3 we estimated genetic pleiotropy between several MetS and MetS related features, by reviewing genetic correlation coefficients assessed in twin and family studies published before 7th of July 2010. We concluded that genetic pleiotropy was greatest between plasma HDL cholesterol and triglycerides (median $r^2_{genetic}$ = -0.45) and between waist circumference and HOMA-IR, a measure of insulin resistance (median $r^2_{genetic}$ = 0.50). This implies that for these combinations it will probably be relatively easy to identify common genetic variants. In most studies we reviewed genetic and environmental correlation coefficients between the different MetS features were, at first sight, similar. This suggests that genes and environment influence the clustering of MetS features through similar mechanisms [9].

The pathophysiology behind the clustering of MetS features is currently not fully understood. Genetic association studies on the clustering of MetS features may contribute to a better understanding of this pathophysiology. In table 1 the single nucleotide polymorphisms(SNP's) for which in this thesis an association with MetS or with a specific combination of MetS features was found, are presented. The SNP's associated with MetS give a general idea of the mechanisms responsible for the clustering of MetS features, whereas the SNP's associated with specific combinations of MetS features provide deeper and more specific insights into the clustering of MetS features. As discussed in the introduction, SNP's could influence the clustering of MetS features via three different models: model 1, the effect of a SNP on MetS feature 1 is completely mediated by the effect of the genetic variant on MetS feature 2; model 2, a SNP is associated with two MetS features through two independent pathways; model 3, a SNP changes the strength of the association between MetS feature 1 and MetS feature 2. In this thesis we found examples for all three models.

Table 1 Genetic variants associated with the clustering of MetS features

Reference		Study Population	SNPs studied	Endpoint
chapter 4	[13]	review of 88 candidate gene studies	SNPs in 25 genes	metabolic syndrome
chapter 5		1886 participants of EPIC-NL	39 SNPs involved in weight regulation insulin resistance, lipid metabolism and inflammation	metabolic syndrome
chapter 6	[22]	3575 participants of the Doetinchem cohort	373 SNPs occurring in transcriptional pathways of glucose and lipid metabolism	low HDL cholesterol and abdominal obesity
chapter 7	[24]	3244 participants of the Doetichem cohort	353 SNPs occurring in transcriptional pathways of glucose and lipid metabolism	Interaction of glucose levels on BMI

We and others [2-8] showed that it is possible to construct one statistical entity out of the traditional MetS features. This does not necessarily imply that the traditional MetS features are characterized by one pathophysiological factor. The SNP's which were associated with the clustering of MetS features in this thesis are involved in several different mechanisms, including weight regulation, glucose and insulin metabolism, lipid metabolism and inflammation (figure 1). This supports the hypothesis that MetS is caused by multiple underlying interrelated causal mechanisms [27].

The most cited mechanisms responsible for the congregation of MetS features are insulin resistance and weight regulation [27, 28]. For both these two mechanisms we found SNP's that were associated with MetS (chapter 4, chapter 5). Of these SNP's, *FTO* rs9939609 and *MC4R* rs17782312 are mainly known for their role in weight regulation and *TCF7L2* rs7903146 and *IRS1* rs2943634 are mainly known for their role in insulin and glucose metabolism. The association of MetS with *FTO* rs9939609 is most likely completely mediated by the effect of *FTO* rs9939609 on body weight (model 1). Freathy *et al.* found that the association of *FTO* rs9939609 with some MetS features, i.e. glucose and lipid levels, disappeared after adjustment for BMI and that these associations were consistent with those predicted given the *FTO*-BMI and BMI-MetS features associations [29]. *FTO* rs9939609 was associated with MetS in a systematic review of candidate gene studies (chapter 4). However, in a random sample of EPIC-NL study, *FTO* rs9939609 was not associated with MetS or MetS-score, probably due to the relative weak association with body weight. Therefore, we were not able to test the mediating effect of *FTO* rs9939609. In a random sample of the EPIC-NL study the association between MetS and

Significant SNPs	Associated with
APOA5 C56G *APOA5 T1131C*	LPL ↑ → FFA ↓, VLDL production ↓ [14]
APOC3 C482T *APOC3 C455T*	LPL ↓ → FFA ↑, inhibition of ApoE mediated clearance of VLDL [15]
CETP Taq-1B	reverse cholesterol transport ↑ → HDL cholesterol ↓ [12]
FTO rs9939609	disturbed food intake regulation → obesity ↑ [16]
TCF7L2 rs7903146	insulin secretion ↓ + hepatic insulin resistance ↑ → glucose ↑ [17, 18]
IL-6 174G>C	insulin resistance ↑ , FFA ↑, CRP ↑ [19, 20] sympathetic nervous system ↑ → hypertension [21]
MC4R rs17782312	disturbed food intake regulation → obesity ↑ [10] insulin resistance ↑ [10, 11]
IRS1 rs2943634	insulin resistance ↑ [12]
CETP Ile405Val	reverse cholesterol transport ↑ → HDL cholesterol ↓ [12]
APOE Cys112Arg	VLDL ↓ [23]
PPARGC1A Gly482Ser *PPARGC1A Thr528Thr*	lipid oxidation ↓, gluconeogenesis ↓ [25, 26]

MC4R rs17782312 remained after adjustment for waist circumference. This suggests that the association between *MC4R* rs17782312 and MetS is probably, at least partly, independent of the effect of *MC4R* rs17782312 on body weight (model 2). These findings are in line with other, both human and animal, studies in which the *MC4R* rs17782313 SNP or the *MC4R* gene affected insulin resistance independent of body weight [10, 11].
TCF7L2 rs7903146 was associated with MetS in the systematic review of candidate gene studies. TCF7L2 is a member of the T-cell transcription factor family, which plays an important role in the WNT signaling pathway. This pathway is a major component in the regulation of cell proliferation and differentiation, is involved in adipogenesis and required for the development of pancreatic islets during embryotic growth, and through TCF7L2 influences glucagons-like 1 peptide secretion [18]. *TCF7L2* rs7903146 is associated with hepatic insulin resistance and diminished insulin secretion [18]. So far, it is not known if the effect of *TCF7L2* rs7903146 on MetS can be completely explained by the effects on glucose metabolism or if other mechanisms also play a role. Possibly, *TCF7L2* rs7903146 could also affect MetS by disruption of adipogenesis, through interference with the WNT signalling pathway [30].
The *IRS1* rs2943634 SNP was associated with MetS in a random sample of EPIC-NL. Accordingly, an IRS1 knock-out mouse model displayed a non-obese MetS like phenotype consisting of insulin resistance, increased blood pressure, increased triglycerides, decreased HDL cholesterol and decreased lipoprotein lipase (LPL) activity [31]. The dyslipidemic phenotype, both observed in knock-out mice and T-allele carriers of *IRS1* rs2943634, may be caused by impairment in insulin action, resulting in overproduc-

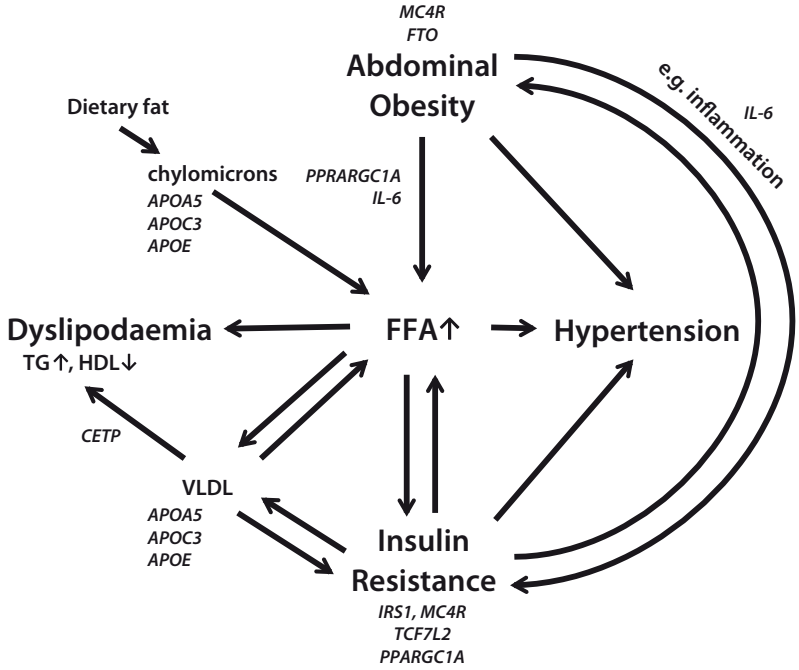

Figure 1 | Mechanisms by which SNP's identified in this thesis effect the co-occurrence of MetS features; TG, triglycerides; HDL, HDL cholesterol; VLDL, very low density lipoprotein; FFA, free fatty acids

tion of VLDL in the liver and decreased clearance of VLDL and chylomicrons (model 1) [31]. The association between IRS1 and hypertension could be related to impairment in endothelium-dependent vascular relaxation [31], possibly in relation to inhibition of the PI3K/Akt signalling pathway caused by a defect in IRS1 (model 2) [32].

Besides insulin resistance and abdominal obesity, also inflammation, a more controversial mechanism, has been suggested to explain the clustering of MetS features [20, 28, 33]. We failed to show an association between a group of SNP's, known for their association with inflammatory biomarkers in GWAS, and MetS in a random sample of the EPIC-NL study (chapter 5). However, we cannot preclude that a few specific inflammatory biomarkers are causative to MetS. For example, when we analysed individual associations of the inflammation SNP's with MetS-score in this random sample, we found an association of *IL6-R* rs4537545 with MetS score (-0.08, 95%CI -0.17; 0.00). Additionally, in a systematic review of candidate gene studies on MetS we found an association between a SNP in the *IL6* gene, *IL6 174G>C* (rs1800795) and MetS (chapter 4). These two studies, in conjunction with some studies which reported an association between interleukin 6 and MetS or MetS related features, such as insulin resistance, FFA levels, triglyceride levels and blood pressure [19-21], support a role for interleukin-6 in MetS development.

Another adipokine which has been proposed to be involved in MetS development is adiponectin [34]. The genetic correlation between adiponectin and MetS (median $r^2_{genetic}$ = -0.32; chapter 3), suggests a link between adiponectin metabolism and MetS. However, in the systematic review we found no evidence for an association between SNP's located in the *ADIPOQ* gene, the gene encoding adiponectin, and MetS. Some recently discovered genetic variants, such as the *ARL15* rs4311394 SNP, may explain part

of this inconsistency [34]. This SNP was discovered in a GWAS on adiponectin, and besides with adiponectin, also associated with insulin, T2D and CHD, all metabolic syndrome related outcomes [34]. To the best of our knowledge, an association between *ARLI5* and MetS has not been reported.

Recently, two GWAS added another dimension to the etiology of MetS [35, 36]. In both these GWAS all SNP's found to be associated with MetS were involved in lipid metabolism. In line with these GWAS, most of the SNP's which were associated with MetS in our systematic review on candidate gene studies were involved in lipid metabolism (chapter 4). More specifically many of these lipid SNP's were located in the *APOAI/C3/A4/A5* gene cluster, in the *BUDI3* gene, which is flanking the *APOAI/C3/A4/A5* gene cluster or in the *ZNF259* gene, which is flanking the *BUDI3* gene. The SNP's located in or close to the *APOAI/C3/A4/A5* gene cluster were: *APOA5* rs2266788 [36], *APOA5 C56G* (rs3135506), *APOA5 T1131C* (rs662799), *APOC3 C482T* (rs2854117), *APOC3 C455T* (rs2854116) (chapter 4), *ZNF259* rs964184 [35], *ZNF259* rs2075290 and *BUDI3* rs10790162 [36]. *APOA5* and *APOC3*, in which several SNP's associated with MetS are located, both affect lipid levels, amongst others, through their influence on LPL. Interestingly, in a recent GWAS *LPL* rs295 was also associated with MetS [36]. In the systematic review (chapter 4) *LPL* S447X (rs328) (r^2_{CEU} 0.49 with *LPL* rs295) was excluded, because the accumulative total study population for this SNP was <4000 subjects. However, in all three candidate gene studies investigating the association between *LPL S447X* and MetS, the *S447* allele was associated with a higher prevalence of MetS [37-39]. In the Doetinchem cohort this SNP was also associated with a MetS-score consisting of abdominal obesity, hyperglycemia, hypertension and low serum HDL cholesterol (P=0.05). However, in a random sample of EPIC-NL *LPL S447X* was neither associated with MetS or with MetS-score. Furthermore, in a random sample of EPIC-NL a group of SNP's, known for their association with lipid levels in GWAS, was also not associated with MetS (chapter 4). The lack of an association with MetS may be explained by the small effect the lipid SNP's had on lipid levels in the EPIC-NL study.

Subgroup analyses revealed that the weak associations between lipid SNP's and lipid levels could not be explained by medication use, sex or a difference between the MORGEN and Prospect study.

In short, we found evidence of an association with MetS for SNP's located in genes involved in glucose and insulin metabolism, weight regulation, lipid metabolism and inflammation. In line with these results, Fontaine-Bisson *et al.* found that that SNP's located in glucose and insulin metabolism, weight regulation and lipid metabolism were associated with T2D, an endpoint of MetS [40].

In chapter 6 and 7 we have investigated the association between specific combinations of MetS features and SNP's in genes, located in transcriptional pathways of glucose and lipid metabolism in the Doetinchem cohort. We again found evidence supporting the link between lipid metabolism and the clustering of MetS features (chapter 6). The *APOE Cys112Arg (rs429358)* and the *CETP Ile405Val (rs5882)* SNP's influenced both abdominal obesity and low HDL cholesterol levels, partly independent of each phenotype (model 1 and model 2). Therefore, these SNP's may explain part of the genetic pleiotropy between HDL cholesterol and waist circumference (median r^2genetic = -0.22) (chapter 3). Furthermore, *APOE Cys112Arg* and *CETP Ile405Val* were associated with a MetS-score consisting of abdominal obesity, hyperglycemia, hypertension and low serum HDL cholesterol (MetS-score/allele *Cys112Arg* 0.12, 95% CI 0.01; 0.21; *Ile405Val* -0.08, 95% CI -0.16; -0.004). In a GWAS of seven studies, another uncorrelated SNP in the *CETP* gene, rs173539, was associated with the prevalnce of MetS, as well as with

the bivariate combination of low HDL cholesterol and high waist circumference [36]. These and our findings support that CETP, which is mainly known for its role in reverse cholesterol transport [41], is involved in the clustering of MetS features. Besides with MetS-score, *APOE Cys112Arg* was also associated with the prevalence of MetS, defined as the presence of three or more of the following four features, abdominal obesity, hyperglycemia, hypertension and low serum HDL cholesterol (PR/allele 1.21, 95% CI 1.03; 1.44). *APOE Cys112Arg* is part of the *APOE ε2/ε3/ε4* haplotype. In the Doetinchem cohort results of the *ε2ε3ε4* haplotype analyses were similar to the results of the *Cys112Arg* SNP. Compared to the *ε3/ε3* haplotype, the *ε4/-* haplotype tended to increase MetS prevalence (PR 1.21, 95%CI 0.98; 1.50). Also in the meta-analyses of candidate gene studies the *ε4/-* haplotype tended to increase the risk of MetS (pooled OR *ε4/-* vs. *ε3/ε3* 1.61, 95% CI 0.87; 2.97, I^2 = 88.3%). When we updated the meta-analyses with the results of the Doetinchem study the pooled OR became borderline significant (pooled OR *ε4/-* vs. *ε3/ε3* 1.53, 95% CI 0.98; 2.37, I^2 = 86.7%). This suggests that also *APOE*, which has an important function in the clearance of chylomicron remnants and VLDL from the plasma, may play a role in MetS development.

In the Doetinchem cohort, we found an interaction effect of BMI on glucose for two highly correlated SNP's in the *PPARGC1A* gene, *Gly482Ser* (rs8192678) and *Thr528Thr* (rs3755863) (model 3) (chapter 7). PPARGC1A is a transcriptional co-activator that interacts with a broad range of transcription factors involved in a wide variety of biological processes, including glucose metabolism in the liver and muscle, mitochondrial biogenesis, lipid oxidation, and adipocyte differentiation [25]. In our study, the rare allele of both *PPARGC1A* SNP's was significantly associated with lower glucose levels in subjects with a BMI ≤25 kg/m^2. An reverse association was suggested in subjects with a BMI > 28 kg/m^2. A small intervention study, the SLIM study, showed similar though non-significant results. The interaction effect we found was in line with an 8 weeks weight loss trial [42]. These studies support that *PPARGC1A Gly482Ser* and *PPARGC1A Thr528Thr* affect the clustering of MetS features, by modulating the association between weight regulation and glucose metabolism. However, the *PPARGC1A* SNP's were not associated with MetS in the Doetinchem cohort (*Gly482Ser* P=0.24; *Thr528Thr* P=0.27). This exemplifies that not all SNP's involved in the clustering of MetS features, are also associated with MetS itself.

Methodological considerations

MetS is a very heterogeneous phenotype. As nicely pointed out by Professor Bradford Hill [43], it is more difficult to find a cause, either genetic or environmental, for a heterogeneous phenotype, such as MetS, than for a more specific phenotype, such as hypertension. Furthermore, as the predominant MetS subtype may differ between populations it may be difficult to replicate associations with MetS in an independent study sample. For example, the pre-dominant MetS subtypes in the Rucphen family study contain abdominal obesity, dyslipidemia and hypertension [44], whereas in the EPIC-NL study the pre-dominant MetS stubtypes all contain hyperglycemia. Therefore, a genetic variant associated with a MetS subtype containing hyperglycemia may be associated with MetS in EPIC-NL, but not in the Rucphen family study. A way to get around the heterogeneity problem is to study specific combinations of MetS features, as is done in the analyses of the Doetinchem cohort (chapter 6 and 7).

Besides this heterogeneity issue, also the relatively low heritability of MetS (10%-30% [44-46]) hampers the detection of associations between genetic variants and MetS. It may be therefore more fruitful to focus on specific combinations of MetS features with much genetic variance in common. The amount of genetic variance shared between MetS features can be estimated with genetic correlation coefficients. A disadvantage of genetic correlation coefficients is that they are usually subject to rather large sampling errors and therefore seldom very precise [9]. By summarizing genetic correlation coefficients between MetS features across several studies, we reduced this sampling error and gave more precise estimates of genetic correlation coefficients between the MetS features (chapter 3).

Not only for MetS, but for any other complex trait, it is difficult to find underlying genetic variants that determine its risk. For example, the unexplained genetic variance for T2D is over 90% [47]. To the best of our knowledge, the unexplained genetic variance for MetS is unknown. However, as the number of genetic association studies on MetS is much smaller than on T2D, the unexplained genetic variance for MetS is likely to be high as well. One of the barriers for the detection of genetic variants associated with complex traits is a low study power, resulting in a large number of false negatives. Firstly, this low study power is caused by the small effect size many SNP's have. For example, in a recent GWAS on MetS, the largest odds ratio(OR) was 1.3 [36]. Secondly, study power is low due to the adjustment for multiple testing, and consequently the small alphas. For example, a GWAS with 4549 cases and 5679 controls has a power of less than 5% to detect an OR of 1.10 for a SNP with a MAF of 0.30 with a P-value of $1.0*10^{-6}$ [48]. Also in the random sample of EPIC-NL the power to detect an association between MetS and 39 SNP's of interest would have been very low, if we had tested all SNP's individually and adjusted for multiple testing afterwards. Using the stringent Bonferroni correction ($P=1.28*10^{-3}$) the power to detect a 1.2 prevalence ratio with MetS for a SNP with a MAF of 0.30 would have been 7%. To increase study power, we therefore studied the joint effect of five groups of SNP's, using Goeman's global test (chapter 5) [49]. Disadvantage of this approach is that a large individual effect of a SNP on MetS can be missed, due to the fact that all the other SNP's in the group are truly not associated with MetS.

In the Doetinchem cohort, we adjusted for multiple testing with the false discovery rate (FDR), when testing 353 SNP's individually for their interaction effect with BMI on glucose levels (chapter 7). The FDR, which is less stringent than the Bonferroni test, refers to the proportion of false positive tests among all positives [50]. To increase

study power in these analyses we relaxed the cut off value for the FDR from the traditional 0.05 to 0.20, like other candidate gene studies did [51, 52]. This has also been done in GWAS in which for example P-value=$1.0*10^{-6}$ or higher was applied, instead of P-value=$1.0*10^{-8}$, which correspond to 0.05 false positives [48, 50].
The downside of less stringent cut off values is an increase in false positives. To differentiate the true positive findings from the false positive findings replication in an independent population is common practise [53]. We replicated the interaction effect between *PPARGC1A Gly482Ser* and BMI on glucose levels observed in the Doetinchem cohort in the SLIM study (chapter 7). For the analyses on multiple MetS features, results did not replicate in the SLIM study (chapter 6). However, for these findings supportive evidence was available in literature. Failure to replicate an association does not necessarily imply that the initial finding is false positive. Inconsistent replication may be caused by various forms of between-study heterogeneity, including differences in genetic ancestry and environmental influences [53]. These sources of heterogeneity may also explain part of the heterogeneity observed in the meta-analysis on candidate gene studies (chapter 4). For example, ethnicity explained nearly all heterogeneity present in the meta-analysis on *APOA5 T1131C* (rs662799).
Two important biases for genetic association studies exist: population stratification and publication bias [50, 54]. Population stratification refers to a situation in which the study population includes subgroups of individuals that are on average more related to each other, than to other members of the wider study population [50]. Population stratification may confound genetic associations, resulting in false positive findings. Participants of the EPIC-NL study (chapter 5), as well as the Doetinchem cohort (chapter 6, 7), were randomly selected from a population mainly consisting of Dutch Caucasian individuals. In the EPIC-NL study all non-Caucasian participants were excluded by a principle component analyses of the genetic population structure. Also related individuals were excluded [55]. Therefore, population stratification has probably not been an issue in the EPIC-NL study. The Doetinchem cohort still contains a few non-Caucasian and a few related participants. However, as they are only few, the gene pool is expected to be fairly homogeneous [56]. Therefore, population stratification has probably not been a major issue in the Doetinchem cohort.
Although the Egger's and Begg's test did not indicate publication bias in any of the meta-analyses on candidate gene studies, publication bias may still have affected the systematic review (chapter 4). First, the power of the Egger's and Begg's test to detect publication bias in our meta-analyses was relatively low. Second, publication bias has not been tested in the narrative review. As negative data are often not reported, we may have overestimated the genetic associations [54, 57]. Therefore, as was the case with the updated meta-analysis on PPARG Pro12Ala and T2D [58], an update of the meta-analyses on MetS may show weaker associations in few years' time, especially after inclusion of data from GWAS.

Implications for public health

Lifestyle therapy, focusing on weight reduction and increased physical activity, may prevent or treat multiple MetS features simultaneously [27]. However, as adherence to lifestyle therapy is poor and long-term success is modest [59], drug treatment may be necessary. Currently no drugs that target multiple MetS features simultaneously are

available. Therefore, if lifestyle therapy is ineffective, no other option is left than to focus on treatment of individual MetS features.

The concept of MetS has already impacted clinical practice. The attention drawn to the co-occurrence of MetS features has motivated physicians, who diagnosed one of the MetS features, to screen for additional ones [60, 61]. Nowadays screening for the MetS features is, or should be, part of routine clinical assessment. Lifestyle intervention is also part of the treatment options for the individual MetS features. Therefore, it is unclear at the moment whether additional value can be obtained for clinical practice by making the actual diagnosis of MetS, i.e. the presence of three or more features. Diagnosing MetS may have several advantages. It may direct physicians towards prescribing lifestyle therapies that address all MetS features simultaneously, instead of using medication [62, 63]. Furthermore, diagnosis of MetS may motivate patients to make lifestyle changes that prevent progression of MetS to T2D or CVD [64]. Whether diagnosis of MetS will indeed result in the intended behavioral changes of patients and physicians described above, are hypotheses which remain to be tested [64].

Currently no drug for MetS is available. A drug for MetS would target pathophysiological factors responsible for the co-occurrence of MetS features. These pathophysiological factors could be identified by genetic research. Although genetic variants related to complex disease generally have small effects, these genetic variants may still pinpoint potential targets for drug development. Thiazolidinediones and sulfonylureas are examples of drugs which target loci for which a genetic association have been found [65]. Thiazolidinediones and sulfonylureas are diabetic medication, which are ligands for PPARG [66] and for the K_{ATP} channels encoded by KCNJ11 [67], respectively. *PPARG Pro12Ala (rs1801282)* [68, 69] and *KCNJ11 E23K (rs5129)* [69, 70] are the corresponding genetic variants. Although the drugs were on the market before the genetic associations were revealed this example exemplifies the concept that drug development based on genetic associations could be fruitful. Currently, no drug targeting one of the genes associated with MetS is on the market. However, for some of them drugs are being developed. For example, the pharmaceutical industry has developed a small-molecule agonist for MC4R [71]. This drug decreases food intake, but is also associated with an increased blood pressure, an increased heart rate, penile erections and flushing [72]. Due to these side-effects MC4R agonist are ineffective as anti-MetS drug and probably not useful as anti-obesity drug [73]. Furthermore, the discovery of small-molecule activators of the insulin receptor, a target upstream of IRS-1, may also lead to the development of a new drug for MetS [73].

Applications of research on the genetics of MetS seem to be more easily attainable in the field of drug development, than in the field of risk prediction. First, MetS did not add predictive ability to a risk score for diabetes consisting of age, sex, ethnicity, fasting glucose, systolic blood pressure, HDL cholesterol, BMI and parental or sibling history of diabetes [74, 75]. MetS also did not add predictive ability to the Framingham risk score for CVD [75-77]. As MetS itself does not seem to improve traditional risk scores, it is questionable whether genetic variants for MetS will improve these scores. Second, so far genetic variants have added little predictive ability to risk scores for chronic diseases, such as T2D [78] and CVD [79, 80]. Third, as the currently known MetS variants have all been previously identified in studies on individual MetS features, inclusion of genetic variants for MetS will not add information to a risk score for T2D and CVD, which already includes genetic variants for the individual features.

Recommendations for future research

Genetic association studies on complex diseases, such as MetS and its features, have identified hundreds of common genetic variants with relatively small effects. However, these common genetic variants only explain a small proportion of the heritability estimates [81]. So many more genetic variants associated with the clustering of MetS features remain to be discovered.

Up till now most studies on genetic variants associated with the clustering of MetS features, focused on MetS itself, as defined by one of the traditional MetS definitions. However, several improvements to the current MetS definition are conceivable, such that this definition better reflects the clustering. Then it would probably be much easier to identify genetic variants associated with the clustering of MetS features.

Our CFA MetS model with the traditional MetS features had an acceptable model fit. This implies that this model reflected the correlation matrix of the MetS features, i.e. the clustering of MetS features, reasonably well. However, in the CFA MetS model the factor loadings of the MetS features differed, whereas in the current MetS definition the MetS features all have an equal weight. To the best of our knowledge, it has not been previously reported whether a CFA MetS model with equal weights for all MetS features has an acceptable model fit. In the subset of the MORGEN study and in a random sample of EPIC-NL, the model fit was unacceptable when the factor loadings of a one-factor CFA MetS model were kept equal (MORGEN subset CFI 0.92, RMSEA 0.13, SRMR 0.081; random sample of EPIC-NL CFI 0.87, RMSEA 0.1136, SRMR 0.088). This implies that in our study populations a CFA MetS model, in which all factor loadings are equal, badly reflects the clustering of the MetS features. Therefore, a MetS definition with weighted features would probably reflect the clustering of MetS features better than the traditional MetS definition.

Another improvement to the MetS definition could be to design a continuous one, e.g. based on a CFA MetS model. As a continuous MetS definition better reflects the clustering of MetS features and is more powerful, than the traditional bivariate MetS definition, it will be probably easier to identify genetic variants associated with clustering of MetS features with such a continuous MetS definition. Similarly, if the determinant of interest is associated with all MetS features, the conventional MetS-score is more powerful than the bivariate MetS definition. Other advantages of a continuous MetS definition are that with such a definition less information is lost [1, 82]. A continuous MetS definition, could eventually lead to a new MetS definition for clinical practice, by the development of a nomogram, in which each MetS feature is divided in multiple strata [82]. In this nomogram the different MetS features are divided into multiple strata, and not into two strata as is the case with current MetS definition. Therefore a categorical or bivariate MetS definition read from this nomogram will be more refined than the current MetS definition.

In order to increase the predictive ability of MetS for T2D and CVD, it has been proposed to add features to the current definition of MetS. However, for many of these features it is not known whether such a revised MetS model acceptably represents the correlations between the features. More research is necessary to establish which MetS definition optimally predicts T2D and CVD, while still representing one statistical entity. Furthermore, it should be investigated if addition of novel MetS features is cost effective.

Although the one-factor CFA MetS model had an acceptable model fit, the model fit was not perfect. Hence, the correlations between the MetS features, i.e. the clustering

of the MetS features, can not be fully explained by one MetS factor. In order to do so, studies on specific combinations of MetS features should be performed. An advantage of the study of specific combinations of MetS features is that they represent less heterogeneous phenotypes than MetS. Furthermore, it may be much easier to detect genetic variants for a specific combination of MetS features, which are highly genetically correlated, than for MetS, which has a relatively low heritability. We suggest to start with the analysis of available GWAS for the associations between SNP's and those combinations of MetS features, which are highly genetically correlated, such as HOMA-IR and waist circumference.

An alternative approach from studying the clustering of MetS features directly, would be to focus on metabolic traits which are likely to explain part of the clustering of MetS features, such as abdominal obesity, HOMA-IR and FFA. Probably, some of the genetic variants associated with these metabolic traits are also associated with the clustering of MetS features. For example, *FTO* rs9939609 is associated with both abdominal obesity and MetS. Genetic association studies on HOMA-IR and FFA are scarce, i.e. candidate gene studies on these traits are relatively few [83], no GWAS on FFA has been published and only a few GWAS on HOMA-IR are available [84, 85]. Therefore, GWAS on FFA and HOMA-IR, and subsequent association analysis of loci discovered in these GWAS with MetS, may lead to the discovery of novel genetic loci associated with the clustering of MetS features.

One of the ways to find more genetic variants associated with the clustering of MetS features is to study different endpoints, e.g. use a revised MetS definition. However, part of the "heritability gap", i.e. the unexplained genetic variance, may also be filled by the study of alternative genetic variants. Up till now most studies focused on common genetic variants. However, research on rare variants with small or intermediate effects may fill part of the "heritability gap", as nicely illustrated by two recent studies on HDL cholesterol [86] and celiac disease [87]. The study on celiac disease indicates that these rare variants may even be located in genes, in which no common genetic variants have been detected [87]. So, research on rare genetic variants may not only help to close the heritability gap, but also lead to discovery of additional disease genes. Second, structural variations other than SNP's are only measured in a few studies. Therefore, research on copy number or epigenetic variations may explain part of the unidentified genetic variance [81, 88]. Third, gene-gene or gene-environment interactions may also explain part of the unidentified genetic variance [81]. A principle problem of research on gene-environment interactions is the misclassified or biased information on the environment exposure [89]. The study of more reliable environmental exposure markers, e.g. plasma nutrient levels instead of nutrient intake measures, may therefore facilitate the detection of gene-environment interactions. Furthermore, as the sample size required to detect an interaction effect is roughly at least four times the sample size that is needed to evaluate the main effect, the power to detect an interaction effects, either gene-gene or gene-environment, is limited [89]. To address this power issue several novel, more powerful, statistical tests to detect interaction effects have been developed, such as the case-only design, multifactor dimensionally reduction, tree based approaches, data-mining approaches and Bayesian modeling approaches [90, 91]. Nevertheless, the research output from these techniques, i.e. the number of confirmed interaction effects, has been limited so far.

In the above mentioned research suggestions, we focussed on further studies through which more genetic variance, responsible for the clustering of MetS features, can be explained. We suggested to study alternative endpoints, alternative genetic variants or

to focus on interaction effects. Up till now most genetic research on the clustering of MetS features suggests that the clustering of MetS features is not caused by one, but by several mechanisms. Therefore, it would make sense to define several subtypes of MetS based on the different mechanisms involved in the clustering of MetS features. For diabetes, the differentiation on a pathophysiological basis between type 1 and type 2 made by Roger Himsworth in 1936 [28], resulted eventually in differential treatment for type 1 and type 2 diabetes, i.e. insulin treatment for type 1 and sulfonylureas treatment for type 2 diabetes [92]. Possibly a differentiation between several pathophysiological distinct MetS subtypes would also result in subtype specific treatment and prevention strategies. In order to be able to define these subtypes, our understanding of the pathophysiological processes involved in MetS development should be improved, either by genetic association studies or by other mechanistically oriented studies.

Conclusion

The clustering of MetS features can be studied by one MetS factor or by specific combinations of MetS features. In case one MetS factor is used, this MetS factor should be powerful and reasonably well represent the correlation matrix between the different MetS features. Our results suggest that it may be best to design a continuous MetS definition in which the features have different weights. In order to increase predictive ability for T2D and CVD of MetS, hsCRP may be added, if cost effective. We showed that one MetS factor will not be able to fully explain the clustering of MetS features. In order to do so, specific combinations of MetS features should be studied. Based on the review of genetic correlation coefficients, genetic pleiotropy was largest between plasma HDL cholesterol and triglycerides (median $r^2_{genetic}$ = 0.50) and between waist circumference and HOMA-IR (median $r^2_{genetic}$ = -0.45). For these two pairs of MetS and MetS related features, it will probably be relatively easy to detect genetic variants.

This thesis shows that the SNP's associated with the clustering of MetS features are involved in weight regulation, glucose and insulin metabolism, lipid metabolism and inflammation. Many of the SNP's involved in lipid metabolism are located in, or in close proxy to, the *APOA1/C3/A4/A5* gene cluster. Interestingly, although the genetic correlation between adiponectin and MetS was relatively high, the number of SNP's associated with both adiponectin and MetS was small. Probably, these SNP's remain to be discovered. The SNP's associated with the clustering of MetS features, could influence this clustering in different ways. First, such as observed for *FTO* rs9939609, the effect on one MetS feature could mediate the effect on other MetS features, therewith affecting the clustering. Second, a SNP could be associated with multiple MetS features through independent pathways, such as observed for *MC4R* rs17782313, *APOE Cys112Arg* (rs429358) and *CETP Ile405Val* (rs5882). Third, a SNP could modulate the association between two MetS features, e.g. *PPARGC1A Gly482Ser* (rs8192678) modulated the association between BMI and glucose.

The SNP's associated with the clustering of MetS features are involved in several different mechanisms. This suggests that, although the MetS features can be represented with one statistical entity, there are multiple underlying mechanisms explaining its development. Therefore, it would make sense to define several subtypes of MetS based on the different mechanisms explaining the clustering of MetS features, like was done for e.g. diabetes mellitus.

Up till now the translation of genetic research into public health relevance has been limited. In the future, discovery of pathophysiological factors associated with multiple MetS features, identified by genetic research, may hopefully lead to the development of new preventive and treatment strategies, targeting multiple MetS features simultaneously.

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Povel CM

Summary
Samenvatting

Summary

Abdominal obesity, hyperglycemia, hypertriglyceridemia, low serum HDL cholesterol levels and hypertension frequently co-occur within individuals. According to the most recent definition, people in whom three or more of these clinical features co-occur are considered to have metabolic syndrome (MetS). People with MetS are at a twofold increased risk for developing coronary heart disease (CHD) and at a fivefold increased risk for developing type 2 diabetes (T2D) in the next five to ten years. In Western societies the prevalence of MetS among adults is high, around 25-30%, and is expected to rise.
Currently, it is unclear how, i.e. by which endpoints, the clustering of MetS features can be studied best. Also the pathophysiology behind the clustering of MetS features is not fully understood. Genetic association studies on MetS or on specific combinations of MetS features may provide more insight in this pathophysiology.

In chapter 2 we tested the model fit of several one-factor MetS models consisting of traditional and novel MetS features, such as hsCRP, uric acid, albumin or liver enzymes, using confirmatory factor analysis in the EPIC-NL case-cohort study (random sample *n*=1928; incident T2D cases *n*=545, incident CVD cases *n*=1312). Three one-factor MetS models had a good model fit and represented one entity, i.e. a standard model consisting of the traditional MetS features, a standard model excluding blood pressure and a standard model including high sensitive C-reactive protein (hsCRP). This suggests that MetS defined according to one of these three models, represents a single disorder, at least from a statistical point of view. Of the three models, the model which additionally included hsCRP predicted T2D and CVD the best. Therefore, inclusion of hsCRP in future MetS definitions may be considered.
In chapter 3 we have reviewed 9 twin and 19 family studies, describing in total 239 genetic correlation coefficients between MetS and MetS related features. Genetic correlations were strongest between waist circumference and HOMA-IR (median $r^2_{genetic}$=0.50), HDL cholesterol and triglycerides (median $r^2_{genetic}$=-0.45), and between adiponectin and MetS (median $r^2_{genetic}$=-0.32). This implies that for these combinations of MetS features, it will probably be relatively easy to identify common genetic variants.
In chapter 4 and 5 we identified genetic variants associated with MetS. In chapter 4 we systematically reviewed candidate gene studies on MetS published before the 2nd of June 2010. In total we included 88 studies on 25 genes in this review. The minor alleles of rs9939609 *(FTO)*, rs7903146 *(TCF7L2)*, *C56G (APOA5)*, *T1131C (APOA5)*, *C482T (APOC3)*, *C455T (APOC3)* and *174G>C (IL6)* were more prevalent in subjects with MetS than in subjects without MetS, whereas the minor allele of Taq-1B *(CETP)* was less prevalent in subjects with MetS. Most single nucleotide polymorphisms (SNP's) associated with MetS were located in genes involved in lipid metabolism. This suggests that besides insulin resistance and weight regulation, lipid metabolism may also play a central role in MetS development.
In chapter 5 we analyzed whether SNP's shown to be associated with inflammatory biomarkers, waist circumference, insulin resistance, serum HDL cholesterol or triglycerides in GWAS, were associated with MetS and MetS-score in a random sample of the EPIC-NL study (*n*=1886). On the group level, the waist circumference SNP's were significantly associated with the prevalence of MetS, and the insulin resistance SNP's with the MetS-score. The groups of lipid and inflammation SNP's were neither associated with

MetS nor with the MetS-score. The lack of an association between the group of lipid SNP's and MetS may be explained by the small effect of these SNP's on lipid levels in the EPIC-NL study. These results in EPIC-NL support the hypothesis that weight regulation and insulin resistance are involved in MetS development. Individual SNP's which were associated with MetS were *MC4R* rs17782312, involved in weight regulation, and *IRS1* rs2943634, related to insulin resistance. These SNP's may explain part of the genetic variation in MetS.

In chapter 6 and 7 we have investigated the association between specific combinations of MetS features and 373 SNP's of genes located in transcriptional pathways of glucose and lipid metabolism in the Doetinchem cohort. In chapter 6 we have studied whether these SNP's were related to multiple MetS features. Two SNP's, *CETP Ile405Val* (rs5882) and *APOE Cys112Arg* (rs429358), were associated with both the prevalence of low HDL cholesterol levels and the prevalence of abdominal obesity. For both SNP's, the association with HDL cholesterol was partly independent of the association with abdominal obesity and partly mediated by obesity. This indicates that the associations with HDL cholesterol and abdominal obesity work, at least partly, through independent pathways.

In chapter 7 we studied whether 353 of the aforementioned SNP's were associated with glucose levels, in interaction with body mass index (BMI). Two highly correlated SNP's in the *PPARGC1A* gene, *Gly482Ser* (rs8192678) and *Thr528Thr* (rs3755863), showed a significant interaction with BMI. The rare allele of both SNP's was significantly associated with lower glucose levels in subjects with a BMI $\leq$ 25kg/m^2. An inverse association was suggested in subjects with a BMI $\geq$ 28kg/m^2. A small intervention study, the SLIM study, showed similar, though non-significant, results. Based on these results it can be speculated that people homozygous for the rare allele of the *Gly482Ser* and *Thr528Thr* SNP's are more susceptible to develop hyperglycemia in an obesogenic environment, but less susceptible to develop hyperglycemia in a non-obesogenic environment.

In conclusion, our results suggest that the traditional MetS features, with or without hsCRP, can acceptably be represented by one statistical entity. However, in order to fully explain the clustering of MetS features, specific combinations of MetS features, especially those which are highly genetically correlated, notably HOMA-IR and waist circumference and HDL cholesterol and triglycerides, should be studied. A SNP could influence the co-occurrence of two MetS features in different ways, i.e. the effect a SNP has on one MetS feature can be explained by the effect the SNP has on another MetS feature, a SNP influences two MetS features through two independent pathways or a SNP may change the strength of an association existing between two MetS features. We showed that SNP's associated with the clustering of MetS features are involved in several different mechanisms. For example rs7903146 *(TCF7L2)* and *IRS1* rs2943634 are involved in glucose metabolism, C56G *(APOA5)* and C482T *(APOC3)* in lipid metabolism, *MC4R* rs17782312 and *FTO* rs9939609 in weigh regulation and *174G>C (IL6)* in inflammation. This suggests that, although the MetS features may represent a single statistical entity, there are multiple, related, mechanisms underlying its development.

Samenvatting

Abdominale obesitas, hyperglycemia, hypertriglyceridemia, een laag serum HDL-cholesterol en een hoge bloeddruk komen vaak samen voor. Volgens de meest recente definitie, hebben mensen met drie of meer van deze kenmerken het metabool syndroom (MetS). Mensen met MetS hebben een twee maal verhoogd risico op type 2 diabetes (T2D) en een vijf maal verhoogd risico op coronaire hart ziekten. In de Westerse wereld is de prevalentie van MetS hoog, ongeveer 25-30%. Men verwacht dat deze prevalentie nog verder zal stijgen.
Op dit moment is het onduidelijk hoe, oftewel met welke eindpunten, de clustering van MetS kenmerken het best bestudeerd kan worden. Ook de pathofysiologie onderliggend aan deze clustering is nog niet volledig ontrafeld. Genetische associatie studies naar MetS of naar specifieke combinaties van MetS kenmerken kunnen meer inzicht geven in deze pathofysiologie.

In hoofdstuk 2 hebben we met behulp van "confirmatory factor analyses" een aantal verschillende één-factor MetS modellen geanalyseerd in het EPIC-NL case-cohort (mensen uit de random steekproef *n*=1928; incidente T2D patiënten *n*=545; incidente hart- en vaatziekten patiënten *n*=1312). Deze modellen waren opgebouwd uit traditionele en potentieel nieuwe MetS kenmerken, zoals hoog gevoelig C-reactief proteïne, urinezuur, albumine of leverenzymen. Drie één-factor MetS modellen, namelijk een standaard model bestaande uit de traditionele MetS kenmerken, een standaard model inclusief hoog gevoelig C-reactief proteïne en een standaard model zonder bloeddruk waren statistisch valide en stelde een entiteit voor. Dit betekent dat MetS gedefinieerd volgens één van deze drie modellen, in ieder geval vanuit statistisch perspectief, één ziekte is. Het standaard model inclusief hoog gevoelig C-reactief proteïne voorspelde van deze drie valide modellen type T2D en HVZ het beste. Gebaseerd op dit resultaat, is de mogelijkheid om hoog gevoelig C-reactief proteïne op te nemen in de definitie van het metabool syndroom, het overwegen waard.
In hoofdstuk 3 hebben we de resultaten van 9 tweeling en 19 familie studies, waarin 239 genetisch correlatiecoëfficiënten tussen MetS- en MetS gerelateerde kenmerken beschreven waren, samengevat. De genetische correlaties waren het sterkst tussen middelomtrek en HOMA-IR (mediaan $r^2_{genetisch}$=0.50), tussen HDL-cholesterol en triglyceriden (mediaan $r^2_{genetisch}$=-0.45) en tussen adiponectine en MetS (mediaan $r^2_{genetisch}$=-0.32). Dit betekent dat het voor deze combinaties van MetS kenmerken waarschijnlijk relatief gemakkelijk is om gemeenschappelijke genetische varianten in het humane DNA te vinden.In hoofdstuk 4 en 5 hebben we genetische varianten geïdentificeerd die geassocieerd zijn met MetS.
In hoofdstuk 4 hebben we de resultaten van kandidaat-gen studies gepubliceerd voor 2 juni 2010 samengevat. We hebben 88 studies, waarin 25 genen beschreven waren, besproken. Het minst voorkomende allel van rs9939609 *(FTO)*, rs7903146 *(TCF7L2), C56G (APOA5), T1131C (APOA5), C482T (APOC3), C455T (APOC3)* en *174G>C (IL6)* had een hogere prevalentie in mensen met MetS dan in mensen zonder MetS. Het minst voorkomende allel van Taq-1B *(CETP)* had juist een hogere prevalentie in mensen met MetS. De meeste genetische varianten die geassocieerd waren met MetS waren afkomstig uit genen die betrokken zijn bij het vetmetabolisme. Dit kan er op duiden dat behalve insulineresistentie en gewichtsregulatie ook het vetmetabolisme een rol kan spelen bij het ontstaan van MetS.

In hoofdstuk 5 hebben we bestudeerd of genetische varianten waarvan bekend is dat ze in genoombrede associaties studies geassocieerd zijn met inflammatoire biomarkers, insulineresistentie, serum HDL-cholesterol of triglyceriden, ook geassocieerd zijn met MetS of met MetS-score in een steekproef van deelnemers uit EPIC-NL (*n*=1886). Op groepsniveau waren de genetische varianten die oorspronkelijk gerelateerd waren met middelomtrek geassocieerd met de prevalentie van het MetS en waren de genetische varianten die oorspronkelijk gerelateerd waren met insulineresistentie geassocieerd met MetS-score. De groep met genetische varianten afkomstig uit het vet of inflammatie metabolisme waren niet geassocieerd met MetS, noch met MetS-score. De afwezigheid van een associatie tussen genetische varianten afkomstig uit het vetmetabolisme en MetS kan misschien verklaard worden door de zwakke associatie tussen deze genetische varianten en lipiden niveaus in de EPIC-NL studie. De resultaten van de EPIC-NL studie ondersteunen de hypothese dat gewichtsregulatie en insulineresistentie betrokken zijn bij de ontwikkeling van MetS. Individuele genetische varianten die geassocieerd waren met MetS waren *MC4R* rs17782312, een genetische variant betrokken bij gewichtsregulatie, en *IRS1* rs2943634, een genetische variant gerelateerd aan insuline resistentie. Deze genetische varianten kunnen mogelijk de genetische component van MetS gedeeltelijk verklaren.

In hoofdstuk 6 en 7 hebben we in het Doetinchem cohort de associatie tussen specifieke combinaties van MetS kenmerken en 373 genetische varianten gelegen in genen afkomstig uit glucose en lipiden transcriptie mechanisme onderzocht. In hoofdstuk 6 hebben we bestudeerd of deze genetische varianten gerelateerd zijn met meerder MetS kenmerken. Twee genetische varianten, *CETP Ile405Val* (rs5882) en *APOE Cys112Arg* (rs429358), waren geassocieerd met zowel de prevalentie van een laag HDL-cholesterol als met de prevalentie van abdominale obesitas. Voor beide genetische varianten was de associatie met HDL-cholesterol gedeeltelijk afhankelijk en gedeeltelijk onafhankelijk van de associatie met abdominale obesitas. Dit betekent dat de associatie met HDL-cholesterol en de associatie met abdominale obesitas, in ieder geval gedeeltelijk, door onafhankelijke mechanismes te weeg wordt gebracht.

In hoofdstuk 7 hebben we bestudeerd of 353 van de eerder genoemde genetische varianten in interactie met BMI geassocieerd waren met glucose niveaus. Twee hoog gecorreleerde genetische varianten uit het *PPARGC1A* gen, namelijk *Gly482Ser* (rs8192678) en *Thr528Thr* (rs3755863), vertoonden een significant interactie effect met BMI. Het minst voorkomende allel van beide genetische varianten was significant geassocieerd met lagere glucose niveaus in deelnemers met een BMI ≤ 25kg/m^2. Er leek een inverse associatie te zijn in deelnemers met een BMI ≥ 28kg/m^2. Een kleine interventie studie, de SLIM studie, liet een soortgelijk, maar niet significant resultaat zien. Gebaseerd op deze resultaten kan men speculeren dat mensen die homzygoot zijn voor het minst voorkomende allel van de *Gly482Ser* en *Thr528Thr* genetische varianten extra gevoelig zijn om hyperglycemia te ontwikkelen in een obesogene leefomgeving, maar juist minder gevoelig zijn om hyperglycemia te ontwikkelen in een niet-obesogene leefomgeving.

Concluderend, onze resultaten suggereren dat de traditionele MetS kenmerken, met of zonder hoog gevoelig C-reactief proteïne, één statistische entiteit voorstellen. Maar om de clustering van MetS kenmerken volledig te kunnen verklaren moeten specifieke combinaties van MetS kenmerken bestudeerd worden. Het is vooral belangrijk om MetS kenmerken, die hoog genetische gecorreleerd zijn, zoals HOMA-IR en middelomtrek en HDL-cholesterol en triglyceriden, nader te bestuderen. Een genetische variant kan er op verschillende manieren voor zorgen dat twee MetS kenmerken vaak samen voorkomen. In de eerste plaats kan het effect dat een genetische variant heeft op een MetS kenmerk volledig verklaard worden door het effect dat de genetische variant heeft op een ander MetS kenmerk. In de twee plaats kan een genetische variant twee MetS kenmerken via twee onafhankelijke mechanismes beïnvloeden. In de derde plaats kan een genetische variant de sterkte van de associatie tussen twee MetS kenmerken veranderen. We hebben laten zien dat genetische varianten die geassocieerd waren met clustering van MetS kenmerken betrokken zijn bij verschillende mechanismes. Bijvoorbeeld rs7903146 *(TCF7L2)* en *IRS1* rs2943634 zijn betrokken bij het glucose mechanisme, *C56G (APOA5)* en *C482T (APOC3)* bij het lipiden metabolisme, *MC4R* rs17782312 en *FTO* rs9939609 bij gewichtsregulatie en *174G>C (IL6)* bij inflammatie. Dit suggereert dat, hoewel de MetS kenmerken een statistische entiteit voorstellen, er meerdere gerelateerde mechanismes aan het MetS ten grondslag liggen.

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GWAS

HOMA-IR

APOE

MORGEN

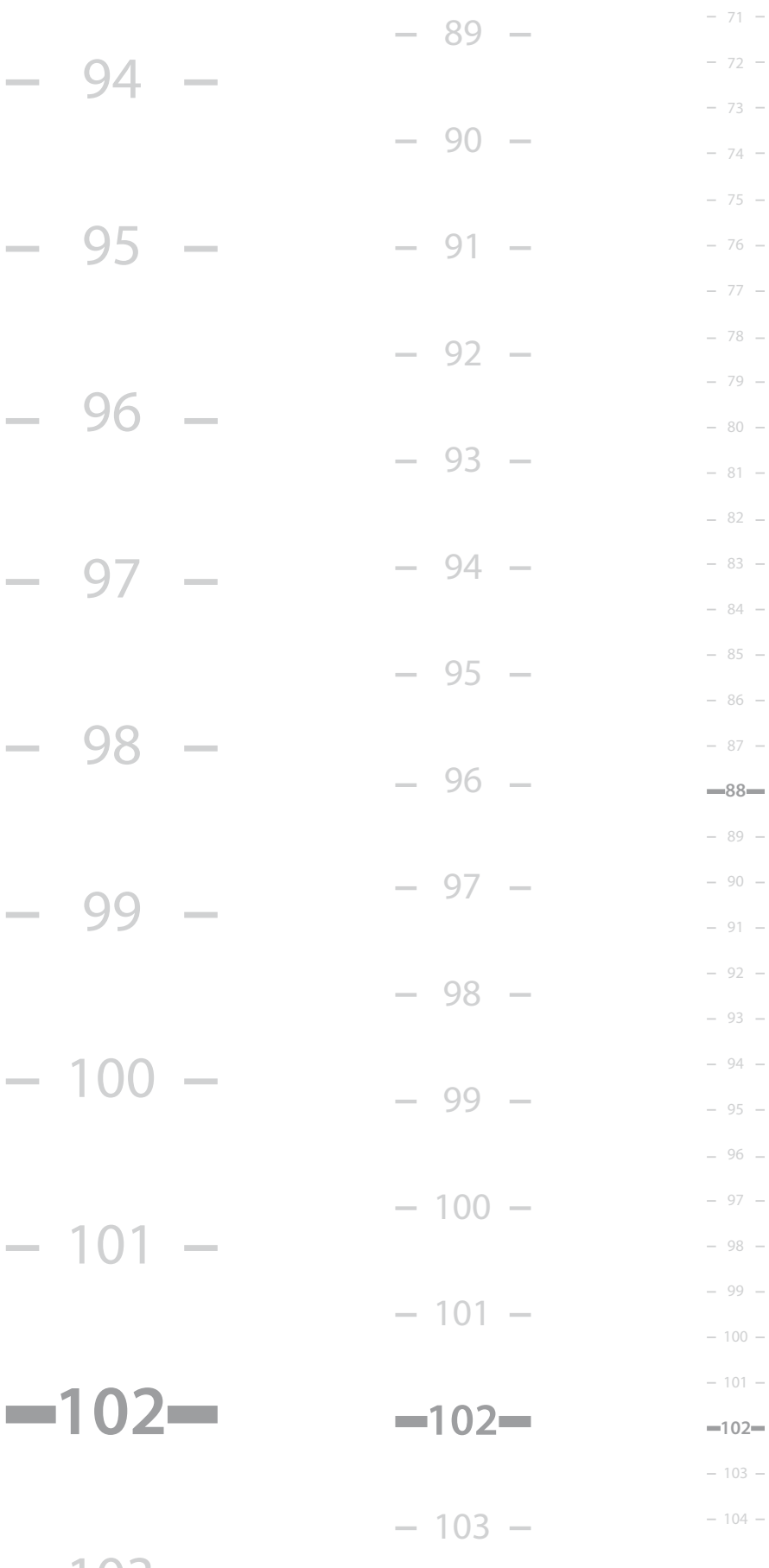
CETP

Abbreviations

Abbreviations

Adip	adiponectin
ALT	Aminotransferase
APOE	Apolipoprotein-E
AST	Aspartate aminotransferase
ATGL	Adipose tissue triglyceride lipase
BMI	Body mass index
BP	Blood pressure
CETP	Cholesteryl ester transfer protein
CFA	Confirmatory factor analysis
CFI	Comparative fit index
CHD	Coronary heart disease
CPAI	Cambridge Phyiscal Activity Index
CRP	C-reactive protein
CVD	Cardiovascular diseases
CVA	Cerebro Vascular Accident
DBP	Diastolic blood pressure
EFA	Explanatory factor analysis
EPIC	European Investigation into Cancer and Nutrition
FDR	False discovery rate
FFA	Free fatty acids
FPG	Fasting plasma glucose
FVII	Factor VII
GGT	Gamma glutamyltransferase
GWAS	Genome wide association study
HbA1C	Haemoglobin A1c
HDL	High Density Lipoprotein
HMW	High molecular weight
HOMA-IR	Homeostasis model assessment insulin resistance
hsCRP	High sensitive C-reactive protein
HSL	Hormone sensitive lipase
HWE	Hardy Weinberg Equilibrium
ICAM-1	Intracellular adhesion molecule-1
INS	Insulin
IR	Insulin resistance
LDL	Low Density Lipoprotein
LMW	Low molecular weight
LPL	Lipoprotein lipase

MAF	Minor allele frequency
MORGEN	Monitoring Project on Risk Factors for Chronic Diseases
MetS	Metabolic Syndrome
NO	Nitric oxide
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor 1
PR	Prevalence ratio
RMSEA	Root mean square error of approximation
SBP	Systolic blood pressure
SD	Standard deviation
SLIM	Study on Lifestyle intervention and Impaired glucose tolerance Maastricht
SNP	Single nucleotide polymorphism
SRMR	Standardized root means square residual
STRS	Short tandem repeats
TG	Triglyceride
t-PA	Tissue plasminogen activator
VLDL	Very Low Density Lipoprotein
WC	Waist circumference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Dankwoord

Dankwoord

Nu, 4 jaar na mijn eerste werkdag als AIO, mag ik mijzelf doctor noemen. Doctor is een persoonlijke titel, maar bij het verkrijgen ervan hebben heel veel mensen mij geholpen. Ieder op zijn of haar eigen manier. Zeker is dat zonder de motiverende woorden, wetenschappelijke input, luisterende oren en gezellig praatjes, dit boekje er niet geweest was. Van alle mensen, die voor mij de afgelopen jaren van betekenis zijn geweest, wil ik er hieronder een aantal naar voren halen.

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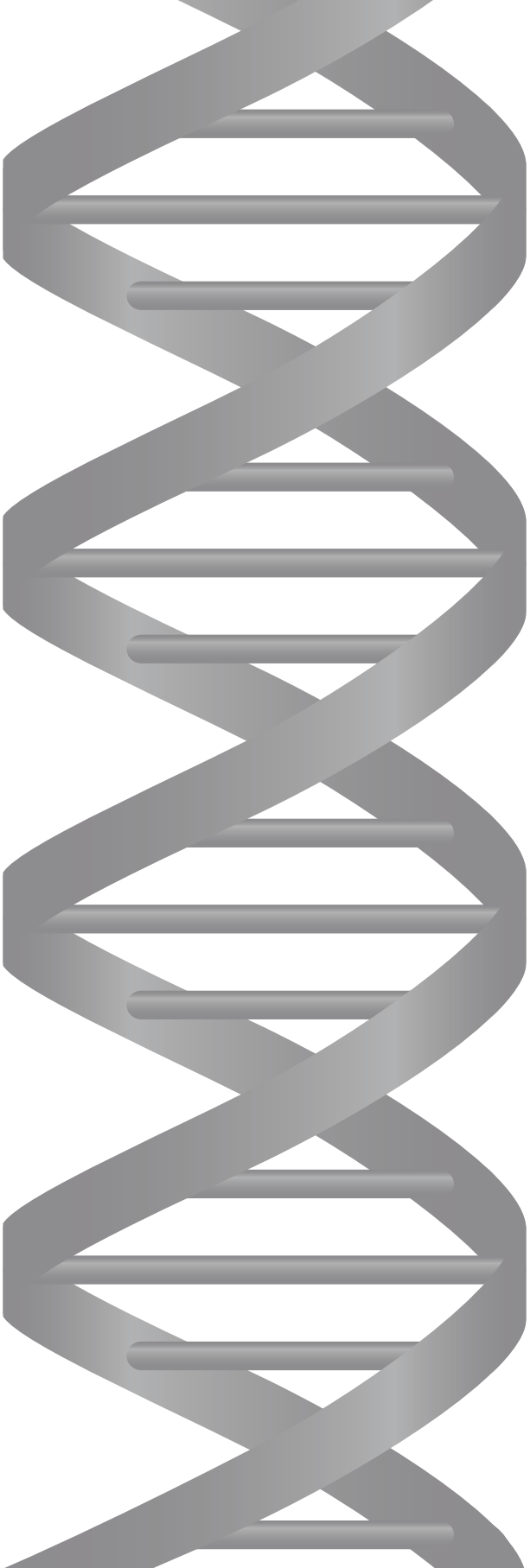

Povel CM

About the author

Curriculum Vitae

Cécile Povel was born on March 13th, 1984 in Leidschendam, the Netherlands. After completing secondary school at the "Christelijk Gymnasium" in Utrecht, she started her studies in Nutrition and Health at Wageningen University. She obtained her Master's degree (cum laude) with a major in Epidemiology and Public Health and a minor in Food Law in 2008. Her Master's thesis Food Law at Wageningen University resulted in a publication "Scientific Substantiation of Health Claims The Soft Core of the Claims Regulation", in the European Food and Feed Law Review. Her Master's thesis Epidemiology at Wageningen University resulted in a publication "Hemachromatosis(HFE) genotype and atherosclerosis: Increased susceptibility to iron-induced vascular damage in C282Y carriers?" in Atherosclerosis. She conducted two internships: one in Food Law at Unilever R&D, Vlaardingen and one in Epidemiology at Karolinksa Institute, Stockholm, Sweden. In 2008, Cécile was appointed as PhD student at the Division of Human Nutrition of Wageningen University and at the Centre for Nutrition and Health (CVG) of the National Institute for Public Health and the Environment (RIVM). She investigated which metabolic endpoints should be studied in order to explain the clustering of metabolic syndrome features, i.e. abdominal obesity, hyperglycemia, hypertriglyceridemia, low HDL cholesterol levels and hypertension. By studying genetic variants associated with the clustering of metabolic syndrome features, she aimed to better understand the pathophysiology behind this clustering. As part of her PhD thesis, she wrote two reviews, as well as several epidemiological papers. During her PhD project, Cécile joined the educational program of graduate school VLAG, attended several international conferences and was involved in teaching. Furthermore, she was a member of the research committee at the Division of Human Nutrition and member of the organising committee of the PhD study tour to Denmark, Sweden and Finland in 2009.

List of publications

Publications in peer-reviewed journals

- Cécile Povel, Bernd van der Meulen, Scientific Substantiation of Health Claims The Soft Core of the Claims Regulation, European Food and Feed Law Review, 2|2007, p 82-90
- Povel CM, Feskens EJM, Imholz S, Blaak EE, Boer JMA, Dollé ME, Glucose levels and genetic variant across transcriptional pathways: interaction effects with BMI, Int J Obes, 34(5), 2010, p 840-845
- Engberink MF, Povel CM, Durga J, Swinkels DW, de Kort WL, Schouten EG, Verhoef P, Geleijnse JM, Hemachromatosis (HFE) genotype and atherosclerosis: Increased susceptibility to iron-induced vascular damage in C282Y carriers?, Atherosclerosis, 211(2), 2010, p520-525
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- Povel CM, Boer JM, Feskens EJM, Shared genetic variance between the features of the metabolic syndrome: Heritability studies, Mol Genet Metab, 104(4), 2011, p 666-669

Submitted papers

- Povel CM, Beulens JW, van der Schouw YT, Dollé MET, Spijkerman AMW, Verschuren WMM, Feskens EJM, Boer JMA, Metabolic syndrome model definitions predicting type 2 diabetes and cardiovascular disease
- Povel CM, Boer JMA, Onland-Moret NC, Dollé MET, Feskens EJM, van der Schouw YT, SNP's involved in insulin resistance, weight regulation, lipid metabolism and inflammation in relation to metabolic syndrome

Abstracts in scientific journals or proceedings

- Povel CM, Feskens EJM, Imholz S, Blaak EE, Boer JMA, Dollé ME, Glucose levels and genetic variant across transcriptional pathways: interaction effects with BMI, Eur J Clin Nutr, 2009, 63 (Suppl. 3), p 14 (Wageningen Nutritional Science Forum, 2009, Arnhem, The Netherlands, poster presentation)
- Povel CM, Feskens EJM, Imholz S, Blaak EE, Boer JMA, Dollé ME, Glucose levels and genetic variant across transcriptional pathways: interaction effects with BMI, In: abstract book of the Annual Meeting of the EDEG, 2009, Wageningen, the Netherlands (poster presentation)
- Povel CM, Feskens EJM, Imholz S, Blaak EE, Boer JMA, Dollé ME, Glucose levels and genetic variant across transcriptional pathways: interaction effects with BMI, Diabetologia, 2009, 52 (Suppl. 1), p 142 (EASD Annual Meeting, 2009, Vienna, Austria, poster presentation)
- Povel CM, Boer JMA, Imholz S, Dolle ME, Feskens EJM, Genetic variants in lipid metabolism are independently associated with multiple features of the metabolic syndrome, In: abstract book of WEON, 2010, Nijmegen, the Netherlands (oral presentation)

- Povel CM, Boer JMA, Reiling E, Feskens EJM, Genetic variants and the metabolic syndrome: a systematic review, In: abstract book of NVDO, 2010, Oosterbeek, the Netherlands (oral presentation)
- Povel CM, Boer JMA, Reiling E, Feskens EJM, Genetic variants and the metabolic syndrome: a systematic review, J Diabetes, 2011, 3 (Suppl. 1), p 126-127 (International Conference on Pre-diabetes and the Metabolic Syndrome, 2011, Madrid, Spain, poster presentation)
- Povel CM, Boer JM, Feskens EJM, Shared genetic variance between the features of the metabolic syndrome: Heritability studies, J Diabetes, 2011, 3 (Suppl. 1), p 126 (International Conference on Pre-diabetes and the Metabolic Syndrome, 2011, Madrid, Spain, poster presentation)
- Povel CM, Boer JMA, Onland-Moret NC, Dollé MET, Feskens EJM, van der Schouw YT, SNPs involved in insulin resistance, weight regulation, lipid metabolism and inflammation in relation to metabolic syndrome, In: abstract book of WEON, 2011, Ijmuiden, the Netherlands (poster presentation)
- Povel CM, Beulens JW, van der Schouw YT, Dollé MET, Spijkerman AMW, Verschuren WMM, Feskens EJM, Boer JMA, Metabolic syndrome model definitions predicting type 2 diabetes and cardiovascular disease, In: abstract book of NVDO, 2011, Oosterbeek, the Netherlands (oral presentation)

Overview of completed training activities

Discipline specific courses and meetings

- Annual conference of the Netherlands Epidemiology Society (WEON), 2008 (Groningen, the Netherlands)
- Nutrigenomics Consortium (NuGo) week, 2008 (Potsdam, Germany)
- SNP's and Human Diseases, 2008 (Rotterdam, the Netherlands)
- Course Epigenesis and epigenetics, 2008 (Wageningen, the Netherlands)
- Wageningen Nutritional Science Forum, 2009 (Arnhem, the Netherlands)
- Annual Meeting of the European Diabetes Epidemiology Group (EDEG), 2009 (Wageningen, the Netherlands)
- Masterclass Nutrigenomics, 2009 (Wageningen, the Netherlands)
- European Association for the Study of Diabetes (EASD) Annual Meeting, 2009 (Vienna, Austria)
- Genetic Epidemiology, 2010 (Utrecht, the Netherlands)
- Annual conference of the Netherlands Epidemiology Society (WEON), 2010 (Nijmegen, the Netherlands)
- Annual meeting of the Dutch Association for Diabetes Research (NVDO), 2010 (Oosterbeek, the Netherlands)
- International conference on pre-diabetes and metabolic syndrome, 2011 (Madrid, Spain)
- Annual conference of the Netherlands Epidemiology Society (WEON), 2011 (IJmuiden, the Netherlands)
- Annual meeting of the Dutch Association for Diabetes Research (NVDO), 2011 (Oosterbeek, the Netherlands)

General courses and activities

- PhD Introduction Course, 2008 (Eindhoven, the Netherlands)
- Philosophy and ethics of food science and technology, 2009 (Wageningen, the Netherlands)
- NWO training day 'Developing your brand' and 'Negotiation', 2009 (Utrecht, the Netherlands)
- Scientific Writing, 2009 (Wageningen, the Netherlands)
- PhD-assessment, 2009 (Wageningen, the Netherlands)
- NWO training day 'Leadership skills for beginners' and 'Networking', 2010 (Utrecht, the Netherlands)
- Interpersonal Communication for PhD students, 2010 (Wageningen, the Netherlands)
- Theme meeting of The Royal Netherlands Academy of Arts and Sciences (KNAW), 2011 (Amsterdam, the Netherlands)
- NWO Science meets press event (bessensap), 2011 (the Hague, the Netherlands)
- Masterclass Multilevel Analysis, 2011 (Wageningen, the Netherlands)

Optional courses and activities

- Preparing a PhD research proposal
- Nutritional Genomics and Genetics Course, 2008 (Wageningen, the Netherlands)
- Organizing and participating in PhD study tour to Denmark, Sweden en Finland, 2009
- Participating in PhD study tour to Mexico and USA, 2011
- Literature and discussion groups 'Journal Club', 'Oldsmobiles', 'Epi-Research', 'Methodology Club', 'Rothman-lunches' and 'CVG lectures', 2008-2012

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Colophon

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