Tagging-SNP haplotype analysis of the secretory PLA2IIa gene PLA2G2A shows strong association with serum levels of sPLA2IIa: results from the UDACS study

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Recent prospective analysis identified secretory phospholipase A2-IIa (sPLA2IIa) as a coronary artery disease (CAD) risk predictor. This study aimed to examine the relationship between serum levels of sPLA2IIa and variation in the sPLA2IIa gene (PLA2G2A) in a cohort of patients with Type II diabetes (T2D) mellitus. Six tagging single nucleotide polymorphisms (tSNPs) accounting for >92% of the genetic variability in PLA2G2A were identified and distinguished six common haplotypes (frequencies >5%). In the 523 Caucasian T2D patients, levels of sPLA2IIa, independent of CRP, were negatively correlated with total antioxidant status \( (P = 0.003) \) and high-density lipoprotein cholesterol \( (P = 0.006) \) in men and correlated with CAD status in women \( (P = 0.002) \) (Odds ratio of top two tertiles versus bottom = 2.50) [95% CI (1.13–5.53) \( P = 0.024 \)]. Overall, tSNP haplotypes showed a highly significant association with sPLA2IIa levels \( (P < 0.0001) \), explaining 6.3% of the variance. The most common haplotype (frequency 14.2%) was associated with 53% higher sPLA2IIa levels \[ 3.25 \text{ ng/ml (±0.14)} \] compared with the combined other haplotypes \[ 2.13 \text{ ng/ml (±0.09)} \], \( P < 0.00001 \). Five of the six tSNPs were associated with significant effects on sPLA2IIa levels but the raising haplotype could not be distinguished by a single tSNP and none are likely to be functional. These data confirm the relationship between elevated sPLA2IIa levels and CAD risk reported in both cases: control and prospective analyses. The strong impact of PLA2G2A haplotype variation on sPLA2IIa levels will help clarify the causality of this association.

INTRODUCTION

Secretory phospholipase A2 group IIa (sPLA2IIa) is a member of a superfamily of enzymes that hydrolyse the sn-2 ester bond of phospholipids and cell membranes, generating non-esterified free fatty acids (NEFAs) and lysophospholipids (1). sPLA2IIa is expressed in many cell types and may be an enzymatic component of the host defence mechanism directed against bacterial invasion and part of the inflammation-associated cellular responses (2). In addition, and more relevant to this study, the presence of sPLA2IIa activity in the arterial wall suggests that it may play a role in atherogenesis (3).

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In fact sPLA2-IIa has been shown to have pro-atherogenic properties both in the circulation and within the arterial wall (3). In the circulation, sPLA2-IIa hydrolysis of low-density lipoprotein (LDL) generates oxidation susceptible, small-dense LDL (sd-LDL) particles, with altered configuration of apolipoprotein B (4), leading to LDL receptor (LDLR) independent uptake, promoting atherogenesis (5). In the process of hydrolysing LDL, both lysophospholipids (precursors of pro-inflammatory mediators of leucotrienes and prostaglandins) (6) and NEFAs, including arachidonic acid, can be released, with downstream pro-inflammatory consequences (7). Acute phase high density lipoprotein (HDL) is a better substrate for sPLA2-IIa than normal HDL, suggesting a possible role in the inflammatory response and in the metabolism of acute phase lipoproteins (8).

In the arterial wall, sPLA2-IIa-modified lipoproteins show increased susceptibility to lipid peroxidation (9), producing oxidized lipoproteins that enhance macrophage growth (10). These modified lipoproteins bind more tightly to extra-cellular proteoglycans, which leads to their enhanced retention in the arterial wall (4,11,12), an early marker of atherogenesis (13). The products of sPLA2-IIa hydrolysis, oxidized NEFAs and lysophosphatidylcholine, induce further aggregation and fusion of lipoproteins, leading to accumulation within the extra-cellular matrix and eventual internalization in macrophages within the intima (14). Although immunohistochemical studies have identified sPLA2-IIa in normal arteries, its extra-cellular distribution and cell expression is increased in early and late atherosclerotic lesions, further implicating it in atherosclerosis (15–17). The pro-atherogenic role of sPLA2-IIa has been confirmed in mouse models, and mice transgenic for the human sPLA2-IIa gene (PLA2G2A) show a dramatic increase in atherosclerosis, on both high fat and chow diets (18,19). These PLA2G2A transgenic mice in addition had raised LDL- and total cholesterol, and sPLA2-IIa was present in the atherosclerotic plaques on the surface of macrophages. To identify whether this was the source of increased atherosclerosis, bone marrow from PLA2G2A transgenic mice was transplanted into ldlr−/− mice fed a high fat diet. There was no effect on plasma lipoprotein levels, however, mice showed an increase in the extent of atherosclerosis, suggesting that the macrophage-expressed sPLA2-IIa contributed to the lesion formation (20).

sPLA2-IIa is an acute phase protein expressed in response to a variety of pro-inflammatory cytokines (3,21,22). Circulating levels of sPLA2-IIa are higher in coronary artery disease (CAD) patients compared with apparently healthy individuals (23,24) and are associated with increased risk of future CAD, in prospective analysis (25), but whether this is a causal relationship remains to be determined.

In this study, we have examined the hypothesis that serum sPLA2-IIa levels are significantly determined by variation in the sPLA2-IIa gene, PLA2G2A, using a tagging single nucleotide polymorphism (tSNP) approach. These tSNPs can infer the allelic state of all the common SNPs in the gene with a high coefficient of determination, thus covering maximum genetic variability. The study cohort was composed of patients with Type II diabetes (T2D), a group with increased CAD risk, with well-characterized measures of oxidative stress and LDL particle size.

### RESULTS

Table 1 summarizes the baseline characteristics of the Caucasian men and women with T2D from UDACS with T2D, in relation to the presence/absence of CAD. Those who had CAD were significantly older and had a longer duration of diabetes. The higher usage of statins, angiotensin converting enzyme (ACE) inhibitors and aspirin potentially explains their lower diastolic blood pressure (BP), LDL-C and total cholesterol levels compared with T2D-free men and women. Serum sPLA2-IIa levels were not statistically significantly different comparing those with CAD and those free of CAD. However, compared with the men, women had significantly higher sPLA2-IIa levels (3.94 and 5.96 ng/ml, respectively, \(P < 0.0001\)). In the men, sPLA2-IIa levels were significantly negatively correlated with total antioxidant status (TAOS) and HDL, independent of age, CAD status, statin use and CRP and were positively correlated with CRP after adjustment for age, CAD status and statin use (Table 2A). In the women, the proportion of CAD events significantly correlated with sPLA2-IIa, even after adjustment for age, statin use and CRP, with women in the top two tertiles having statistically significantly higher sPLA2-IIa levels than those in the bottom tertile, [Odds ratio (OR) of top two tertiles versus bottom tertile = 2.50; 95% CI (1.13–5.53) \(P = 0.024\) unadjusted and 4.82 (1.79–13.0) \(P = 0.002\) after adjustment] (Table 2B). Although TAOS was negatively correlated with sPLA2-IIa (\(P < 0.006\)), this did not remain statistically significant after adjustment.

### Table 1. Baseline characteristics (mean and SD) of Caucasian patients with T2D from UDACS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No CAD n = 383</th>
<th>CAD* n = 136</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65.5 (11.3)</td>
<td>69.5 (9.7)</td>
<td>0.0003</td>
</tr>
<tr>
<td>BMI (kg/m²)^b</td>
<td>28.2 (5.5)</td>
<td>29.5 (4.7)</td>
<td>0.67</td>
</tr>
<tr>
<td>HbA1c (%)^b</td>
<td>7.7 (1.7)</td>
<td>7.5 (1.5)</td>
<td>0.27</td>
</tr>
<tr>
<td>Glucose (mmol/l)^b</td>
<td>10.02 (4.40)</td>
<td>9.58 (4.25)</td>
<td>0.31</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.19 (1.07)</td>
<td>4.71 (1.12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL (mmol/l)^b</td>
<td>2.81 (0.93)</td>
<td>2.32 (0.89)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL (mmol/l)^b</td>
<td>1.30 (0.38)</td>
<td>1.23 (0.37)</td>
<td>0.06</td>
</tr>
<tr>
<td>TG (mmol/l)^b</td>
<td>1.90 (1.06)</td>
<td>1.92 (1.07)</td>
<td>0.84</td>
</tr>
<tr>
<td>SHBG (mmHg)^b</td>
<td>141.5 (20.6)</td>
<td>140.0 (20.9)</td>
<td>0.47</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.2 (11.4)</td>
<td>78.4 (10.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)^d</td>
<td>8 [4–16]</td>
<td>11 [6–17]</td>
<td>0.005</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>57.2% (219)</td>
<td>66.2% (90)</td>
<td>0.07</td>
</tr>
<tr>
<td>Smoking (% current)</td>
<td>17.0% (64)</td>
<td>12.0% (16)</td>
<td>0.18</td>
</tr>
<tr>
<td>TAOS (%)^a</td>
<td>44.9 [36.7–52.5]</td>
<td>42.9 [34.1–50.7]</td>
<td>0.13</td>
</tr>
<tr>
<td>Ox-LDL/LDL (U/mmol)^b</td>
<td>16.8 (7.8)</td>
<td>18.6 (10.3)</td>
<td>0.08</td>
</tr>
<tr>
<td>PPD/MPD (nm²)^c</td>
<td>0.991 (0.016)</td>
<td>0.993 (0.018)</td>
<td>0.35</td>
</tr>
<tr>
<td>Sd-LDL (%)^b</td>
<td>71.9 [58.5–81.4]</td>
<td>71.5 [54.9–80.7]</td>
<td>0.74</td>
</tr>
<tr>
<td>CRP (mg/l)^d</td>
<td>1.66 (1.42)</td>
<td>1.77 (1.59)</td>
<td>0.49</td>
</tr>
<tr>
<td>sPLA2-IIa (ng/ml)^b</td>
<td>3.08 (2.20)</td>
<td>3.45 (2.62)</td>
<td>0.12</td>
</tr>
<tr>
<td>Total Women</td>
<td>3.81 (2.66)</td>
<td>4.47 (2.89)</td>
<td>0.17</td>
</tr>
<tr>
<td>Men</td>
<td>2.62 (1.81)</td>
<td>3.02 (2.36)</td>
<td>0.12</td>
</tr>
<tr>
<td>Statin (%)</td>
<td>23.0</td>
<td>60.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ACE inhibitor (%)</td>
<td>26.5</td>
<td>38.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>21.6</td>
<td>43.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* \(n = 7\) had missing CHD data.

^a Log-transformed.

^b Square root transformed.

^c Median [IQR].

^d Peak partial diameter LDL/mean particle diameter LDL.
Using a web-based database with complete resequencing data for PLA2G2A (egp.gs.washington.edu/data/pla2g2a/pla2g2a.genotyping.html), six tSNPs were identified which together explained 92% of the haplotype variability in PLA2G2A (Table 3). The location of these tSNPs [one in the promoter region, two silent SNPs in the coding sequence (exons 2 and 4), one in intron 3 and two in the 3′-untranslated region, UTR] is presented in Figure 1, together with their pairwise linkage disequilibrium (LD). The tSNPs fall into three LD blocks.

The univariate analyses of the tSNPs with intermediate phenotypes are presented in Supplementary Material, Table S2 (A–F). Four out of the six tSNPs (2655T/C, 763C/G, 1983G/A, 5128T/G) showed strong associations with sPLA2IIa levels only (P < 0.0001), whereas with a fifth SNP (1022G>T) the association was less strong (P = 0.01). In addition, −655T>C showed strong association with LDL cholesterol levels (P = 0.007). However, as these SNPs were primarily identified for tagging purposes, i.e. chosen to cover the genetic variability of the gene, only the haplotype analysis is considered in detail here. Of the potential 64 haplotypes defined by six tSNPs, 23 inferred haplotypes were observed in the sample. Of these haplotypes, six occurred at frequencies >5% and accounted for 68% of the observed haplotypes. Eleven tSNP haplotypes occurred at frequencies between 1 and 5% and six occurred at frequencies <1% (see Supplementary Material, Table S3). The frequencies and associated sPLA2IIa levels for the six haplotypes occurring at frequencies >5% are presented in Table 4. Overall,

<table>
<thead>
<tr>
<th>Tertile of sPLA2-IIa (ng/ml)</th>
<th>P-value for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>&lt;1.95</td>
<td>1.95–3.30</td>
</tr>
<tr>
<td>n = 107</td>
<td>n = 102</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adjusted for age, CAD status and statin use.
<sup>b</sup>Adjusted for age, CAD status, CRP and statin use.
<sup>c</sup>Median [IQR].
<sup>d</sup>Log-transformed.
<sup>e</sup>Square root transformed.
<sup>f</sup>Peak particle diameter LDL/mean particle diameter LDL.
haplotypic variation in \textit{PLA2G2A} was associated with a highly significant effect on sPLA\textsubscript{2}IIa levels ($P < 0.0001$). The most frequently occurring haplotype H1 (CCGGAT) was associated with 53\% higher sPLA\textsubscript{2}IIa levels [3.26 (± 0.14) ng/ml] than all the other five haplotypes combined ($P < 0.00001$). The sPLA\textsubscript{2}IIa levels for these five haplotypes did not differ significantly from each other ($P = 0.34$) with a mean level of 2.13 (± 0.13) ng/ml. Haplotype variation in \textit{PLA2G2A} explained 6.3\% of the variance in sPLA\textsubscript{2}IIa levels. These haplotypes showed no significant association with any other intermediate trait (data not shown), and there was no frequency difference of any of the haplotypes comparing those with or without CAD.

In an attempt to identify a potentially functional SNP associated with the sPLA\textsubscript{2}IIa-raising effect of H1, a cladogram representing the evolutionary relatedness of the haplotypes was drawn up using the website http://www.fluxus-engineering.com (Fig. 2). H1 is separated from H6 by two changes, whereas H2 and H3 are separated from H6 by a single change. Thus no single SNP could distinguish H1 from the other haplotypes to suggest a single-functional SNP.

**DISCUSSION**

In this study, we report the strong association of \textit{PLA2G2A} tSNP haplotypes with serum sPLA\textsubscript{2}IIa levels. The University College London Diabetes and Cardiovascular (UDACS) study was chosen for this genetic analysis of \textit{PLA2G2A} because of the detailed measures of oxidative stress and LDL size, variables that are known to be influenced by sPLA\textsubscript{2}, and which might show association with \textit{PLA2G2A} variation. As reported previously in the EPIC study (25), in this present study, women had significantly higher sPLA\textsubscript{2}IIa and CRP than men. These higher levels in EPIC were independent of hormone replacement therapy (25). In women, sPLA\textsubscript{2}IIa levels correlated with CAD risk, with an OR of 2.50 [95\% CI (1.13–5.53) $P = 0.02$] in the top two tertiles combined, compared with the bottom tertile, which remained significant even after adjustment for CRP ($P < 0.0002$). In the men this trend was evident, but did not reach statistical significance. In the men, sPLA\textsubscript{2}IIa levels showed strong negative correlation with HDL cholesterol, seen previously in the EPIC study, and with TAOS, a net measure of plasma-oxidative stress, with higher TAOS, equating to lower the oxidative stress. Although TAOS is not a highly specific measure of plasma-oxidative stress, for a large number of samples it is a practical, inexpensive assay and there is evidence supporting the use of plasma TAOS as a marker of plasma-oxidative stress. Although TAOS is not a highly specific measure of plasma-oxidative stress, for a large number of samples it is a practical, inexpensive assay and there is evidence supporting the use of plasma TAOS as a marker of plasma-oxidative stress (26) with, for example, a strong correlation ($r = -0.65$) between the plasma TAOS and the gold-standard measure of oxidative stress, estrified F2-isoprostane levels ($P = 0.003$) (27). The inverse relationship between sPLA\textsubscript{2}IIa and TAOS is supported by a recent study demonstrating that macrophage-specific over-expression of sPLA\textsubscript{2}IIa, after bone marrow transplantation from sPLA\textsubscript{2}IIa transgenic mice, accelerated atherogenesis in \textit{ldlr}–/– mice, with an increase in oxidative stress as measured by F2-isoprostanes (28). Thus our results add weight to the concept that the increased-oxidative stress associated with high sPLA\textsubscript{2}IIa levels provides an additional mechanism for the pro-atherogenic role of...
sPLA₂IIa, although in this study this association was statistically significant only in the women.

The primary aim of this study was to examine the impact of variation in the \textit{PLA2G2A} gene on serum sPLA₂IIa levels. Serum mass measures by ELISA are specific for sPLA₂IIa and show no cross-reaction with any other sPLA₂; however, no sPLA₂IIa activity assay is currently available. No common SNPs in \textit{PLA2G2A} have been previously studied with respect to CAD, although \textit{PLA2G2A} has been studied as a candidate gene for adenomatous polyposis coli (29).

Tagging-SNP analysis is a method of maximizing the study of the genetic variability irrespective of the functionality of individual SNPs. Six common haplotypes were identified, with the most common haplotype occurring at a frequency of 14%, reflecting the rather weak LD across the gene.

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Tagging-SNP analysis is a method of maximizing the study of the genetic variability irrespective of the functionality of individual SNPs. Six common haplotypes were identified, with the most common haplotype occurring at a frequency of 14%, reflecting the rather weak LD across the gene. This was confirmed by block analysis carried out using the HAPLOVIEW website (http://www.broad.mit.edu/mpg/haploview/), which identified that the SNPs fall into three LD blocks. Overall, the haplotypes were associated with highly significant effects on sPLA₂IIa levels, \( P < 0.00001 \), confirming that the variation in \textit{PLA2G2A} was contributing to the sPLA₂ variance. This contribution to the variance did not change appreciatively (6.4%), if we included all the observed haplotypes (data not shown). Although genetic contribution of \textit{PLA2G2A} to the variance in sPLA₂IIa levels is relatively low, it is in the same order of magnitude as the variance in cholesterol ester transfer protein (CETP) concentration explained by functional variants of \textit{CETP} (30). Frisdal \textit{et al.} (30) reported that the two-functional SNPs in the CETP promoter which explain the intronic Taq1B association, namely \(-629C > A\) and \(-1137C > T\), explain 10.1% of the variance in CETP concentration.

The common \textit{PLA2G2A} haplotype, H1, was associated with 53% higher sPLA₂IIa levels compared with the pooled other haplotypes, which suggests that this haplotype might be associated with increased CAD risk. However, its low frequency and the relatively small sample size in UDACS mean that this study is underpowered to address the question.

The cladogram analysis carried out provides an unrooted evolutionary tree (31) to attempt the identification of a functional SNP(s), or in the case of tSNPs, the ‘bin’ in which the functional SNP might occur. Haplotype H1 differs from H2 (and all the four other haplotypes) by the presence of the minor and common alleles, respectively, of 5’-variants \(-655C/763C\) compared with \(-655T/763G\) present in the other five haplotypes. No other single SNP was unique to H1 that could explain the sPLA₂IIa-raising effect. This suggests that there is more than one-functional SNP accounting for this sPLA₂-raising effect and further genotyping will be required to identify this.

Several studies (23–25) have found an association between sPLA₂IIa levels and CAD risk, however, as with the association of lipoprotein associated-PLA₂ and risk (32–34), causality has not been formally established (35). In traditional epidemiological studies, the association between phenotype (a risk factor) and disease is often biased by confounding and reverse causation, i.e. the raised-risk factor is a result of the disease state not causal of it (36,37). Although statistical adjustment makes some allowance for confounding, residual confounding is a concern, because in any study not all confounders are known or measured, and those which are measured are sometimes done with errors, making complete adjustment difficult. Associations of sPLA₂IIa with CAD events could be overestimated in traditional observational studies. Conversely, the association may also be prone to underestimation, as a result of over-adjustment. Genotype, however, is not subject to confounding because it is determined at conception by the random inheritance of one of each parental allele, thus common genetic variants are potentially useful tools for overcoming the confounding. If sPLA₂IIa really does increase the risk of CAD events, individuals with alleles that raise sPLA₂IIa should have an increased risk of events similar to their effect on sPLA₂IIa level. Moreover, if sPLA₂IIa really exerts effects on CAD risk through, for example, BP, then these individuals should have a higher BP. Mendelian randomization is an approach which examines the triangular relationship among genotype, phenotype and disease and enables the determination of causality (37,38). These present results strongly suggest that variation in \textit{PLA2G2A} is having its effects through sPLA₂IIa levels directly, but UDACS is inadequately powered to detect an association between genotype and CAD risk. However, these studies provide the groundwork, by identifying the strong relationship between tSNP haplotypes and sPLA₂IIa variance, for future \textit{PLA2G2A} haplotype investigations in large prospective or case-control studies, to determine the relationship among \textit{PLA2G2A} genotype, serum sPLA₂IIa levels and CAD risk, to clarify this issue of causality.

**MATERIALS AND METHODS**

**Study design**

UDACS study consist of 1014 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) 2001–2 (629 men; 532 Caucasians with T2D). All patients had diabetes according to WHO criteria (39). Analysis was restricted to the Caucasian subjects with T2D to remove possible heterogeneity within the sample. Six of these patients had sPLA₂IIa levels more than 3 standard deviations (SD) from the mean and were excluded from further analysis, as it might imply underlying infection. Thus 526 Caucasian patients with T2D (313 men and 213 women) were examined further. CAD status was not available for seven of these patients. Information about medication was available, particularly statin usage, ACE inhibitors and aspirin. Aspirin as an inhibitor of COX-1 and COX-2 affects platelet function by inhibiting the enzyme prostaglandin. sPLA₂IIa also feeds into the prostaglandin pathway and therefore may be ultimately affected by aspirin usage.

**Clinical measurements**

CAD event was recorded if any patient had positive coronary angiography/angioplasty, coronary artery bypass, cardiac thallium scan, exercise tolerance test, myocardial infarction or symptomatic/treated angina, detailed elsewhere (26). Routine plasma traits were measured (26) including plasma oxidized LDL by ELISA (Mercodia, Uppsala, Sweden), expressed as...
the ratio of oxidized LDL divided by total LDL to generate a specific measure of LDL oxidation (40). Plasma TAOS, which is inversely related to oxidative stress, was measured by a photometric microassay (41). LDL particle size and peak particle diameter were measured as previously described (42,43). The percentage small-dense LDL (sd-LDL) is derived from the percentage of LDL subclasses I and II from the four subclasses I–IV obtained by ultracentrifugation (44). Serum sPLA2IIa levels were measured by a commercially available ELISA (Cayman Chemical Company, Ann Arbor, MI, USA). The intra- and inter-assay coefficients of variation were 6.0 and 10.3%, respectively. Full ethical approval was granted by the UCHL NHS Trust and all patients included in the study had consented.

DNA extraction, tagging-SNP identification and genotyping

DNA was extracted using the salting out method (45). tSNPs were identified using the STRAM algorithm (46) on the PHASE (47) output from the National Institute of Environmental Health Sciences SNP database website http://egp.gs washington.edu/genes.html. Six tSNPs of PLA2G2A were identified (rs1774131, rs11573156, rs3753827, rs2236771, rs876018, rs3767221). All SNPs were genotyped using TaqMan technology (Applied Biosciences, ABI, Warrington UK). Reactions were performed on 384-well microplates and analysed using ABI TaqMan 7900HT software. Primers and MGB probes are detailed in Supplementary Material, Table S1.

Statistical methods

The Hardy–Weinberg equilibrium and the LD ($D'$) of tSNPs were assessed using THESIAS (48,49). All analyses were performed on normally distributed data after appropriate transformation (log or square root). Results are presented as mean and SD. Parametric or non-parametric (Kruskal–Wallis) analysis of variance was used, when appropriate, to compare the changes of the continuous variables across the SNPs categories. Multiple regressions were used to calculate the adjusted R-square for the proportion of the variance explained by the model. For the categorical variables, Pearson’s chi-square or Fisher’s exact tests were used depending on the expected values of each category in the two-way table. Adjusted $P$-values were obtained from the analysis of covariance for continuous data and logistic regression for categorical data. Haplotypes were inferred using both THESIAS (48,49) and PHASE (47) excluding individuals with missing values. The haplotypic pair for each subject was calculated by PHASE (47) and only the haplotypes with frequencies >5% were used for further analysis. Because of multiple testing, the significance level was taken as $P < 0.01$, instead of an inappropriately conservative Bonferroni-like adjustment of the $P$-values (50,51).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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