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Genetic variants within the *LPIN1* gene, encoding lipin, are influencing phenotypes of the metabolic syndrome in humans

Wiedmann S MSc[1,4], Fischer M MD[1,4], Koehler M[1], Neureuther K MSc[1], Riegger G MD[1], Doering A MD[2], Schunkert H MD[3], Hengstenberg C MD[1,5], Baessler A MD[1,5]

[1]Clinic for Internal Medicine II, University of Regensburg, Regensburg, Germany
[2]GSF-National Research Center for Environment and Health, Institute for Epidemiology, Neuherberg, Germany
[3]Clinic for Internal Medicine 2, University of Schleswig-Holstein, Lübeck, Germany
[4]These authors contributed equally to this work
[5]To whom correspondence should be adressed.

Running Title: *LPIN1* gene and metabolic syndrome in human

Corresponding authors:
Andrea Baessler, M.D. or Christian Hengstenberg, M.D.
Clinic for Internal Medicine 2
University of Regensburg
Franz-Josef-Strauss-Allee 11
93053 Regensburg
E-mail: andrea.baessler@klinik.uni-regensburg.de
christian.hengstenberg@klinik.uni-regensburg.de

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ABSTRACT

Objective: Lipin, a novel molecular protein expressed by adipocytes, has marked effects on adipose tissue mass, insulin sensitivity, and glucose homeostasis. Thus, we hypothesized that genetic variants within *LPIN1* are associated with traits of the metabolic syndrome (MetS).

Research Design and Methods: 15 SNPs covering the *LPIN1* gene region were genotyped in an age- and sex stratified sample of the general population (MONICA study Augsburg, DNA and phenotypes of 1,416 Caucasians). Ten of these SNPs were also genotyped for replication in an independent sample of 1,030 subjects recruited throughout Germany. The MetS was defined via the sum of its core components, and additionally, by a factor score derived from factor analysis. Permutation based methods were used to test the association between genetic *LPIN1* variants and metabolic traits for empirical significance.

Results: Linkage disequilibrium (LD) analysis revealed three LD blocks encompassing *LPIN1*. We identified three associated three-marker haplotypes: one common haplotype (26.8% frequency) increases the risk for the MetS (OR=1.6 95% CI: [1.2-2.2]), while the other two being less common (5.7% and 4.0%) are strongly associated with lower blood pressure levels (systolic BP: 127±18 vs. 135±20 mmHg, p=0.0001), a lower BMI (24.6±3.6 vs. 26.9±4.1 kg/m^2, p=3.7*10^{-7}) and waist circumference (82±12 vs. 90±12, p=3.2*10^{-8}), lower HbA1c levels (5.1±0.7 vs. 5.3±0.9, p=0.0002) as well as a lower MetS factor score (-0.67±1.00 vs. 0.04±1.24, p=1.4*10^{-7}). Furthermore, the frequencies of arterial hypertension (23.7 vs. 46.4%, p=0.00001), obesity (12.9 vs. 30.8%, p=0.0003), diabetes (2.2 vs. 8.2%, p=0.041), and the presence of ≥3 MetS components (3.3 vs. 13.7%, p=0.002) were significantly lower than in subjects not carrying one of these protective haplotypes. Strong associations were also observed in the replication sample using the same haplotypes, but with effects in the opposite direction.

Conclusions: These data suggest that allelic variants of the *LPIN1* gene have significant effects in human metabolic traits and thus implicate lipin in the pathophysiology of the metabolic syndrome.

Obesity has been described as the central causative component in the development of the metabolic syndrome (MetS). In fact, increased adiposity is associated with many metabolic alterations, including insulin resistance, dyslipidemia, and hypertension, all key components of the MetS. It is now evident that adipose cells secrete multiple bioactive molecules collectively referred to as adipokines, and many of these have been implicated in the association between central adiposity and cardiovascular pathology. From the wide range of adipokines identified over the past few years, it is apparent that adipose tissue is a secretory organ of considerable complexity that is closely integrated into overall physiological and metabolic control. Both in humans and in animal models, it has been shown that either increased adipose tissue mass as seen in obesity or abnormally low amounts of adipose tissue as seen in lipodystrophy, lead to metabolic dysregulation and insulin resistance (1-3). Thus, factors that influence adipose tissue mass and function exert important effects on metabolic homeostasis (4).

Human and rodent data provide evidence that lipin is one crucial factor involved in the development and function of adipose tissue (5, 6). Lipin is a novel protein that is prominently expressed in peripheral tissues, namely adipose tissue and skeletal muscle. It has been identified through positional cloning of the mutated gene (*LPIN1*) in a mouse model of lipodystrophy (fatty liver dystrophy mouse, fld) which is characterized by lipin deficiency, reduction of adipose tissue mass, mild hyperglycemia and insulin resistance (5,7). In contrast, enhanced lipin expression in adipose tissue of lipin transgenic mice results in increased expression of lipogenic genes, increased lipid storage and accelerated diet-induced obesity (8). These findings suggest that lipin promotes lipodystrophy in its absence as well as obesity when present at high levels (8). Thus, lipin might be critical for adipocyte differentiation, the maintenance of mature adipocyte function and lipogenesis and thereby may have profound effects in the pathogenesis of obesity, adipocyte gene expression and metabolic alterations associated with obesity (9).

Recent findings report on a strong negative correlation between lipin mRNA expression, fasting glucose and insulin levels, as well as insulin resistance in both human and mouse adipose tissues (10,11). Moreover, intragenic polymorphisms and haplotypes exhibited associations with serum insulin levels and body mass index, highlighting the importance of lipin in glucose homeostasis and obesity (10), both critical contributors to the MetS. Interestingly, the gene encoding lipin, *LPIN1*, is localized on chr2p, a genomic region that has previously been linked to fat mass and plasma leptin levels (12-15).

Based on these data, we hypothesized that genetic variations across *LPIN1* contribute to the expression of phenotypes related to the metabolic syndrome and carried out a comprehensive genetic association analysis. We systematically explored the linkage disequilibrium (LD) and haplotype structures of the *LPIN1* genomic region with single-nucleotide polymorphisms (SNPs) and assessed the role of common sequence variants and haplotypes in the metabolic syndrome as well as its components in a representative sample of the general population. In addition, we sought to replicate our results in an independent cohort recruited throughout Germany.

RESEARCH DESIGN AND METHODS

Study Populations.

General Population. The subjects participated in the echocardiographic substudy (total n=1,674) of the third MONICA Augsburg survey 1994/1995 (monitoring of trends and determinants in cardiovascular disease). The third survey represents a gender- and age-stratified random sample of all German residents of the Augsburg area and consists of individuals 25 to 74 years of age (mean 51.8±13.8), including 851 (50.7%) women

and 827 (49.3%) men and about 300 subjects for each 10-year increment. The study design, sampling frame, and data collection have been described in detail elsewhere (16). Briefly, all the participants completed a detailed questionnaire on demography, medication, history of concomitant diseases and cardiovascular risk factors, lifestyle, health behaviour, and psychological factors. Anthropometric data were obtained by detailed physical examination. The clinical characteristics of this study population are summarized in *Table 1*. Unfortunately, fasting insulin, fasting glucose, and fasting triglyceride levels were not available in this MONICA sample. The study was approved by the local ethics committee and all participants gave written informed consent.

Replication cohort. Our replication cohort consisted of spouses, brothers-in-law and sisters-in-law of members of the German Myocardial Infarction Family Study. The ascertainment strategy and study design have been described in detail elsewhere (17). Briefly, MI kindreds were ascertained through index patients, who were identified by screening more than 200,000 patient charts in 14 cardiac in-hospital rehabilitation centers distributed throughout Germany. Index patients had all suffered from MI before the age of 60 years. If at least one sibling had suffered from MI or had severe coronary artery disease (CAD, percutaneous transluminal coronary angioplasty (PTCA) or bypass surgery (CABG) before the age of 70 years), the nuclear family (index patient, available parents, all siblings as well as all spouses) was contacted and invited to participate in the study. The study protocol was approved by the Ethics Committee of the University of Regensburg, Germany, and all participants gave informed consent.
The cohort for the present replication study consisted of unrelated Caucasian individuals (n=1,029, n=380 men, n=649 women) who were examined by the same protocol as the affected family members and had no evidence of CAD by history and physical examination. Descriptive statistics for this sample are presented in *Table 2*. Contrary to the MONICA sample, fasting triglyceride levels were available in this sample.

SNPs and Genotyping.
SNPs. Fifteen single nucleotide polymorphisms (SNPs) covering the *LPIN1* gene and intergenic regions were genotyped in the MONICA study sample. From the 1,674 subjects, both DNA samples and high-quality phenotypic data were available in 1,411 subjects. Information for SNPs (rs number, polymorphic site and localization within the gene) was taken from SNP public databases (dbSNP; available at http://www.ncbi.nlm.nih.gov/SNP), GeneWindow (http://genewindow.nci.nih.gov), and from Applied Biosystems (www.appliedbiosystems.com). SNPs were selected with priority to HapMap SNPs (http://www.hapmap.org), tagging the gene based on an r^2 cutoff =0.8 and a minor allele frequency (MAF) >5%. However, if no predesigned Taqman® assay for these tagSNPs was availabe, we selected alternative SNPs using the following selection criteria: validated SNPs with MAF >5%, submitted multiple times or discovered by the TSC (The SNP Consortium Ltd), location in conserved coding or non-coding sequences that are in high LD and as close as possible next to the respective HapMap tagSNP. Of the 15 selected SNPs, four covered a region of 76 kb past the 5' end of the gene, one was located in exon 10, six were in the introns, one was in the UTR 3´ and three were within 890 kb past the 3' end of the gene (*Figure 1*). In total, a region of 281 kb was covered with SNPs. The coding SNP (rs33997857) led to a non-synonymous amino acid substitution (V494M). The seven intergenic SNPs were picked to determine the extent of LD and to explore the impact of sequence variations in noncoding and intergenic regions on the disease. In the replication sample a reduced set of ten SNPs were genotyped due to the LD structure (rs6748533, rs6707885, rs893346,

rs2577256, and rs1036668 were not genotyped).

Genotyping. SNPs were genotyped using 5´-exonuclease activity (TaqMan) assay on a HT7900 (Applied Biosystems, Darmstadt, Germany). SNP assays were ordered from Applied Biosystems either as Custom TaqMan SNP genotyping assays or predesigned TaqMan SNP genotyping assays. Probes were labelled with the fluorophores, FAM or VIC. Genotyping was done on 384-well plates prepared with the GENESIS Freedom pipetting robot from Tecan (Crailsheim, Germany). The Universal PCR Master Mix from Applied Biosystems was used in a 5-µl total reaction volume with 10 ng DNA per reaction. Allelic discrimination was measured automatically on the ABI Prism HT7900 (Applied Biosystems) using the Sequence Detection Systems 2.1 software (autocaller confidence level 95%). 10% of all genotypes were repeated in independent PCR reactions to check for consistency and to ensure intra- and inter-plate genotype quality control. No genotyping discrepancies were detected between the repeated samples. Call rates were ≥ 98.0%.

Definitions. Despite increasing literature about the MetS, it is not completely understood why its components cluster in individuals. In an attempt to answer this question, many investigators have used factor analysis to identify patterns underlying the co-occurrence of MetS components (18-23). These analyses support the current clinical definitions of the MetS in such that the core components central obesity, insulin resistance, blood pressure, and lipid measurements are linked by one single underlying factor (23). Moreover, in the present investigation the use of current proposed definitions for the MetS (24) has been hampered by the fact that blood samples have not been taken routinely in the fasting state in MONICA. Accordingly, we used factor analysis to describe the MetS by means of a factor score, computed for each subject derived from the following variables: waist circumference (cm), type 2 diabetes/prediabetes (yes/no), mean arterial blood pressure (mmHg), HDL cholesterol (mg/dl). Here, type 2 diabetes/prediabetes was defined as a history of type 2 diabetes and/or serum HbA1c >6.0% (25). The factor score represents the degree of association between the original measured variables and the unmeasured underlying factor ("MetS-Factor"); the higher the score, the more pronounced is the expression of the MetS. The resulting MetS factor score is normally distributed with a mean of 0.0 ± 1.2 and ranges between –4.9 to +5.8 (*Online Supplemental Figure 1* [available at http://diabetes.diabetesjournals.org]). As a quantitative trait it does not need an arbitrary cut-off to define affection status, and can thus reduce phenotypic misclassification. As demonstrated in the *Online Supplemental Figure 1*, the MetS factor score strongly correlates with the number of the core components and was significantly higher in obese than in non-obese individuals ($p<0.0001$), in hypertensives than in normotensives ($p<0.0001$), in subjects with diabetes than in subjects without diabetes ($p<0.0001$), as well as in subjects with dyslipidemia as compared to subjects without dyslipidemia ($p<0.0001$).

Additionally, we used the presence of ≥3 MetS components to define the MetS. The following criteria were used in this regard: hypertension was defined as systolic blood pressure (SBP) ≥140 mm Hg, diastolic blood pressure (DBP) ≥90 mm Hg or intake of antihypertensive medication; type2 diabetes/prediabetes as history of diabetes and/or serum HbA1c >6.0% (25) obesity as body mass index (BMI) >30 kg/m^2 (24) and/or waist circumference >102 cm in men and >88 cm in women (26) and dyslipidemia as high density lipoprotein (HDL) <40 mg/dl in men and <50 in women (26).

Statistical analysis.

For each of the 15 SNPs, we tested whether the observed allele frequencies departed from Hardy-Weinberg proportions. Statistically inferred haplotypes were

derived using the PHASE software (27). Using the haplotype file format from PHASE output, we assessed LD between all pairs of SNPs using the HaploBlockFinder software (28), applying the definition of Lewontin's standardized disequilibrium coefficient (*D´).* A haplotype block was defined as a region in which all pairwise *D´* values were >0.8 (29). Individual SNPs were tested for association using linear and logistic regression analyses. Haplotypes with frequencies ≥2% were tested using the haplotype trend regression method (30). This test fits a model of additive effects of haplotypes under the hypothesis of no haplotype effects and under the assumption of Hardy-Weinberg equilibrium. Permutation based procedures, permuting the dependent variable, were performed to test for empirical significance. The permuted p-value is the fraction of permuted tests which get a better p-value than the original test did.

Factor analysis was used to identify a specific cluster of MetS components on the basis of correlation between original measures. Factor extraction was conducted using the method of principle components. Eigenvalues were used to condense the variance in a correlation matrix and represent that amount of variance accounted for by a factor. Variables with eigenvalues of 1.0 or higher are traditionally considered worth analyzing. Here, only one factor with eigenvalue >1.0 could be extracted based on the original measured variables included in this study. A factor score that is a combination of the original measured variables representing the predicted value of the underlying MetS factor, was computed for each individual. This score was then considered as dependent variable in quantitative trait regression analysis. Power calculations were computed over a range of parameters for the linear trend test (31, 32) indicating that our sample size is sufficient to achieve 80% power for at least half of the genetic model parameter settings (*Online Supplemental Figure 3*). Additionally, we divided the study population into tertiles of MetS factor score distribution and calculated tertile 3 / tertile 1 risk ratios using crude and adjusted logistic regression models. A two-sided *P* value $P<.05$ was considered significant. All p-values reported represent nominal p-values; empirical p-values are reported when specifically indicated.

RESULTS

Phenotypic characteristics. The clinical and anthropometric characteristics of the MONICA population are presented in *Table 1.* The participants present with the expected characteristics of our study sample of the general population. The MetS defined by the presence of ≥3 components could be observed in 10.3 % in men and 12.5 % in women.

Linkage disequilibrium evaluation and haplotype structure in the study population. *Figure 1* depicts the gene structure and all SNPs used in this study, including their position and general characteristics based on the March 2006 release of the UCSC Genome Browser (available at http://www.genome.ucsc.edu). No deviations from the expected Hardy-Weinberg proportions were detected for any of the SNPs. LD analysis revealed slight LD spanning the whole gene region and three high LD blocks (*Figure 1, Online Supplemental Figure 2).* Specifically, two three-marker haploblocks each forming four common haplotypes and one two-marker haploblock forming two common haplotypes could be identified. The first three-marker haploblock (haploblock 1: rs6726960, C__25965595_10, rs1469952) is located within 18.6 kb between intron 1 and intron 5 of the *LPIN1* gene and forms four haplotypes with frequencies ≥2.0%. The second three-marker haploblock (haploblock 2: rs33997857, rs6744682, rs6708316), is located within 9.9 kb between exon 10 and intron 13 and forms four haplotypes with frequencies ≥4%. The two-marker haploblock (haploblock 3: rs2716610, rs1050800) is positioned within 6.4 kb between intron 17 and the UTR3´. Altogether, the fifteen SNPs spanning the *LPIN1* gene and intergenic regions formed

three different haploblocks with ten haplotypes with frequencies ≥2% in this study sample.

Association of individual SNPs with the MetS and its components. We tested all individual SNPs for association with the MetS and its single components. Single SNP association analyses are summarized in *Figure 2.* Noticeable, yet inconsistent association results were found between single SNPs and parameters of the MetS particularly in men with blood pressure, and HDL cholesterol levels. In women these associations were less pronounced.

Association of haplotypes with the MetS and its components. Based on our LD analysis we next screened for association between haplotypes and the MetS factor score as well as with single components of the MetS. *Figure 3* depicts the results of these association tests. We found a highly significant association peak between the MetS factor score, BMI, waist circumference and blood pressure levels and allelic combinations of a three-marker haploblock across exon 10 and intron 13 of the *LPIN1* gene beginning at SNP marker rs33997857. This haploblock is consistent with "haploblock 2" based on our LD analysis and is composed of the exonic-SNP leading to a codon change (V494M) and two intronic-SNPs. After performing 10,000 permutations the MetS factor score ($p<0.0001$), the existence of ≥3 MetS components ($p=0.0004$), waist circumference ($p<0.0001$), blood pressure levels ($p<0.0001$), and HbA1c levels ($p<0.0001$) remained significantly associated with these haplotypes (all p-values based on permutation testing).

We likewise tested for association between allelic variants and MetS phenotypes across "haploblock 1" and "haploblock 3". In essence, we did not find any evidence of relationship here (data not shown).

As a consequence, we investigated each allelic variant of "haploblock 2" in more detail. Association results of the respective haplotypes are shown in *Table 3* with the exception of the most common haplotype consisting of all major SNP alleles. This haplotype did not show any evidence of association neither with the MetS factor score nor with any of its components (data not shown). Subjects carrying the "122 haplotype" (frequency 26.8%) were more often obese and diabetic, and had higher HbA1c and LDL cholesterol levels than subjects not carrying this haplotype implying susceptible characteristics of this haplotype. Moreover, the MetS factor score as well as the frequency of having three or more MetS core components were significantly higher in carriers of this susceptible haplotype.

In contrast, individuals carrying "haplotype 121" (frequency 4.0%) or "haplotype 112" (frequency 5.7%) had markedly lower blood pressure levels, a lower BMI and waist circumference, lower HbA1c and LDL cholesterol levels as well as a lower MetS factor score corresponding to non-susceptible or protective effects of these haplotypes. Accordingly, the frequencies of arterial hypertension, obesity, diabetes, and the presence of three or more MetS components were significantly lower in subjects carrying these protective haplotypes than in subjects without.

Figure 4 depicts the relative frequencies of the presence of the susceptible and protective haplotypes with and without MetS. In this analysis the protective haplotypes were pooled because of their low frequencies and similar impact on the metabolic phenotypes. Two traits of the MetS with their corresponding odds ratios are present: tertile 3 vs. tertile 1 of the MetS factor score distribution as well as presence of ≥3 MetS components vs. ≤2 MetS components. Using sex adjusted logistic regression analysis both the susceptible and the protective haplotypes showed significant association with the MetS, independently of the definition used.

Replication of association in an independent cohort. To confirm our findings, we additionally tested for association in a cohort of healthy spouses of

coronary artery disease patients. The characteristics of study participants were comparable to the sample from the MONICA general population with the exception of a lower frequency of obese subjects and a higher proportion of women (*Table 2*). Linkage disequilibrium structure and haplotype frequencies were also comparable ("111" (59.4%), "122" (23.3%), "112" (8.2%), "121" 6.7%) . As depicted in *Figure 5* "haploblock 2" was also significantly associated with traits of the MetS with the strongest effect on fasting triglycerides (which were not available in the MONICA sample) and HDL cholesterol levels. The MetS factor score and the trait "diabetes" also showed a significant association with alleles of haploblock 2. However, fasting triglycerides were higher in subjects presenting haplotype 112, and HDL cholesterol levels were lower in subjects with the haplotypes 121 or 112 (*Table 4*). Moreover, type 2 diabetes was also more frequently found in subjects with haplotpye 121. As a consequence, the MetS factor score was higher in subjects with these rare haplotypes, and thus, the effects are in the opposite direction as compared to those in the MONICA sample.

DISCUSSION

The present study offers a comprehensive analysis of common genetic variants across the *LPIN1* gene region with the metabolic syndrome (MetS) and its individual components. We have identified *LPIN1* as a gene that is significantly associated with traits of the MetS in the general population of the Augsburg area in Germany and have found association of the same haplotypes in a second sample recruited throughout Germany, however, with effects in the opposite direction. The similarity in the minor allele frequencies in both studies and the fact that cluster of adjacent SNPs show the same pattern suggests that this discrepancy may not be due to technical issues. We cannot exclude spurious associations in the two studies, however, similar observations of associations with odds ratios in opposite direction have been made previously (33). Recently, Lin et al. (2007) have used theoretical modeling to demonstrate that these associations may indeed represent confirmations of true associations and that multilocus effects and variation in interlocus correlations contribute to this "flip-flop phenomenon" (33). Thus, considering also previous reports about *LPIN1*, our results may still demonstrate that the LPIN1 gene plays a critical role in molecular pathways affecting human metabolic traits.

We were led to pursue *LPIN1* because, first, the physiological role of the *LPIN1* gene appears to be in the regulation of fat mass and body weight (5-8). Second, recent findings report on strong negative correlations between lipin mRNA expression and parameters of insulin sensitivity in both human (10,11) and mouse adipose tissues (10). These findings are supported by two recently released reports, showing subcutaneous lipin mRNA levels to be decreased in subjects with either obesity (6,11), impaired glucose tolerance (11) or in women with the MetS (6). In contrast, lipin mRNA levels were positively correlated with an increasing insulin sensitivity index and were up-regulated following weight reduction in obese (6).

Suviolahti et al. have recently identified for the first time an association between SNPs across *LPIN1* and insulin levels as well as with body mass index in Finnish families with dyslipidemia and in a Finnish obesity case-control study, respectively (10). A comparison of our data with those of the Suviolahti group reveals that we have genotyped three identical SNPs (rs893346, rs2716610, rs1050800). Suviolahti et al. reported moderate differences in allele frequencies in obese vs. lean subjects for SNP rs2716610 and moderate differences in fasting insulin levels for SNP rs893346. None of these SNPs showed reliable associations with any of our phenotypes. However, one of the Suviolahti SNPs which was associated with BMI is located in the immediate 3' vicinity of our haploblock, which analogously is associated with BMI in the general population of MONICA.

According to the HapMap data these markers are in strong linkage disequilibrium implying a possible replication of the findings.

While in our replication sample HDL cholesterol and triglyceride levels as well as the presence of type 2 diabetes and the MetS factor itself were clearly associated with alleles of the same haploblock, the continuous traits BMI and blood pressure levels only showed weak evidence of association with these haplotypes in this population sample. This lack may possibly be due to unrecognized phenotypic differences across study cohorts. However, population stratification was not explicitly tested in our study. Gene-environmental interactions might be different in addition to the lower prevalence of obesity as well as the higher percentage of female individuals in the replication sample.

Furthermore, the proportional contribution of each of the single components to the MetS may differ individually and in populations. In addition, multiple susceptibility genes may exist (genetic heterogeneity), of which only a subset are required for disease. Nevertheless, we find metabolic phenotypes that show association in two independent populations with p-values far below the recently revised recommendation of the experiment–wise significance level of $p<0.001$ quoted to be sensible in genetic association studies (34). Moreover, considering the findings of Suviolahti et al. the association could be shown in multiple independent populations making false positive findings unlikely.

Evidence for a role of lipin in traits of glucose metabolism accumulated with the recent reports of association of lipin mRNA levels and glucose transport and GLUT4 mRNA expression levels (6). As we do not have fasting glucose and insulin levels in our study samples we were not able to approve these findings in our study.

The biological mechanism underlying this genetic association may be due to an altered gene expression. Indeed, strong negative correlations were observed between human lipin mRNA levels and glucose (r=-0.81), insulin (r=-0.74), triglyceride levels (r=-0.64), and the HOMA-IR index (r=-0.82) of insulin resistance (10). Moreover, lipin mRNA levels were strongly correlated with GLUT4 mRNA levels (r=0.69) (6). Interestingly, our haplotype comprised one coding SNP leading to a non-synonymous amino acid substitution and two intronic SNPs. However, with respect to the coding SNP, the same "1" allele occurred in all the haplotypes, indicating that this coding SNP is not responsible for the observed associations. This observation supports the hypothesis that the proper causal mutation is located elsewhere in this LD-block or is in LD with it. Additional functional studies will be needed to clarify whether individuals carrying these different haplotypes show differences in the expression of lipin.

As we do not have fasting glucose, insulin and triglyceride levels in our MONICA study cohort, we were not able to define the MetS according to one of the currently proposed definitions. This, as well as the fact that study participants were recruited more than 10 years ago might explain the low prevalence of the MetS in our study population. However, several MetS definitions are in use: the WHO (1999), the EGIR (1999), the ATPIII (2001) as well as the most recently encouraged IDF criteria (2005). These definitions differ in their measures and cut-offs and it is still a matter of debate which of them best represents the MetS. Additionally, it is still unclear why its components cluster in individuals. Several recently performed factor analyses (18-23) support the current clinical definitions of the MetS in such that the core components central obesity, insulin resistance, blood pressure, and lipid measurements are linked by one single underlying factor (23). Accordingly, we used factor analysis to describe the MetS by means of a factor score, computed for each subject. As a quantitative trait it does not need an arbitrary cut-off to define affection status, and can thus reduce phenotypic misclassification. Together with the confirmatory association findings using individual components and their sum, we are

confident, that our MetS factor score represents an adequate measure of the metabolic state in our study participants.

In summary, our work offers a comprehensive analysis of the LD structure, common genetic variants, and haplotypes within the *LPIN1* gene region. We suggest an association of common genetic variants of the *LPIN1* gene with the metabolic syndrome and its associated phenotypes thus raising the possibilty of lipin contributing to the pathophysiology of the metabolic syndrome.

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Conflict of Interest statement: All authors declare that they have no competing financial interests.

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TABLE 1. Clinicial and anthropometric characteristics of the MONICA study population according to gender

	Men (n=721)	**Women (n=690)**
Age (years)	52± 14	52 ± 14
BMI (kg/m^2)	27.0 ± 3.6	26.4 ± 4.7
Waist circumference (cm)	95 ± 10	83 ± 11
Obesity * (%)	25.8	33.5
HDL cholesterol (mg/dl)	48 ± 14	60 ± 17
Dyslipidaemia * (%)	29.0	29.0
Systolic BP (mm Hg)	137 ± 19	131 ± 21
Diastolic BP (mm Hg)	83 ± 12	79 ± 11
Arterial hypertension *(%)	49.2	40.4
HbA1c (%)	5.2 ± 0.9	5.3 ± 0.8
Diabetes mellitus * (%)	7.3	8.4
>= 3 MetS components * (%)	10.3	12.5

Data are means ± SD unless otherwise indicated. * Definitions are as defined in text.

TABLE 2. Clinical and anthropometric characteristics of subjects in the replication sample

	Men (n=380)	**Women (n=649)**
Age (years)	60 ± 10	56 ± 9
BMI (kg/m^2)	27.3 ± 3.8	27.0 ± 4.5
Obesity * (%)	16.8	19.1
HDL cholesterol (mg/dl)	54 ± 12	66 ± 15
Fasting triglycerides (mg/dL)	157 ± 130	125 ± 68
Dyslipidaemia * (%)	15.6	17.0
Arterial Hypertension * (%)	45.5	40.3
HbA1c (%)	5.6 ± 0.8	5.5 ± 0.6
Diabetes mellitus * (%)	11.7	7.5
>= 3 MetS components * (%)	8.9	8.3

Data are means ± SD unless otherwise indicated. * Definitions are as defined in text.

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TABLE 3. Association of three-marker haplotypes from "haploblock 2" with traits of the MetS

	Haplotype 122			**Haplotype 121**			**Haplotype 112**		
	present	**not present**	***P* value**	**present**	**not present**	***P* value**	**present**	**not present**	***P* value**
	(n=647)	**(n=769)**		**(n=93)**	**(n=1323)**		**(n=143)**	**(n=1273)**	
Hypertension traits									
Systolic BP (mmHg)	135 ± 21	133 ± 19	n.s.	127 ± 18	135 ± 20	0.0001	128 ± 18	135 ± 20	0.00008
Diastolic BP (mmHg)	81 ± 12	80 ± 12	n.s.	78 ± 12	81 ± 12	0.032	78 ± 11	81 ± 12	0.0016
Hypertension * (%)	47.1	43.0	n.s.	23.7	46.4	0.00001	32.2	46.4	0.001
Obesity traits									
BMI (km/m^2)	26.8 ± 4.2	26.6 ± 4.1	n.s.	24.6 ± 3.6	26.9 ± 4.1	$3.7*10^{-7}$	25.3 ± 3.8	26.9 ± 4.2	0.00002
Waist circum. (cm)	90 ± 12	89 ± 13	n.s.	82 ± 12	90 ± 12	$3.2*10^{-8}$	85 ± 12	90 ± 12	0.00001
Obesity * (%)	32.8	27.0	0.019	12.9	30.8	0.0003	16.9	31.1	0.0005
Diabetes traits									
Diabetes * (%)	9.3	6.6	0.059	2.2	8.2	0.041	6.5	8.0	n.s.
HbA1c (%)	5.3 ± 0.9	5.2 ± 0.8	0.012	5.1 ± 0.7	5.3 ± 0.9	0.0002	5.1 ± 0.6	5.3 ± 0.9	0.013
Dyslipidaemia traits									
LDL chol. (mg/dl)	147 ± 43	142 ± 43	0.021	135 ± 47	145 ± 43	0.032	130 ± 41	146 ± 43	0.00002
HDL chol. (mg/dl)	54 ± 16	54 ± 17	n.s.	56 ± 19	54 ± 16	n.s.	53 ± 18	54 ± 16	n.s.
Dyslipidaemia * (%)	30.4	27.9	n.s.	30.4	28.9	n.s.	33.1	28.6	n.s.
MetS traits									
Factor Score	0.10 ± 1.23	-0.11 ± 1.24	0.002	-0.67 ± 1.00	0.04 ± 1.24	$1.4*10^{-7}$	-0.42 ± 1.28	0.03 ± 1.22	0.00005
≥3 components * (%)	16.0	10.5	0.007	3.3	13.7	0.002	8.6	13.5	n.s.

TABLE 4. Association of three-marker haplotypes from "haploblock 2" with traits of the MetS in the replication study sample

	Haplotype 122			**Haplotype 121**			**Haplotype 112**		
	present	**not present**	***P* value**	**present**	**not present**	***P* value**	**present**	**not present**	***P* value**
	(n=429)	**(n=601)**		**(n=102)**	**(n=928)**		**(n=136)**	**(n=894)**	
Systolic BP (mmHg)	133.5 ± 16.8	135.3 ± 17.5	n.s.	136 ± 17	134.4 ± 17.3	n.s.	139 ± 18	134 ± 17	0.04
Diastolic BP (mmHg)	80.4 ± 9.7	81.7 ± 9.9	n.s.	80 ± 8	81 ± 10	n.s.	82 ± 12	81 ± 9	n.s.
Hypertension * (%)	40.6	43.4	n.s.	46.1	41.8	n.s.	47.8	41.4	n.s.
BMI (km/m^2)	26.7 ± 4.3	26.6 ± 4.1	n.s.	27.5 ± 4.2	26.6 ± 4.2	0.037	27.3 ± 3.2	26.6 ± 4.3	n.s.
Diabetes * (%)	8.9	9.1	n.s.	19.8	7.9	0.00007	8.6	12.0	n.s.
TG (mg/dl)	133 ± 76	140 ± 109	n.s.	145 ± 102	136 ± 96	n.s.	175 ± 182	131 ± 75	0.00008
HDL chol. (mg/dl)	63 ± 15	60 ± 15	0.003	57 ± 12	62 ± 15	0.008	56 ± 14	62 ± 15	0.00008
Factor Score	-0.66 ± 1.09	-0.52 ± 1.15	n.s.	-0.12 ± 1.33	-0.63 ± 1.09	0.002	-0.32 ± 1.16	-0.62 ± 1.12	0.044

Data are means ± SD unless otherwise indicated. n.s., not significant. Asymptotic p values are given. * Definitions are as defined in text. The most frequent hapotype, "haplotype 111" did not show any evidence of association with these phenotypes and is thus not shown in the Table.

FIGURE LEGENDS

FIG. 1. Structure of the *LPIN1* region and position of the 15 genotyped SNPs including general SNP characteristics as given in the dbSNP database and the UCSC Genome Browser (March 2006 assembly). *LPIN1* gene region deposited grey. Information for SNP C__25965595_10 is taken from Applied Biosystems (www.appliedbiosystems.com) and GeneWindow (http://genewindow.nci.nih.gov). *MAF, minor allele frequency. Haplotypes were inferred using PHASE 2.0 (27) and haploblocks were derived from the Haploblockfinder (28) genetical analysis software. Only haplotypes with frequencies ≥2% are shown.

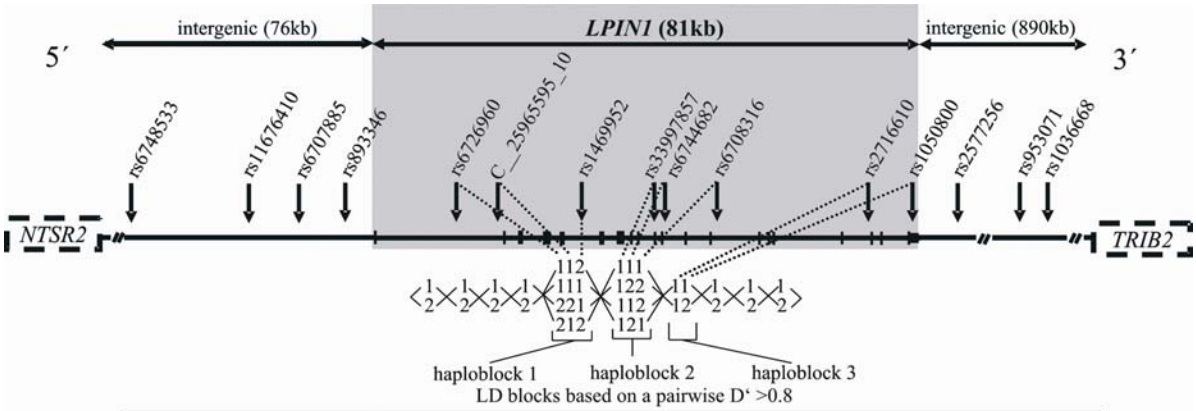
FIG. 2. Association between individual SNPs and traits of the MetS in men (upper panel) and women (lower panel). The strength of association is represented by –log(p values).

FIG. 3. -log(p-value) plot by the haplotype trend regression method. Meaningful haplotype block structures based on linkage disequilibrium analysis are indicated ("haploblock 1", "haploblock 2" and "haplotype 3"). Three-marker haplotypes from "haploblock 2" (encompassing SNPs rs33997857, rs6744682 and rs6708316) show highly significant associations with traits of the MetS.

FIG. 4. Relative frequencies of the susceptible and the protective haplotypes in subjects within the first and third tertile of the MetS factor score distribution (the first tertile implies individuals with the lowest evidence of a MetS, while the third tertile encloses those with the highest evidence for a MetS) as well as in subjects with ≥3 and <3 MetS components (arterial hypertension, insulin resistance, abdominal obesity, dyslipidemia). The corresponding odds ratios for susceptible and protective haplotype carriers with respect to these MetS criteria are mentioned.

FIG. 5. -log(p-value) plot by the haplotype trend regression method in the replication cohort. Using the same three-marker haploblock as in the MONICA sample, the significant associations between three-marker alleles from "haploblock 2" (bold, beginning with SNP rs33997857) and traits of the MetS could be confirmed.

Figure 1



SNP no.	rs- or Assay-ID-number of SNPs	Position	MAF	Call rate [%]	Gene region	Polymorphism (Codon change)
1	rs6748533	11,769,325	0.14	99.4	Intergenic 5′	C/G
2	rs11676410	11,787,218	0.17	98.9	Intergenic 5′	G/A
3	rs6707885	11,794,067	0.16	99.5	Intergenic 5′	A/G
4	rs893346	11,800,212	0.08	99.3	Intergenic 5′	G/A
5	rs6726960	11,816,382	0.22	99.7	Intron 1	T/C
6	C__25965595_10	11,825,293	0.20	99.7	Intron 1	T/C
7	rs1469952	11,834,970	0.46	99.1	Intron 5	G/A
8	rs33997857	11,844,689	0.03	98.7	exon 10	A/G (ATG,GTG)
9	rs6744682	11,846,779	0.31	99.1	Intron 11	T/A
10	rs6708316	11,854,609	0.33	98.7	Intron 13	C/G
11	rs2716610	11,876,833	0.16	98.0	Intron 17	T/C
12	rs1050800	11,883,265	0.17	99.0	UTR3′	T/C
13	rs2577256	11,889,941	0.49	98.9	Intergenic 3′	C/T
14	rs953071	11,924,066	0.40	99.2	Intergenic 3′	C/T
15	rs1036668	12,050,089	0.23	98.0	Intergenic 3′	G/T

Figure 2

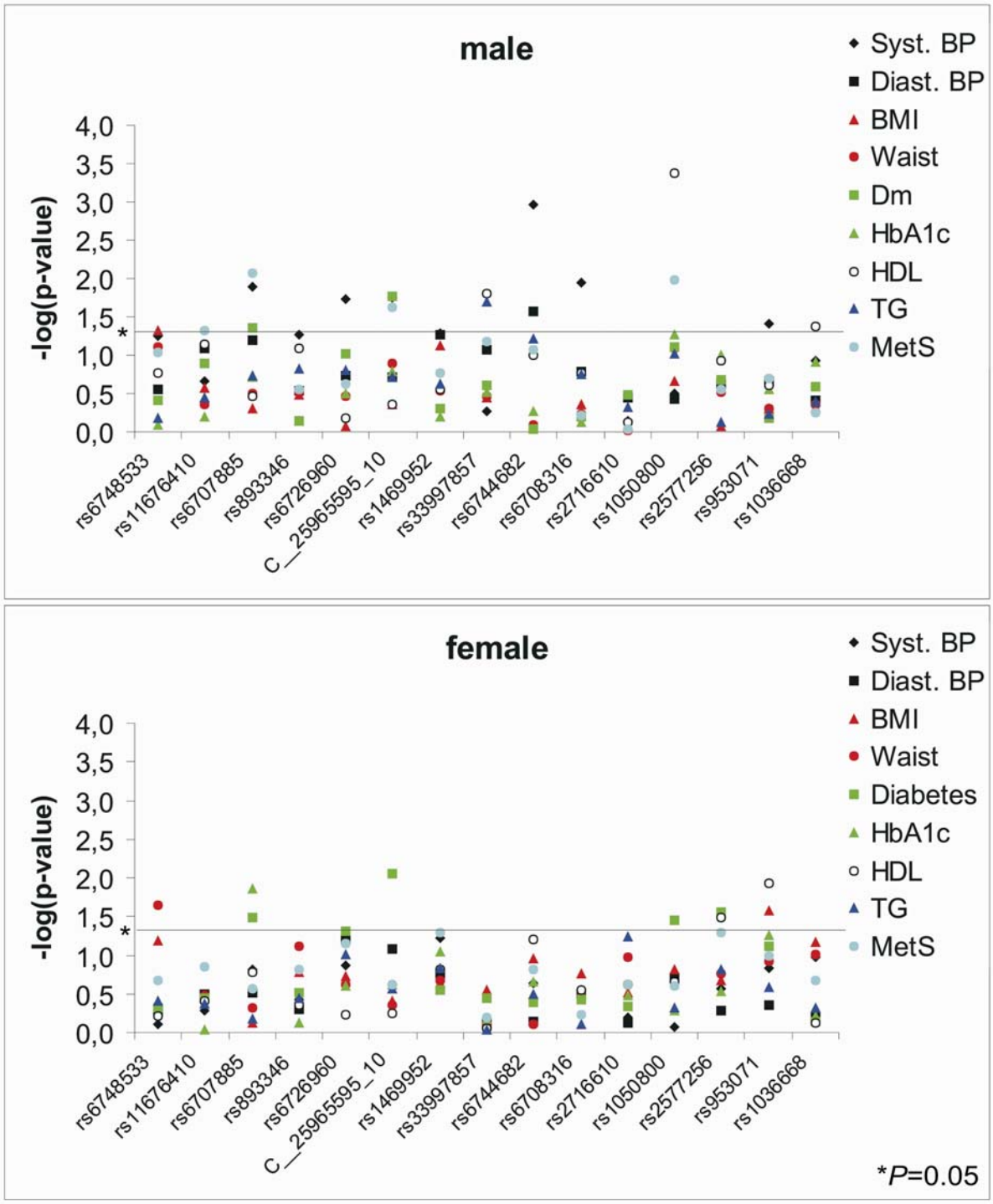

male
-log(p-value)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
rs6748533
rs11676410
rs6707885
rs893346
rs6726960
C__25965595_10
rs1469952
rs33997857
rs6744682
rs6708316
rs2716610
rs1050800
rs2577256
rs953071
rs1036668
Syst. BP
Diast. BP
BMI
Waist
Dm
HbA1c
HDL
TG
MetS
female
-log(p-value)
Diabetes
*P=0.05

Figure 3

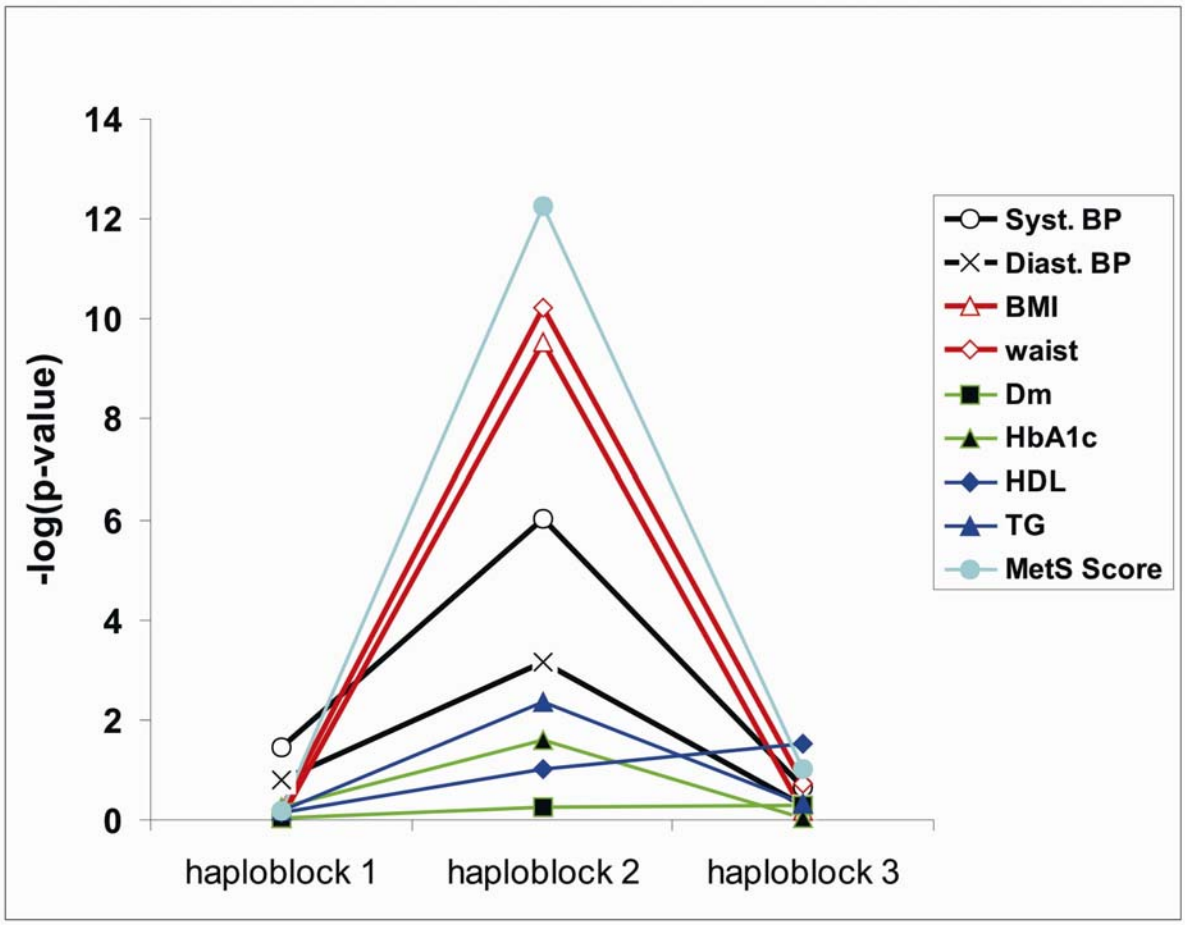

14
12
10
8
6
4
2
0
-log(p-value)
haploblock 1
haploblock 2
haploblock 3
Syst. BP
Diast. BP
BMI
waist
Dm
HbA1c
HDL
TG
MetS Score

Figure 4

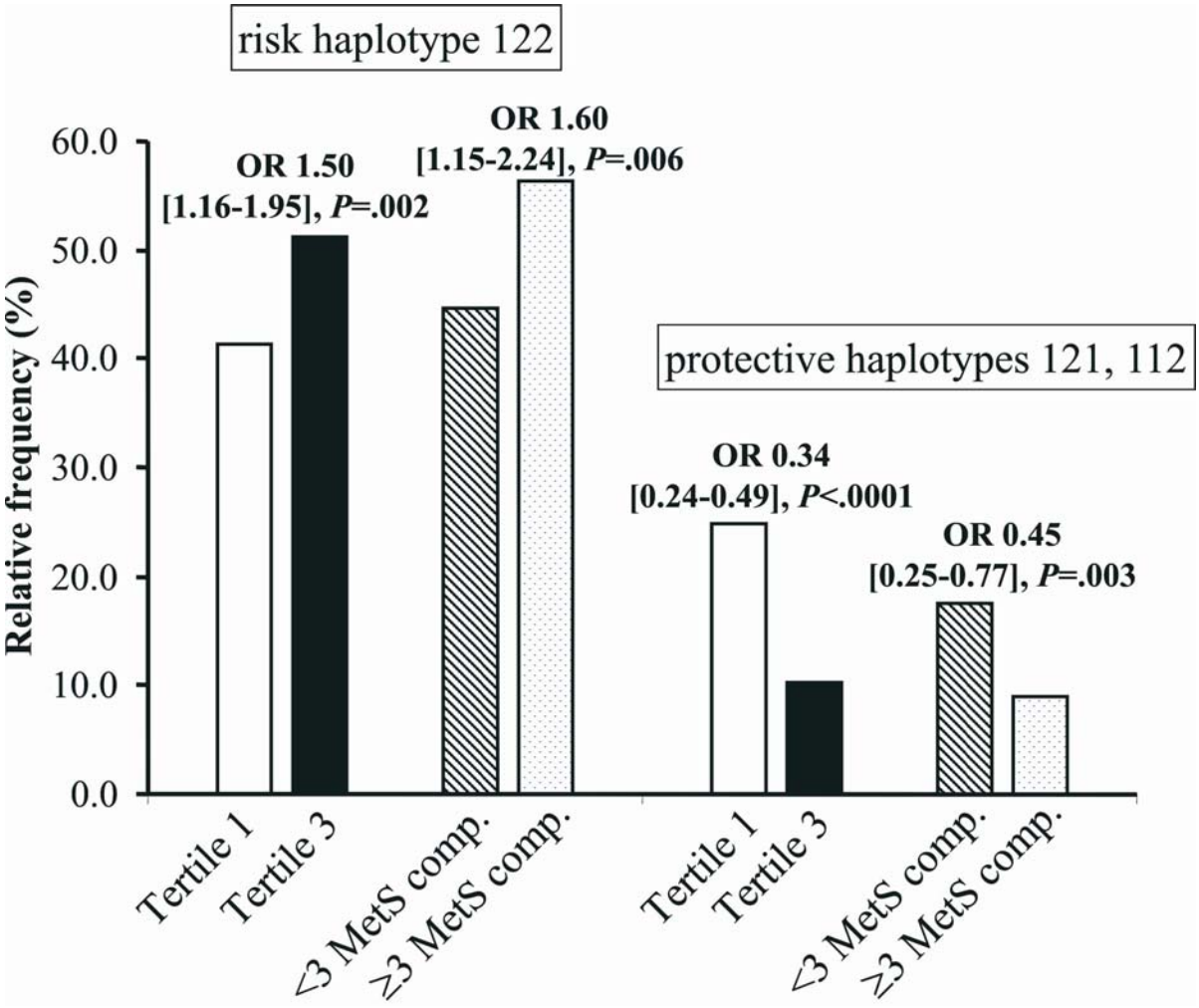
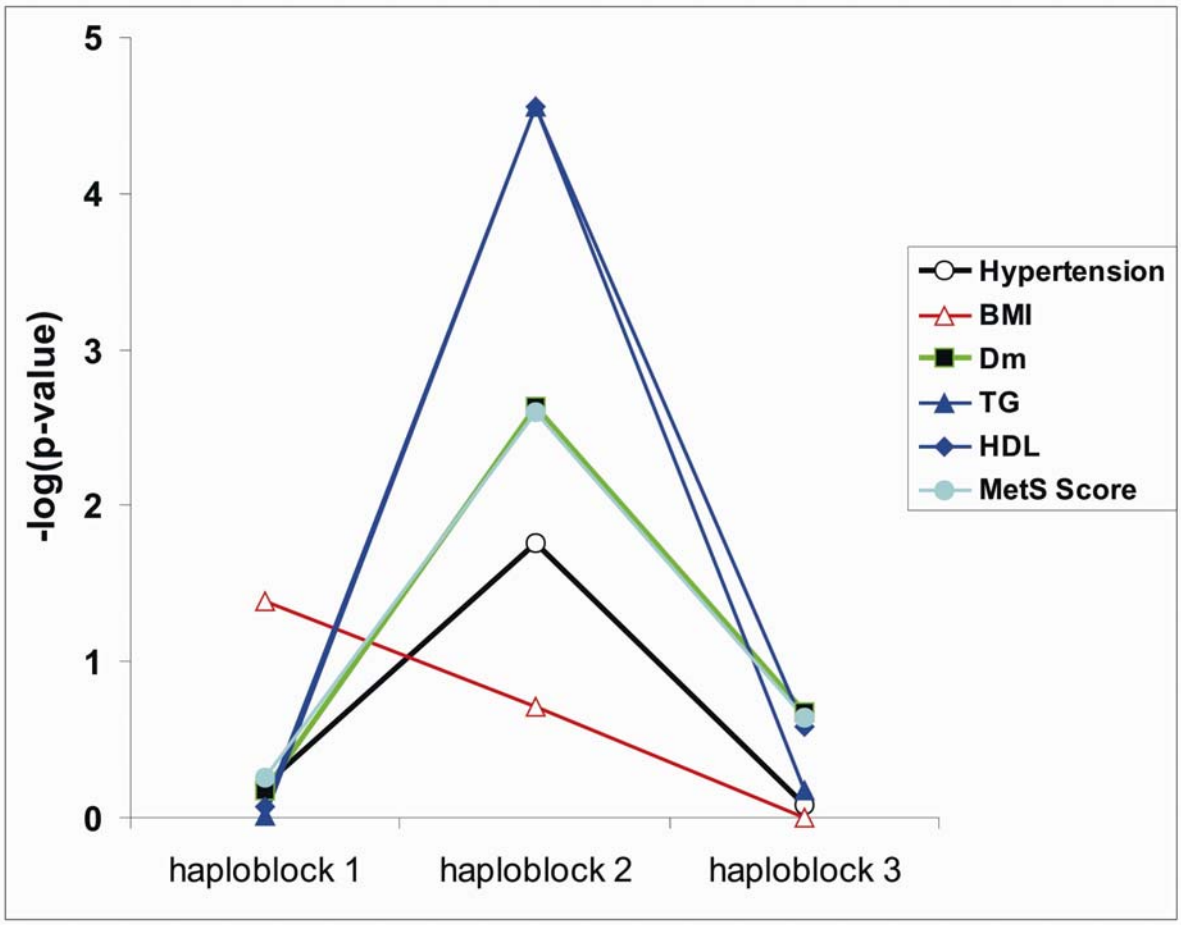

risk haplotype 122
OR 1.50
[1.16-1.95], P=.002
OR 1.60
[1.15-2.24], P=.006
protective haplotypes 121, 112
OR 0.34
[0.24-0.49], P<.0001
OR 0.45
[0.25-0.77], P=.003
Relative frequency (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Tertile 1
Tertile 3
<3 MetS comp.
≥3 MetS comp.
Tertile 1
Tertile 3
<3 MetS comp.
≥3 MetS comp.

Figure 5


5
4
3
2
1
0
-log(p-value)
haploblock 1
haploblock 2
haploblock 3
Hypertension
BMI
Dm
TG
HDL
MetS Score