Tagging SNP haplotype analysis of the secretory PLA2-V gene, *PLA2G5*, shows strong association with LDL and oxLDL levels, suggesting functional distinction from sPLA2-IIA: results from the UDACS study

Peter T.E. Wootton¹⁺, Nupur L. Arora¹⁺, Fotios Drenos¹, Simon R. Thompson¹, Jackie A. Cooper¹, Jeffrey W. Stephens², Steven J. Hurel³, Eva Hurt-Camejo⁴,⁵, Olov Wiklund⁵, Steve E. Humphries¹ and Philippa J. Talmud¹,*

¹Division of Cardiovascular Genetics, Department of Medicine, Royal Free and University College Medical School, 5 University Street, London WC1E 6JF, UK, ²The Medical School, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, UK, ³Department of Diabetes and Endocrinology, UCL Hospitals, London W1T 3AA, UK, ⁴AstraZeneca, R&D, Molecular Pharmacology, Mölndal S-43183, Sweden and ⁵Wallenberg Laboratory for Cardiovascular Research, I, Göteborg SE-413 45, Sweden

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Animal and human studies suggest that both secretory PLA2 (sPLA2)-V and sPLA2-IIA (encoded, respectively, by the neighbouring *PLA2G5* and *PLA2G2A* genes) contribute to atherogenesis. Elevated plasma sPLA2-IIA predicts coronary heart disease (CHD) risk, but no mass assay for sPLA2-V is available. We previously reported that tagging single nucleotide polymorphism (tSNP) haplotypes of *PLA2G2A* are strongly associated with sPLA2-IIA mass, but not lipid levels. Here, we use tSNPs of the sPLA2-V gene to investigate the association of *PLA2G5* with CHD risk markers. Seven *PLA2G5* tSNPs genotypes, explaining >92% of the locus genetic variability, were determined in 519 patients with Type II diabetes (in whom *PLA2G2A* tSNP data was available), and defined seven common haplotypes (frequencies >5%). *PLA2G5* and *PLA2G2A* tSNPs showed linkage disequilibrium (LD). Compared to the common *PLA2G5* haplotype, H1 (frequency 34.9%), haplotypes H2–7 were associated with overall higher plasma LDL (*P* < 0.00004) and total cholesterol (*P* = 0.00003) levels yet lower oxLDL/LDL (P = 0.006) and sPLA2-IIA mass (*P* = 0.04), probably reflecting LD with *PLA2G2A*. Intronic tSNP (rs11573248), unlikely itself to be functional, distinguished H1 from LDL-raising haplotypes and may mark a functional site. In conclusion, *PLA2G5* tSNP haplotypes demonstrate an association with total and LDL cholesterol and oxLDL/LDL, not seen with *PLA2G2A*, thus confirming distinct functional roles for these two sPLA2s.

INTRODUCTION

Secretory phospholipase A2 (sPLA2) group V (sPLA2-V) enzyme is a member of the superfamily of PLA2 enzymes characterized by their ability to hydrolyse the sn-2 ester bond of phospholipids and cell membranes, generating non-esterified free fatty acids (NEFAs) and lyso-phospholipids (1). The sPLA2-V gene (*PLA2G5*) is tightly linked and in a negative orientation to the sPLA2-IIA gene (*PLA2G2A*) on chromosome 1p34–36.1 (2), with both enzymes sharing structural and functional similarities (3). While the role of sPLA2-IIA in atherogenesis has been well studied, the
involvement of sPLA2-V is less well understood and questions about functional redundancy have been raised (4).

sPLA2-IIA is an acute phase protein, elevated in cell types in response to pro-inflammatory stimuli (3). sPLA2-IIA hydrolyses LDL phospholipids generating oxidation-susceptible, small-dense LDL (sdLDL) particles, with an altered configuration of apolipoprotein B (5), leading to LDL-receptor-independent uptake into the arterial wall (6). Within the arterial wall, these modified LDL particles bind proteoglycans present in the intima and are further modified, leading to increased retention (7). In addition, the release by sPLA2s of potent lipid mediators, in particular arachidonic acid and lyso-phospholipids, promotes pro-inflammatory responses in the arterial wall (reviewed in 8). The involvement of sPLA2-IIA in atherogenesis is confirmed by its localization in atherosclerotic plaques (9,10).

Observational studies have also identified sPLA2-IIA as a marker for coronary heart disease (CHD) risk (11), with elevated levels associated with an increased probability of recurrent coronary events (12,13). In prospective analysis, serum levels of sPLA2-IIA were associated with a higher risk of future CHD in healthy individuals independent of classical and inflammatory markers (14). In animal models, sPLA2-IIA transgenic mice show an increased susceptibility to atherosclerosis (15,16).

Less information exists regarding the potential pro-atherogenic actions of the functionally similar sPLA2-V enzyme. In vitro, sPLA2-V is able to hydrolyse HDL and LDL phosphatidylcholine (PC) 20 times more efficiently than sPLA2-IIA (17), thus hydrolysing the most common phospholipid species present in lipoproteins and cell surface membranes (18), leading to arachidonic acid and lyso-PC generation (19). Since several atherogenic susceptible mouse strains have a naturally occurring mutation in Pla2g5, other sPLA2s have been implicated in the development of atherosclerosis in these strains. sPLA2-V is a likely candidate, and recent immunohistochemical analysis (supported by mRNA expression studies) has shown sPLA2-V to be associated with smooth muscle cells and foam cells in the lipid cores of both human and mouse atherosclerotic lesions (20). LDL receptor knock-out mice, either over-expressing or deficient in Pla2g5, provide the first in vivo evidence that sPLA2-V contributes to atherosclerosis (21).

Although these studies support an independent role for sPLA2-V in atherogenesis, currently no commercially available assay for sPLA2-V mass exists, and therefore human observational studies to confirm the CHD risk association cannot be undertaken.

By utilizing a tagging single nucleotide polymorphism (tSNP) haplotype approach, we have made use of common genetic variations to test the hypothesis that variation in PLA2G5 influences both plasma lipid levels and oxidative stress. These tSNPs were genotyped in a cohort of patients with Type II diabetes (T2D) mellitus, a group associated with a higher CHD risk, where oxidative stress and lipid measurements were available (22). In addition, we have sPLA2-IIA mass measures and PLA2G2A tSNP haplotype data in the same cohort (22), thus enabling us to compare the relationship of these two genes with CHD risk markers.

| Table 1. Baseline characteristics of Caucasian patients with Type II diabetes from UDACS |
| No CHD, N = 383 | CHD, N = 136 | P-value |
| Age (years) | 65.5 (11.3) | 69.5 (9.7) | 0.0003 |
| BMI (kg/m²) | 29.2 (5.5) | 29.5 (4.7) | 0.67 |
| HbA1c (%) | 7.7 (1.7) | 7.5 (1.5) | 0.27 |
| Glucose (mmol/l) | 10.0 (4.40) | 9.58 (4.25) | 0.31 |
| Cholesterol (mmol/l) | 5.19 (1.07) | 4.71 (1.12) | <0.0001 |
| LDL (mmol/l)² | 2.81 (0.93) | 2.32 (0.89) | <0.0001 |
| HDL (mmol/l)² | 1.30 (0.38) | 1.23 (0.37) | 0.06 |
| TG (mmol/l)² | 1.90 (1.06) | 1.92 (1.07) | 0.84 |
| sPLA2-IIA (ng/ml)² | 141.5 (20.6) | 140.0 (20.9) | 0.47 |
| sPLA2-IIA (ng/ml)² | 81.2 (11.4) | 78.4 (10.0) | 0.01 |
| Duration of diabetes (years)² | 8.0 (4–16) | 11.0 (6–17) | 0.005 |

- Log-transformed.
- Square-root transformed.
- Median (IQR).

RESULTS

The baseline characteristics of the Caucasian men and women with T2D, with or without CHD, are shown in Table 1. Those who had CHD were significantly older, and had a longer duration of diabetes, and their higher usage of statins and angiotensin converting enzyme (ACE) inhibitors, most likely explains their lower diastolic blood pressure (BP), LDL and total cholesterol levels compared with CHD-free men and women.

Seven tSNPs (−1437C>A, −1251G>A, −423G>A, 1640C>T, 1742G/ins/del, 11069T/ins/del, 22507T>G) were identified in the PLA2G5 gene region. The genotype distribution of all tSNPs was as expected for Hardy–Weinberg equilibrium as tested by THESIAS (Table 2 gives a summary of the observed allele frequencies). The locations of the chosen tSNPs (three in the promoter region, three within introns and one variant 3’ of exon 5) are shown schematically in Figure 1A. Lewontin’s D’ for each individual tSNP is shown below the SNP map in Figure 1B, demonstrating strong LD across the PLA2G5 gene.

Univariate analyses of tSNPs with total, LDL- and HDL-cholesterol and measures of total antioxidant status, LDL size and oxidized LDL to LDL ratio are presented in Supplementary Material, Tables S2–S8. Of the tSNPs, −1437C>A, 1742G/ins/del and 11069T/ins/del showed significant association with cholesterol and LDL levels (P-values ranged from P < 0.01 to <0.0001); Supplementary Material, Tables S2, S6 and S7, respectively. −423G>A and 1742G/ins/del were also strongly associated with differences in sPLA2-IIA levels (P < 0.01; Supplementary Material, Tables S4 and S6, respectively).
analysis similar to that demonstrated in the haplotype analysis of the closely related PL2G2A gene (22) was not achievable since several different combinational changes of tSNPs were possible for each haplotype. As such, no individual tSNP could be identified as being responsible for the associations described. However, H2–7, which showed significant association with LDL cholesterol, in contrast to H1, have in common the 11609T allele, while H1 is defined by the rare 11609T allele, which also carries the 11609T allele, has trait values most similar to H1. The 11609T allele variant (rs11573248) is located 3 kb 5’ of exon 2, and it is therefore doubtful that it is of functional importance, but is likely to be in strong LD with a yet undetermined functional variant.

**DISCUSSION**

**PLA2G5 tSNP haplotypes are associated with differences in LDL levels and sPLA2-IIA mass**

We have used a genetic approach to distinguish the effects of the sPLA2 enzymes group V and IIA. tSNPs that capture 92% of the locus genetic variation were used to investigate the association of PLA2G5 variants (encoding sPLA2-V) with plasma lipid levels and with markers of oxidative stress, contrasted in the same study to those previously reported for PL2G2A (encoding sPLA2-IIA) (22). The common PLA2G5 tSNP haplotype H1 (with a frequency of 39%), when compared with the rarer haplotypes H2–7, showed strong association with lower plasma levels of LDL (P < 0.00004) and total cholesterol (P < 0.00003), yet showed no association with lipid levels or measures of oxidative stress (22). These data implied that in vivo, sPLA2-IIA is not having a major impact on determining the hydrolysis of lipids in the circulation. In contrast, PLA2G5 tSNP haplotypes are having a major effect on LDL levels, reflected also in levels of total cholesterol, and oxLDL levels.

**Functional differences in sPLA2-V and sPLA2-IIA**

The contrasting effects of PLA2G5 and PL2G2A on LDL levels are supported by in vitro data demonstrating that sPLA2-V is 20 times more active than sPLA2-IIA in its ability to hydrolyse phospholipids (17). The recent studies by Rosengren et al. (20,25) strongly support distinct roles for sPLA2-V and sPLA2-IIA on circulating lipids and in atherogenic lesions. Recombinant sPLA2-V but not sPLA2–IIA hydrolysed lipoprotein phospholipids in human sera and isolated VLDL, HDL and LDL (preferentially in that order, probably due to differences in the sphingomyelin content), accompanied by an increase in lyso-phospholipids (20). This enzymatic difference between the two sPLA2s is suggested to be due to the tryptophan residues in the interface-binding region of sPLA2-V, absent in sPLA2-IIA, which would enable enhanced penetration of sPLA2-V into the phospholipid monolayer (20).

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**Table 2. SNP rs number and minor allele frequency of the PLA2G5 tSNPs in UDACS**

<table>
<thead>
<tr>
<th>tSNP</th>
<th>rs number</th>
<th>Minor allele frequency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1437C&gt;A</td>
<td>rs11573185</td>
<td>0.45 (0.43–0.48)</td>
</tr>
<tr>
<td>−1251G&gt;A</td>
<td>rs2148911</td>
<td>0.07 (0.05–0.08)</td>
</tr>
<tr>
<td>423C&gt;G</td>
<td>rs11573191</td>
<td>0.18 (0.16–0.19)</td>
</tr>
<tr>
<td>1640C&gt;T</td>
<td>rs640002</td>
<td>0.15 (0.13–0.16)</td>
</tr>
<tr>
<td>1742G/ins/del</td>
<td>rs11573203</td>
<td>0.28 (0.26–0.30)</td>
</tr>
<tr>
<td>11069TA/ins/del</td>
<td>rs11573248</td>
<td>0.36 (0.34–0.39)</td>
</tr>
<tr>
<td>2250T&gt;G</td>
<td>rs622450</td>
<td>0.14 (0.12–0.15)</td>
</tr>
</tbody>
</table>
Further differences between sPLA2-V and sPLA2-IIA were seen by Rosengren et al. (20). In studies of C57BL/6 mice and ldlr/apoe double knockout mice, which lack pla2g2a due to deletion of exon 3, sPLA2-V expression was upregulated in the aorta when mice were fed a Western diet. No effect of a high fat diet was seen on sPLA2-IIA expression in the PLA2G2A transgenic mice. However, when mice were treated with inter-peritoneal injection of lipopolysaccharide to promote inflammation, expression of sPLA2-IIA but not sPLA2-V was stimulated. Difference in the localization of these two enzymes in the atherosclerotic plaques of mice and humans further support a distinct functional divergence (20,25).

Inverse relationship between LDL levels and sPLA2

Surprisingly, compared to PLA2G5 H2–7 carriers, H1 carriers had significantly lower LDL cholesterol ($P < 0.00004$), but significantly higher oxLDL/LDL ($P = 0.006$) and borderline higher sPLA2-IIA mass ($P = 0.04$), so in part the lower LDL levels could reflect higher LDL hydrolysis by sPLA2-IIA. This seems unlikely, since as discussed above, PLA2G2A haplotypes that had a strong association with sPLA2-IIA levels showed no effect on LDL cholesterol levels. PLA2G2A and PLA2G5 lie head to tail on chromosome 1p34–36.1 (2) (in a cluster with other sPLA2 genes), thus it is

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Figure 1. (A) Map of the PLA2G5 gene showing the selected tagging SNPs numbered from the start of exon 1. (B) Haploview LD (D’) of tSNPs of both PLA2G5 and PLA2G2A. The darker boxes represent the stronger LD. The D’ for any two SNPs is presented in the box representing their intersection. No number indicates a D’ of 1. The head to tail orientation of the two genes and the tSNPs used in this and our previous study (22) are presented.
possible that variation within this intergenic region may be influencing the expression of both genes. \textit{PLA2G5} SNPs \(2423G>A\) and \(1742G\)in/del are both associated with significant differences in \(\text{sPLA2-IIA}\) levels (see Supplementary Material, Tables), and from Figure 1B it is clear that both these SNPs show significant LD with \textit{PLA2G2A} \(763C>G\), which shows strong statistical significant association with \(\text{sPLA2-IIA}\) levels \((P < 0.0001)\) (see Supplementary Material, Tables in 22). Therefore, it is probable that the association of \textit{PLA2G5} haplotypes with \(\text{sPLA2-IIA}\) mass (explaining only 0.6% of the \(\text{sPLA2-IIA}\) variance) is simply reflecting the LD that exists in this gene cluster. The effect on lipid levels therefore suggest that the association of \textit{PLA2G5} haplotypes with \(\text{LDL}\) levels are due to the haplotypic effects associated with differences in \(\text{sPLA2-V}\) activity/mass itself.

Thus, we speculate that, in comparison to haplotypes H2–7, H1 should be associated with higher \(\text{sPLA2-V}\), and since high \(\text{sPLA2 activity}\) is reported to increase risk in humans (13) and mice (5), this would lead to increased CHD risk. On the face of it this appears counter-intuitive, since the lower \(\text{LDL cholesterol levels}\) in H1 carriers, compared to H2–7, should be associated with decreased risk. However, the lower plasma \(\text{LDL levels}\) seen in the H1 carriers could be the result of \(\text{LDL conversion to sdLDL particles}\) due to increased phospholipid hydrolysis. This would result in increased retention of \(\text{LDL}\) in the arterial wall, increased oxidation and thus lead to increased atherosclerosis. Since \(\text{sdLDL}\) is more prone to oxidation, the oxLDL/LDL ratio should also be higher. We did not see an effect of \textit{PLA2G5} haplotype on \(\text{sdLDL levels}\) but we did see a significant effect of haplotypes on oxLDL/LDL. Carriers of haplotype H1 had oxLDL/LDL levels which were 22% higher \((10.19 \text{ U/mmol)}\) compared to H2–7 \((8.35 \text{ U/mmol}, P = 0.006)\).

There is support for these associations and the predicted outcomes from the study by Mallat \textit{et al.} (13), who measured both \(\text{sPLA2 activity}\) and mass in patients with acute coronary syndrome. While the \(\text{sPLA2-IIA mass assay}\) shows no cross
reactivity with sPLA2-V, the activity assay measures most plasma sPLA2s (i.e. including both -IIA and -V) (13). Those individuals without hyperlipidaemia had significantly higher sPLA2 activity compared to those with hyperlipidaemia (2.88 versus 2.33 nmol/min/ml, respectively; \( P = 0.001 \)), and the cumulative incidence of death or MI according to tertiles of sPLA2 in patients with cholesterol \( \leq 197 \) mg/dl was 8.4% compared to 5.2% in those with cholesterol \( > 197 \) mg/dl (13). Thus, this study also shows an inverse relationship between plasma lipids and sPLA2 activity (13).

**Limitations of the study**

The tSNPs identified for this study were derived from the resequenced data from the NIEHS database and have an \( r^2 = 0.92 \). A comparison cannot be made directly to those tSNPs obtained from the recent HAPMAP release (21). This highlights the issue of tSNP identification in a rapidly changing field where both the databases are constantly upgraded, as well as the algorithms for tSNP identification. The use of additional tSNPs might possibly sub-divide haplotype H1, allowing a clearer identification of the relevant haplotype for functional studies. However, the use of a limited set of tSNPs cannot be a confounder of an association that is identified. In the absence of a specific sPLA2-V assay, we cannot exclude the possibility that these haplotypic effects on LDL cholesterol levels are independent of sPLA2-V activity or mass, and we cannot determine what the association of these \( PLA2G5 \) haplotypes actually are with sPLA2-V levels. In addition, because of the relatively small sample size, the UDACS study was insufficiently powered to detect any associations between \( PLA2G5 \) inferred haplotypes and CHD risk. The association between haplotype H1 and lower LDL levels has been reported here in patients with T2D, and the association may not be the same in non-T2D individuals.

Finally, the strong LD across the gene cluster means that it is not possible to distinguish \( PLA2G5 \) and \( PLA2G2A \) effects entirely, although LD may be lower in non-Caucasians, which could allow for better discrimination of these effects.

**CONCLUSION**

The association of \( PLA2G5 \) haplotypes with markers of CHD provides the foundation for further investigations in case-control and prospective studies, specifically investigating whether these haplotypes are associated with CHD risk and with LDL levels in non-diabetic subjects. Despite these limitations, this study represents the first investigation of genetic variation in \( PLA2G5 \) and its association with markers of CHD and strongly supports the recent studies by Rosengren et al. (20,25) showing a distinct functional difference between the two closely related sPLA2 enzymes sPLA2-V and sPLA2-IIA.

**MATERIALS AND METHODS**

**Study design**

The University College London Diabetes And Cardiovascular Disease Study (UDACS) has been described in detail elsewhere (26,27). In brief, the UDACS consists of 1014 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) 2001–02 (629 men; 600 Caucasians with T2D). All patients had diabetes according to WHO criteria (28). Analysis was restricted to the Caucasian subjects with T2D to remove possible heterogeneity within the sample.

**Clinical measurements**

CHD 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 (27). Routine plasma traits were measured including plasma oxidised LDL (oxLDL) by ELISA (Mercodia, Uppsala, Sweden), expressed as the ratio of oxLDL divide by total LDL to generate a specific measure of LDL oxidation (27,29). Plasma total antioxidant status (TAOS), which is inversely related to oxidative stress, was measured by a photometric assay (30). The percentage sdLDL is derived from the percentage of LDL sub-classes I and II from the four sub-classes I–IV obtained by ultra-centrifugation (31). Serum sPLA2-IIA 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 UCLH NHS trust and all patients included in the study had given written consent.

**DNA extraction, tagging-SNP identification and genotyping**

DNA was extracted using the salting out method (32). Tagging SNPs were identified using the STRAM algorithm (33) on the PHASE (34) output from the National Institute of Environmental Health Sciences SNP database website (http://egp.gs.
rs640022 tSNP was determined using a polymerase chain reaction (PCR) with sense (5'-GGACTGGTGATGGTGGGAGT-3') and anti-sense (5'-CCAGGTATGATGGTGCACAG-3') oligonucleotides flanking the variant of interest. Restriction of the PCR product with the PvuII enzyme results in fragment sizes of 166/20 bp in common homozygotes, 186/166/20 bp in heterozygotes and 186 bp in rare homozygotes. Fragments were resolved using Microtitre Array Diagonal Gel Electrophoresis (MADGE) (35). Two negative controls were included in each PCR run.

Statistical methods

Hardy–Weinberg equilibrium was assessed using THESIAS (23,24). Linkage disequilibrium (LD) as measured by D' was assessed using Haploview (http://www.broad.mit.edu/mpg/haploview/). All analyses were performed on normally distributed data after appropriate transformation (log or square root). Results are presented as mean and standard deviation (SD). Parametric or non-parametric (Kruskall–Wallis) analysis of variance was used, when appropriate, to compare the changes of the continuous variables across the SNPs categories. 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 (23,24) and PHASE (34) excluding individuals with missing values. The haplotypic pair for each subject was calculated by PHASE 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 inappropriate conservatively Bonferroni-like adjustment of the P-values (36,37).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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