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Translational studies of lipoprotein-associated phospholipase A2 in inflammation and atherosclerosis

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Abstract

Objectives—To examine the role of lipoprotein-associated phospholipase A2 (Lp-PLA_2/ PLA2G7) in human inflammation and coronary atherosclerosis.

Background—Lp-PLA_2 has emerged as a potential therapeutic target in coronary heart disease (CHD). Data supporting Lp-PLA_2 are indirect and confounded by species differences; whether Lp-PLA_2 is causal in CHD remains in question.

Methods—We examined inflammatory regulation of Lp-PLA_2 during experimental endotoxemia in human, probed the source of Lp-PLA_2 in human leukocytes under inflammatory conditions, and assessed the relationship of variation in *PLA2G7*, the gene encoding Lp-PLA_2, with coronary artery calcification (CAC).

Results—In contrast to circulating TNFα and CRP, blood and monocyte Lp-PLA_2 mRNA decreased transiently, and plasma Lp-PLA_2 mass declined modestly during endotoxemia. *In vitro,* Lp-PLA_2 expression increased dramatically during human monocyte to macrophage differentiation and further in inflammatory macrophages and foam like-cells. Despite only a

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marginal association of SNPs in *PLA2G7* with Lp-PLA$_2$ activity or mass, numerous *PLA2G7* SNPs were associated with CAC. In contrast, several SNPs in *CRP* were significantly associated with plasma CRP levels but had no relation with CAC.

Conclusions—Circulating Lp-PLA$_2$ did not increase during acute phase response in human, while inflammatory macrophages and foam cells, but not circulating monocytes, are major leukocyte sources of Lp-PLA$_2$. Common genetic variation in *PLA2G7* is associated with sub-clinical coronary atherosclerosis. These data link Lp-PLA$_2$ to atherosclerosis in human while highlighting the challenge in using circulating Lp-PLA$_2$ as a biomarker of Lp-PLA$_2$ actions in the vasculature.

Keywords

Lp-PLA$_2$; PLA2G7; CAC

INTRODUCTION

Lipoprotein-associated phospholipase A2 (Lp-PLA$_2$) has emerged as a potential therapeutic target in coronary heart disease (CHD) and phase III clinical trials are underway. Supporting evidence includes apparent atherogenic biochemical properties; Lp-PLA$_2$ cleaves oxidized phosphatidylcholine on modified LDL producing inflammatory lysophosphatidylcholine and oxidized non-esterified fatty acids (1,2). In addition, enzymatic expression of Lp-PLA$_2$ is up-regulated in human atherosclerosis (3), while circulating levels are associated with incident CHD (4). Promising proof-of-principle pre-clinical and clinical trials have been carried out (5–7). However, whether Lp-PLA$_2$ is causal and whether its inhibition will prevent CHD events remain open questions.

Data for Lp-PLA$_2$ in human atherosclerosis remain indirect and confounded by species differences in physiology and actions. Lp-PLA$_2$ circulates in blood, bound to lipoproteins which modulate its actions. In rodents Lp-PLA$_2$ is carried mostly on high-density lipoprotein (HDL) particles whereas in human the enzyme is bound to low-density lipoprotein (LDL) particles. Thus, confounding may be particularly marked for plasma Lp-PLA$_2$ relative to other inflammatory markers, as regulation of atherogenic lipoproteins is a major influence on circulating Lp-PLA$_2$ levels and activity (8). Indeed, whether circulating Lp-PLA$_2$ is associated with CHD beyond a complete assessment of atherogenic lipoproteins remains uncertain (9).

Arterial Lp-PLA$_2$ biosynthesis by macrophages and foam cells, rather than circulating levels or activity, may determine its atherogenicity (10). Lp-PLA$_2$ expression within the necrotic core and surrounding macrophages of vulnerable and ruptured plaques is increased compared with less-advanced lesions (11), suggesting a potential role in promoting plaque instability. The extent to which human Lp-PLA$_2$ is regulated in circulation by systemic inflammation, however, versus locally controlled in arterial macrophage-foam cells is uncertain. Further, lesion biosynthesis is difficult to measure in human limiting our ability to monitor Lp-PLA$_2$ activity in disease-relevant tissue and to assess vascular efficacy of pharmacological inhibition.

In this report, we examined inflammatory regulation of circulating Lp-PLA$_2$ during experimental endotoxemia in human, probed the source of Lp-PLA$_2$ in human leukocytes under inflammatory conditions, and determined the relationship of genetic variation in phospholipase A2, group VII (*PLA2G7*), the gene encoding Lp-PLA$_2$, to coronary artery calcification (CAC) as well as plasma levels of Lp-PLA$_2$ mass and activity. We found that, unlike blood tumor necrosis factor alpha (TNFα) and C-reactive protein (CRP), circulating Lp-PLA$_2$ did not increase during the acute phase response in human, that inflammatory

macrophages and foam cells, but not circulating or *ex vivo* monocytes, are primary leukocyte sources of Lp-PLA_2, and that common genetic variation in *PLA2G7* is associated with sub-clinical coronary atherosclerosis. These data link Lp-PLA_2 to atherosclerosis in human while providing a human physiological context for the difficulty in using circulating Lp-PLA_2 as a biomarker of disease or pharmacological efficacy in atherosclerosis.

METHODS

Clinical Studies

Human endotoxemia—Healthy volunteers on no medications and no significant medical history (N=32, 50% female; mean age 25.7±3.90) were studied as described previously (12,13) and in the supplement. Serial blood samples were collected before and after intravenous bolus infusion of 3 ng/kg US standard reference endotoxin and were prepared for plasma, whole-blood RNA and monocyte RNA (12).

Genetic association studies—The Penn Coronary Artery Calcification (PennCAC) resource included European-ancestry subjects recruited to three separate studies at U.Penn: the Study of Inherited Risk of Coronary Atherosclerosis (SIRCA; N=799), the Penn Diabetes Heart Study (PDHS; N=782), and the Philadelphia Area Metabolic Syndrome Network (PAMSyN; N=480). These studies are described in detail previously (14,15) and in the supplement. In each study, subjects with clinical atherosclerotic CVD were excluded. *PLA2G7* SNPs were genotyped in all three studies. Plasma Lp-PLA_2 mass and activity data were available in SIRCA and PDHS. Global CAC scores were determined by electron beam tomography (Imatron, San Francisco, CA) according to the method of Agatston(16). For all human studies described, the University of Pennsylvania (U.Penn) Institutional Review Board (IRB) approved each study and written informed consent was provided by all participants.

Laboratory Methods

Human monocyte, macrophage and foam cell studies—Human moncoyte isolation, macrophage ("M1" and "M2" phenotype) differentiation (17) and "foam cell" preparation was performed as described (12) and in supplement. Experiments were performed in batches using freshly-isolated monocytes, macrophages and foam cells derived from the same human volunteer.

Plasma LpPLA2, inflammatory and metabolic markers—Plasma and cell-media levels of Lp-PLA_2 mass and activity, TNFα, and CRP, as well as lipid and biochemical markers were measured as described (13,14,18) and in supplement.

Real-time quantitative PCR and expression quantitative trait locus analysis—Whole-blood, isolated circulating monocyte, and human cultured monocyte, macrophage and foam cell mRNA was subjected to quantitative PCR (qPCR) using primers and probes (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) as described (12) for measurement of Lp-PLA_2, TNFα, and β-actin mRNA (supplement). The relative quantitation $2^{-(\Delta\Delta Ct)}$ method was used to determine fold-change from baseline (19). Exploratory expression Quantitative Trait Locus (eQTL) analysis is described in the supplement.

Genotyping—As described previously (20) and in the supplement, PennCAC participants were genotyped using the ITMAT Broad Care (IBC) CVD candidate gene array, which surveys ~50,000 SNPs in ~2,000 candidate genes (21). SNP data for *PLA2G7* (N=19) and *CRP* (N=16) were selected for current analysis.

Statistical analysis

The effect of endotoxemia on plasma Lp-PLA_2 mass and activity, TNFα, and CRP as well as whole-blood and monocyte mRNA was tested by repeated-measures analysis of variance (ANOVA). ANOVA was applied also to *in vitro* cell data. Post hoc *t*-tests were used to compare specific time-points and treatments. We observed heterogeneity of variance in several variables following LPS challenge, which was to be expected given the known variation in responses to endotoxin. We tested for homogeneity of variance using Levene's test, and in cases where the assumption of homogeneity of variance was violated, we confirmed whether the group differences were significant using Tamhane's post-hoc test.

In PennCAC, CAC scores were transformed by the natural log after adding 1 (Ln(CAC+1)), to correct for skewed distribution. This variable was used as the outcome in a linear regression model, with *PLA2G7* and *CRP* SNPs, adjusting for age, gender and age-gender interaction. For linear regression analysis of SNP associations with plasma proteins, Lp-PLA_2 mass and activity were normally distributed, and therefore used as outcomes while CRP was log-transformed. The linear regression model included adjustments for age, gender, and smoking. Analysis used PLINK v 1.06. Analyses of CAC and plasma proteins were performed separately in each sample and then subjected to meta-analysis. Meta-analysis applied a weighted *Z*-score method using METAL(22) (http://www.sph.umich.edu/csg/abecasis/Metal) as we applied in (23), described in supplement. In analysis of SNP data, we corrected for the number of independent tests within each gene (10 tests for 19 *PLA2G7* SNPs, unadjusted *P* value threshold of 0.005, and 15 tests for 15 *CRP* SNPs, unadjusted *P* value threshold of 0.0033) using the method of Nyholt (24).

RESULTS

Lp-PLA_2 is not induced in a human model of acute phase response

As we described (13,25) endotoxemia produced an acute, febrile illness associated with a marked, transient induction of plasma TNFα (P <0.001), followed by a delayed ~100-fold induction of plasma CRP at 24 hours (P<0.001) (Figure 1A). In contrast, plasma Lp-PLA_2 mass and activity did not increase following LPS (Figure 1B). Indeed, levels of Lp-PLA_2 mass tended to decline (by 18% at 6hours, P<0.01). The mRNA response to LPS in whole-blood for TNFα (Figure 1C) and Lp-PLA_2 (Figure 1D) as well as in circulating monocytes for TNFα (Figure 1E) and Lp-PLA_2 (Figure 1F) was similar to that of plasma proteins. The mRNA levels of Lp-PLA_2 in circulating monocytes were low but detectable (baseline CTs~30, varying from CTs of 28–32 post-LPS).

Lp-PLA_2 expression is induced in inflammatory human macrophages and foam cells

Lp-PLA_2 mRNA levels were low (CTs ~30) in freshly-isolated human monocytes but increased markedly (CTs ~20) following six-days of differentiation to mature macrophages (P<0.0001) (Figure 2A) and increased modestly during further polarization to M1 (P<0.0001) but not M2 macrophages (Figure 2B). Lp-PLA2 protein mass also was induced during differentiation to macrophages, with increases in both the cell-associated protein (P<0.0001) and the secreted protein (P=0.0004) (Figure 2C). Following loading of human monocyte-derived macrophages with acetylated LDL-C for 48hrs, cholesterol ester (128 vs. 0.6 ug CE/mg protein) and total cholesterol (422 vs. 316 ug chol/mg protein) were significantly higher in loaded versus unloaded cells consistent with findings for *in vivo* foam cells (26). Lp-PLA_2 mRNA levels were significantly greater in foam cells compared with mature macrophages (P<0.01) (Figure 3A). Similarly, cell-associated (P=0.05) and secreted (P=0.008) Lp-PLA2 protein levels were higher in foam cells than in macrophages (Figure 3B). There was no Lp-PLA2 protein detectable in the media or acLDL used to treat cells.

Overall, these data are consistent with lack of *in vivo* increase in plasma or monocyte levels of Lp-PLA_2 during the acute phase and suggest that, in human atherosclerosis, Lp-PLA_2 may be generated by macrophages and foam cells rather than circulating leukocytes.

Exploratory interrogation of *PLA2G7* SNP eQTLs for Lp-PLA_2 mRNA expression in publicly-available data revealed nominal associations of several SNPs in the *PLA2G7* region with exon probe levels in PBMCs (best *P*=0.0059, rs12181971) and brain (best *P*=0.008, rs12195701) (27), as well as skin (best *P*=0.021, rs16874962), fat (best *P*=0.019, rs16874962) and lymphoblastoid cells (best *P*=0.037, rs7745519) (MuTHER twin2 study resource (28)). These modest associations, however, were not significant after correction for multiple testing (Supplement). Macrophage and foam cell expression datasets were not available for testing a more atherosclerosis-relevant cell type.

Common polymorphisms in PLA2G7 are associated with coronary calcification but only weakly with plasma Lp-PLA_2 mass or activity

Individually in SIRCA or PDHS samples, there were no significant associations between *PLA2G7* SNPs and Lp-PLA_2 mass or activity. In the combined meta-analysis, only one SNP (rs1805017) had nominal association with Lp-PLA_2 mass (*P*=0.02; *P*=0.2 after Bonferroni correction) (Table 1A). As a positive control, we performed similar analysis of plasma CRP using common *CRP* SNPs. In contrast to findings for *PLA2G7*-Lp-PLA_2, there were significant associations between numerous *CRP* SNPs and plasma CRP in the SIRCA and PDHS samples and in the overall meta-analysis (Table 1B). Nine of sixteen *CRP* SNPs had nominal (*P*<0.05) associations with CRP levels and eight of these SNPs had significant associations after Bonferroni correction.

Association of *PLA2G7* SNPs with CAC was assessed initially in SIRCA&PAMSyN together with follow-up in PDHS. Multiple *PLA2G7* SNPs had nominal associations with CAC in SIRCA&PAMSYN (eleven with *P*<0.05; lowest *P*<0.0001 for rs1421378). Replication signals in PDHS were modest (strongest rs10948300 *P*=0.02) likely due to limited power; however, in PDHS sixteen of nineteen SNPs had effects in the same direction as in SIRCA/PAMSYN ($\chi 2$=8.9, *P*=0.003). Meta-analysis of the combined sample found several SNP associations with CAC (rs9349373, *P*=0.002; rs2216465, *P*=0.002; rs12195701, *P*=0.004) that were significant after Bonferroni correction (Table 2A). Including plasma Lp-PLA_2 mass or activity in the model did not attenuate the association between *PLA2G7* SNPs and CAC. These findings support recent associations of variation in *PLA2G7* with CHD (29–31).

As an expected negative control (14,32,33), we examined *CRP* variant associations with CAC in the same sample and found minimal signal, with one SNP having nominal association in SIRCA&PAMSYN (rs3093068, *P*=0.04); however there were no associations in PDHS nor in combined meta-analysis.

DISCUSSION

We provide novel insight into the pathophysiology of Lp-PLA_2 in human. First, we show that unlike TNFα and CRP, circulating Lp-PLA_2 does not increase during experimental endotoxemia and therefore does not contribute to human acute phase response. Second, we found that inflammatory macrophages and foam cells, but not circulating monocytes or cultured primary monocytes, generate significant Lp-PLA_2. This is consistent with the concept that the majority of Lp-PLA_2 in atherosclerotic plaque is derived from local biosynthesis by inflammatory macrophage and foam cells rather than from circulating leukocytes. Third, we found that common variants in *PLA2G7* are associated with CAC but had limited relation to circulating Lp-PLA_2 mass or activity. This supports an atherogenic

role for *PLA2G7*-Lp-PLA$_2$ in human that may be independent of circulating LpPLA2 mass or activity.

Lp-PLA$_2$ does not contribute to human acute phasee response

We demonstrate that Lp-PLA$_2$ is not an acute phase protein in humans. This is in contrast to rodent models where LPS challenge was shown to induce a rapid increase in plasma and tissue levels of Lp-PLA$_2$ (34). This provides further evidence of fundamental differences between humans and rodents in the physiology and action of Lp-PLA$_2$ (35). Lack of induction in blood and circulating monocytes by endotoxemia *in vivo* also suggests limited, if any, role for circulating leukocyte production of Lp-PLA2 in atherosclerosis. In contrast, marked *in vitro* up-regulation in macrophages and foam cells is consistent with a specific role for local vascular production of Lp-PLA$_2$ in atherosclerosis. While it is possible that local macrophage Lp-PLA$_2$ production in plaque may contribute to a portion of circulating Lp-PLA$_2$, it is unlikely to render circulating levels useful as independent biomarkers of Lp-PLA2 actions in atherosclerosis because published data show that circulating Lp-PLA2 mass and activity do not correlate with plaque Lp-PLA2 in patients undergoing elective carotid endarterectomy (36) and because there is substantial confounding of plasma Lp-PLA2 by circulating lipoproteins regardless of tissue source. Overall, these data suggest that levels of Lp-PLA$_2$ mRNA and protein in blood may be poor surrogates of *PLA2G7* actions in arterial plaque.

Pro-inflammatory macrophages and foam cells, but not monocytes, generate significant Lp-PLA$_2$

We found that Lp-PLA$_2$ expression was markedly increased during the differentiation of monocytes to macrophages, and further induced *in vitro* in “foam cell”-like macrophages. This is consistent with constitutive expression and activity in inflammatory macrophages (37) and foam cells in atherosclerosis. Indeed, Lp-PLA$_2$ expression is increased in atherosclerotic lesions in humans (10). In this environment, secreted Lp-PLA$_2$ can hydrolyze oxidized phospholipids and fatty acids on atherogenic lipoproteins, generating reactive lipid mediators thought to promote plaque instability. Inhibition of Lp-PLA$_2$ suppressed oxidized-LDL-induced macrophage apoptosis (38), a feature of inflammatory plaque. Further, in a porcine model of complex atherosclerosis, suppression of Lp-PLA$_2$ retarded atherosclerosis progression and decreased plaque inflammation, necrosis and fibrous cap erosion (7). Compared to placebo, short-term Lp-PLA$_2$ inhibition in human also reduced several markers of plaque inflammation in carotid lesions examined *ex vivo* (5,6). Overall, these data provide indirect evidence for atherogenic actions of Lp-PLA$_2$ in vascular lesions. Indeed, Lp-PLA$_2$ inhibition is currently being tested in large phase-III clinical trials of CHD in high risk patients (NCT0100072, clinicaltrials.gov).

Genetic variation in PLA2G7 may relate to CHD independent of circulating Lp-PLA$_2$

Several epidemiological studies revealed an association of higher plasma Lp-PLA$_2$ mass and activity levels with risk of CHD (9,39–41). Meta-analyses support a modest CHD relationship independent of traditional risk factors and plasma CRP (39,42,43). Published studies, however, may underestimate the degree of confounding because of incomplete measurement and control for all atherogenic lipoproteins (9). In circulation, Lp-PLA$_2$ associates with both apoB lipoproteins and HDL with the majority found on LDL particles. Since Lp-PLA$_2$ protein and activity are closely linked to circulating apoB lipoproteins (35,44), it is not surprising that genetic factors (e.g. *APOC1, PSRC1, ZNF259*) that regulate plasma apoB lipoproteins are also associated with plasma Lp-PLA$_2$ (45). Parenthetically, we found modest association of lipid-related genes (e.g., *LRP2, LPL, APOA2*) with plasma Lp-PLA$_2$ likely reflecting this indirect post-translational influence (Supplemental Table S1A and B). Interpretation of studies of plasma Lp-PLA$_2$ in CHD is challenging partly because

circulating lipoproteins may grossly confound the association of plasma Lp-PLA_2 with CHD (8) and further because lesion macrophage production may be more relevant to the disease than circulating protein.

While we failed to detect significant association between plasma Lp-PLA_2 and common SNPs in *PLA2G7*, the same *PLA2G7* variants were associated with CAC within our study samples. Our preliminary exploration also revealed only nominal associations of *PLA2G7* SNPs with Lp-PLA_2 mRNA levels in multiple cells and tissues. These eQTL findings should be interpreted cautiously because of limited power, relatively low levels of Lp-PLA_2 expression in tested cells, and (unlike *CRP*) well-characterized *cis*-acting SNPs for *PLA2G7* are lacking. Further, appropriately powered studies are needed to determine whether *PLA2G7* SNPs are related to expression of Lp-PLA_2 in inflammatory macrophages and foam cells, sources that may be most relevant to atherosclerosis. However, our data suggest caution in using circulating leukocyte Lp-PLA_2 mRNA levels as surrogates for effects of *PLA2G7* variation on arterial pathology. Overall, our findings support the concept that *PLA2G7* may relate to atherosclerosis independent of circulating Lp-PLA_2 mRNA and protein.

Published studies of *PLA2G7* in CHD are conflicting. In a meta-analysis of individuals of European ancestry, *PLA2G7* SNPs did not associate with risk of CHD (N~5,000) (41), although there was a relationship between Lp-PLA_2 activity and CHD, and between *PLA2G7* SNPs and Lp-PLA_2 activity. However in a meta-analysis of over 13,000 Asians, a common non-synonymous *PLA2G7* SNP showed evidence of association with CHD (30). Additional non-synonymous SNPs have been associated with carotid plaque in Japanese (31) and recently a loss-of-function variant in *PLA2G7* was shown to protect against CHD in Koreans (29). Due to the absence in Caucasian samples of the functional *PLA2G7* SNP found in Asians (rs76863441 or V279F), we were not able to evaluate the effect of this functional variant in our samples. However, common variation in *PLA2G7* is well covered on the IBC array platform (tag SNP-coverage r^2>0.8 for alleles with MAF≥2% in the gene ±5KB). (21). Therefore, we are confident that we achieved excellent coverage of common variation in this gene region in Caucasians. While ethnic difference in the presence of allelic variation may exist,, most published data suggest a relationship of *PLA2G7* with clinical CHD supporting our CAC findings.

Findings for *CRP* in our samples are consistent with published data and contrast with that observed for *PLA2G7*-Lp-PLA_2. Thus, while a number of SNPs in *CRP* had strong associations with circulating CRP levels, there was no relationship between these same SNPs and CAC. These data are in line with hallmark Mendelian randomization studies of clinical CHD outcomes (32,33) and support a model of confounding or reverse causation for CRP associations with CAC and CHD.

Limitations of the present study, and future outlook

Our study has several limitations. First, our studies are correlative and do not define causality. We have not studied loss-of-function or gain-of-function variants in *PLA2G7* for their relation to CAC or CHD and therefore cannot infer Lp-PLA2 directional actions in atherosclerosis. However, expression data in inflammatory macrophages and foam cells coupled to preliminary studies of Lp-PLA_2 inhibition in human atherosclerosis support an atherogenic role for human *PLA2G7.* Second, recent studies have shown stronger associations of *PLA2G7* with circulating Lp-PLA_2 measures than in our sample. This may relate to our smaller sample size, heterogeneity in the SIRCA and PDHS study samples, or differences in Lp-PLA_2 assays used across studies. The *PLA2G7*-Lp-PLA_2 system, however, may be a poor target for Mendelian randomization studies for several reasons including heterogeneous environmental and genetic influences on circulating levels,

PLA2G7 actions in atherosclerosis are likely independent of circulating Lp-PLA_2, and well-characterized *cis*-acting SNPs to use as instrumental variables for *PLA2G7* are lacking. Finally, although not a direct measure of coronary atherosclerosis, studies show that CAC provides a quantitative estimate of coronary atherosclerosis (46) and is a useful predictor of CHD events (47).

In conclusion, we have demonstrated that Lp-PLA_2, in contrast to CRP, is not an acute phase protein in humans. Lp-PLA_2 has limited expression in circulating leukocytes or unstimulated monocytes *ex vivo* but is induced during differentiation to macrophages and in foam cells. Thus, robust biomarkers of Lp-PLA_2 action in atherosclerosis and of its pharmacological modulation in vascular tissues are lacking. Common variation in *PLA2G7*, but not in *CRP*, is related to the burden of CAC, suggesting that *PLA2G7* may indeed modulate human atherosclerosis. Our data provide support for the atherogenicity of Lp-PLA_2 in human while highlighting the challenges in using plasma Lp-PLA_2 as a biomarker of CHD and in determining drug-dosing and therapeutic efficacy in atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SNP	single nucleotide polymorphism
CAC	coronary artery calcification
CHD	coronary heart disease
LPS	lipopolysaccharide
CRP	C-reactive protein
HDL	high-density lipoprotein
LDL	low-density lipoprotein

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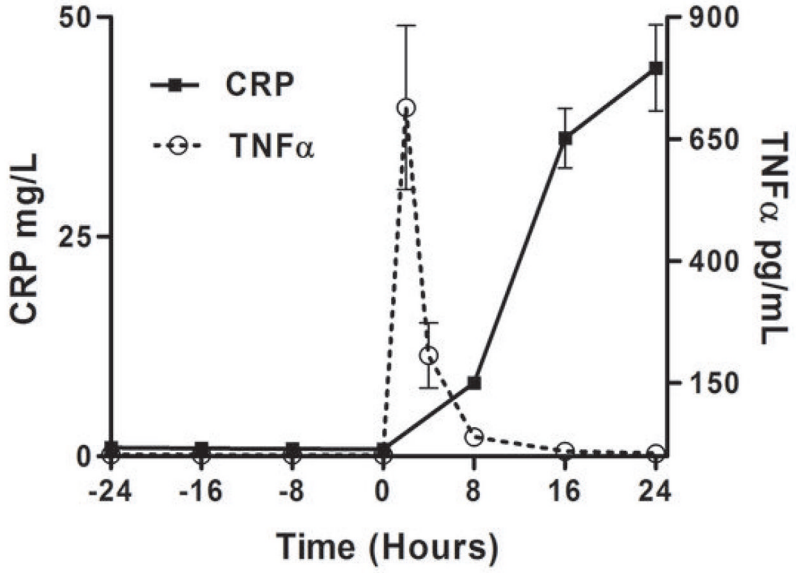
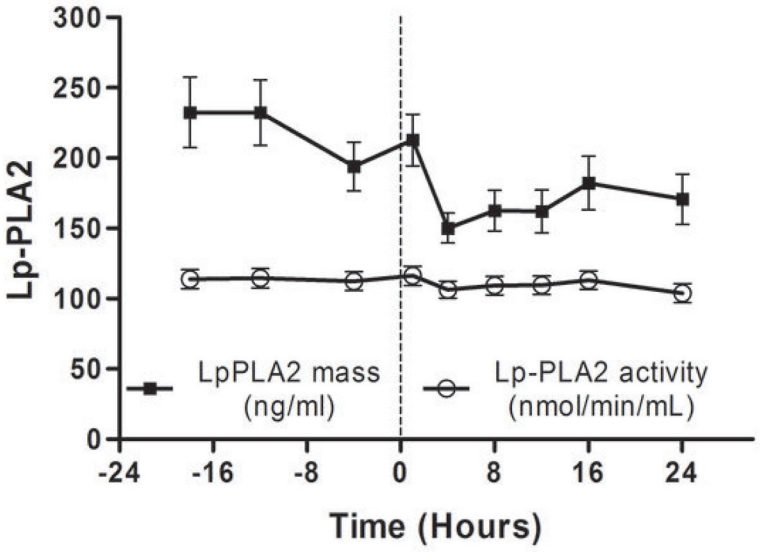
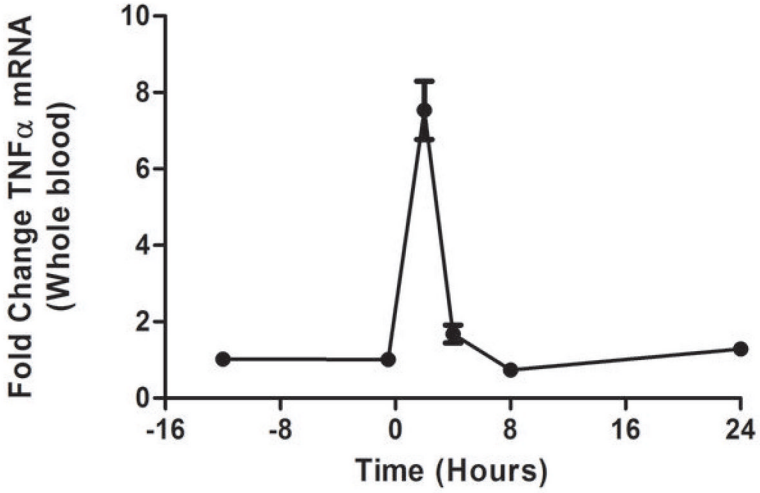
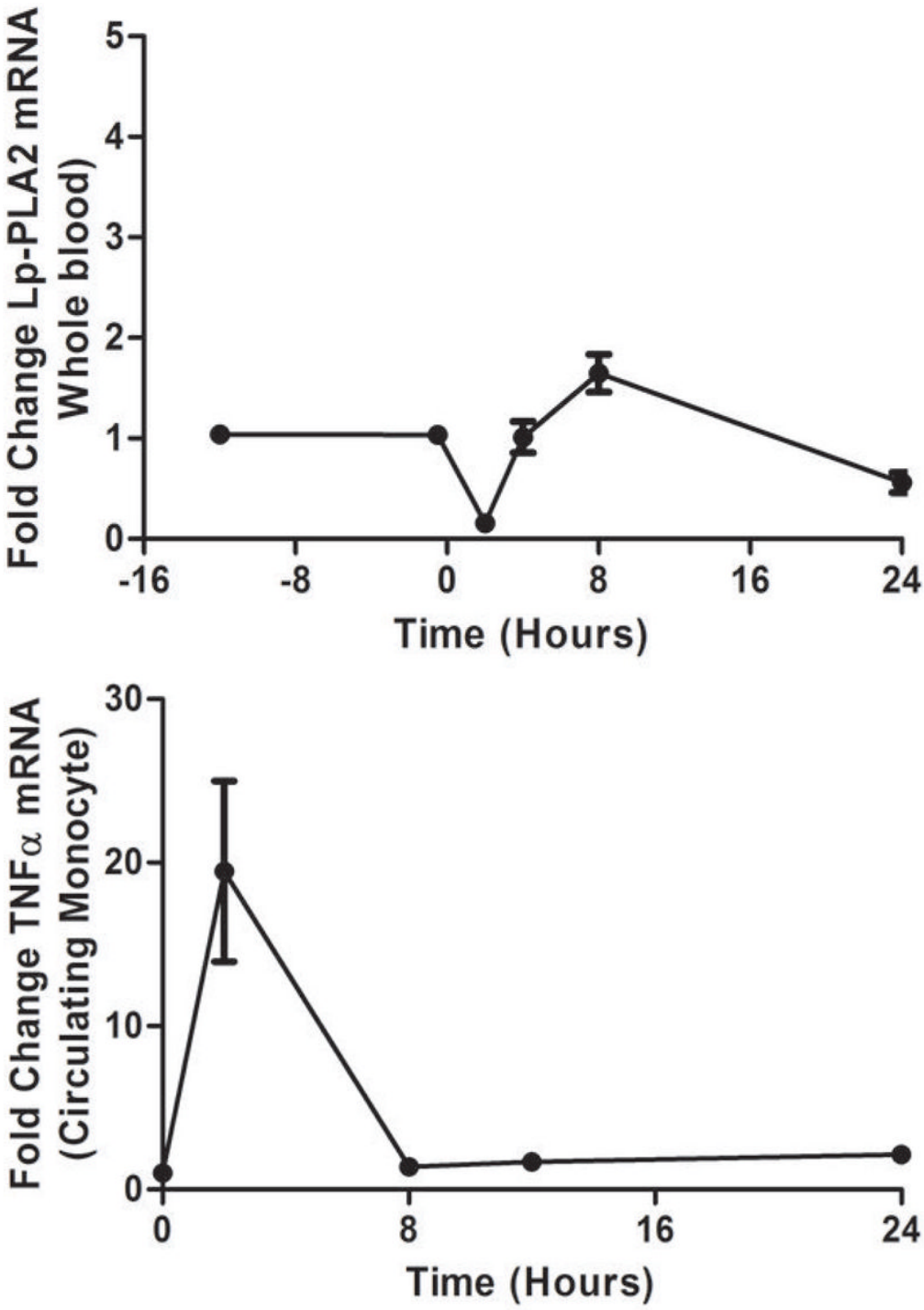








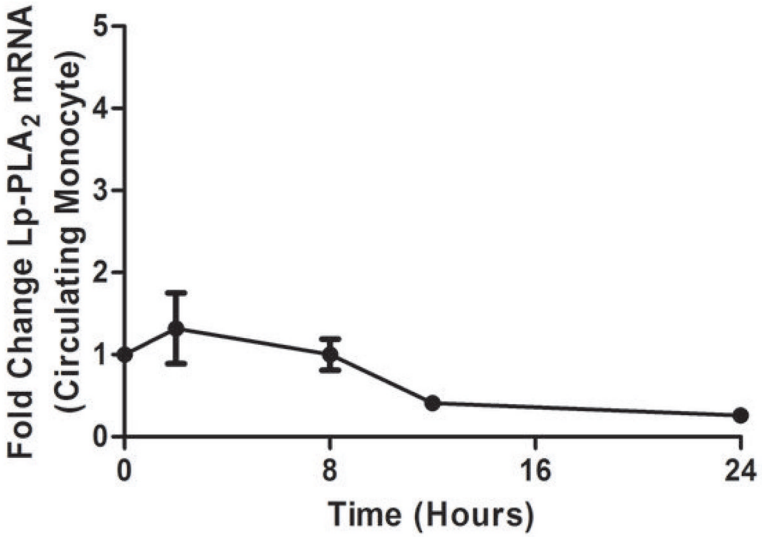
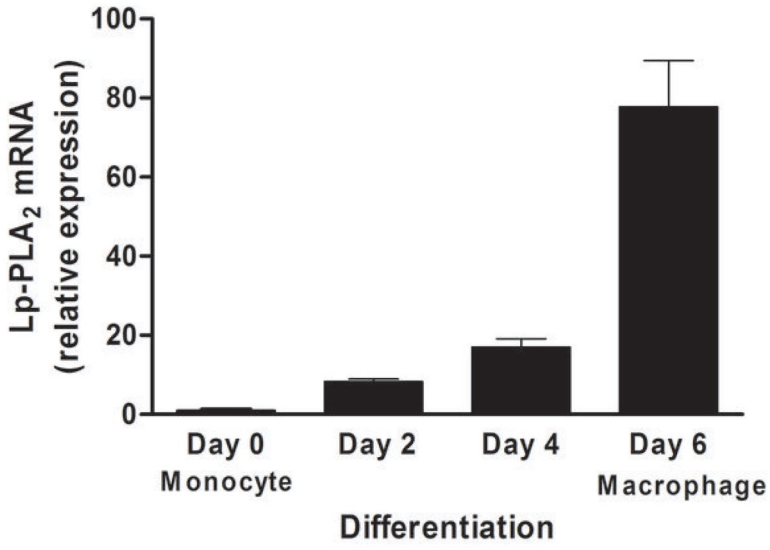
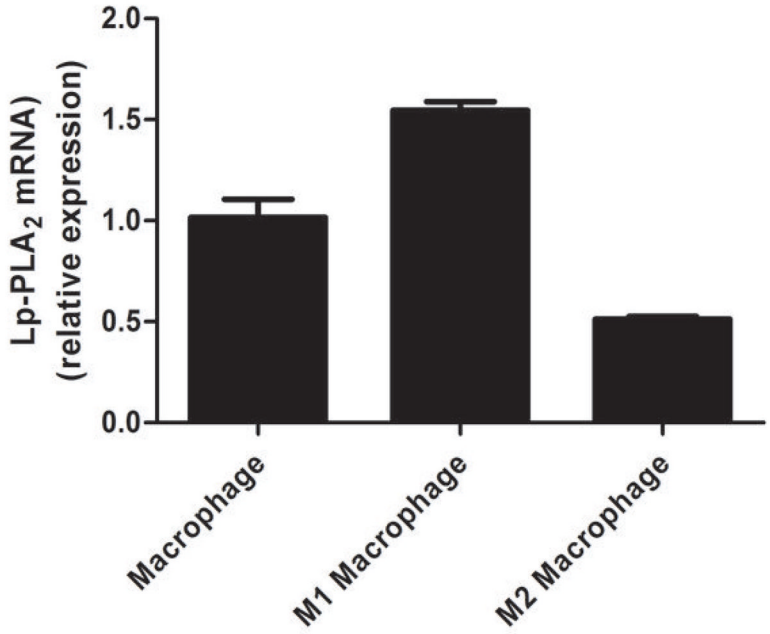


Figure 1. Human endotoxemia does not induce circulating Lp-PLA$_2$ protein or leukocyte Lp-PLA$_2$ mRNA *in vivo*

Endotoxemia (3ng/Kg LPS intravenously) markedly increased plasma levels of (A) TNFα and CRP (P<0.001) but not (B) Lp-PLA$_2$ mass or activity which declined transiently (P<0.01). Following LPS, whole-blood (C) TNFα mRNA was markedly induced but (D) Lp-PLA$_2$ mRNA is not. Similarly, LPS increased circulating monocyte mRNA levels of (E) TNFα but not (F) Lp-PLA$_2$.





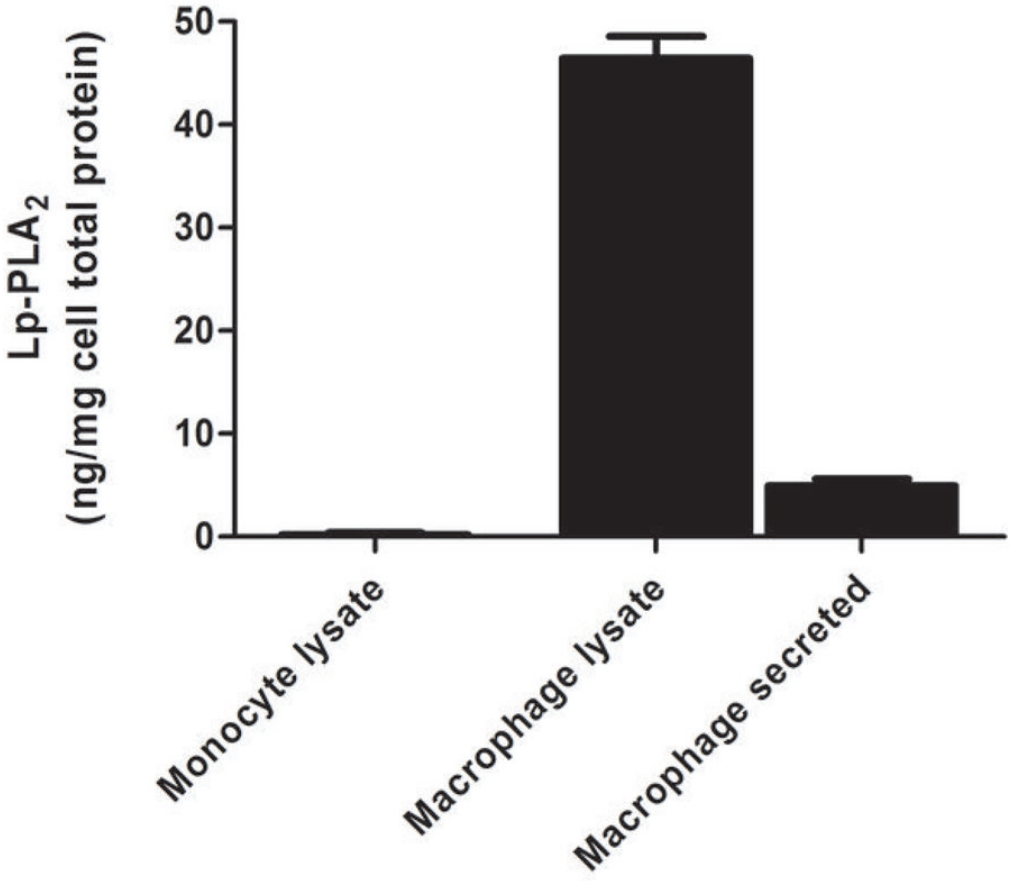
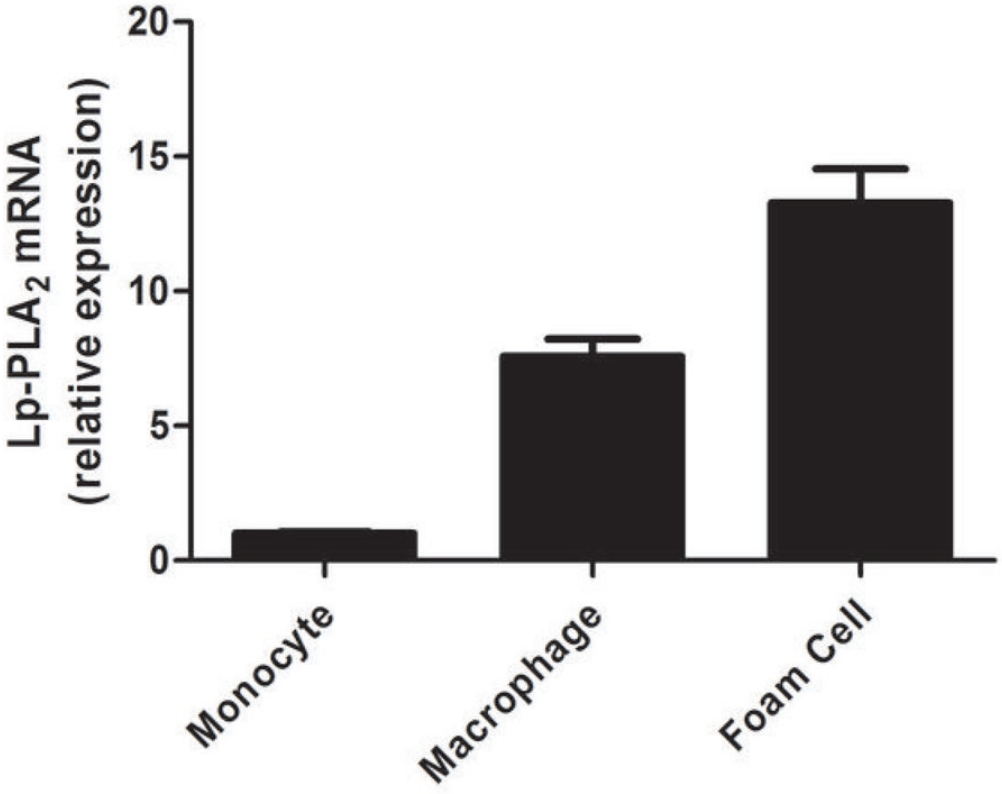


Figure 2. Lp-PLA$_2$ mRNA and protein increase during differentiation of human monocytes to macrophages *in vitro*

Lp-PLA$_2$ mRNA levels increased markedly during differentiation from monocytes to mature macrophages (P<0.0001) (A) and increased modestly during further polarization to M1 macrophages (P<0.001) but fell during M2-polarization (P<0.001) (B). Lp-PLA2 protein mass also increased significantly during differentiation to macrophages, with increases in both the cell-associated protein (P<0.0001) and the secreted protein (P=0.0004) † (C). (ANOVA and Bonferroni post-hoc tests).

† As monocytes were grown in suspension, protein levels were measured in monocyte cell lysates but could not be measured in media.



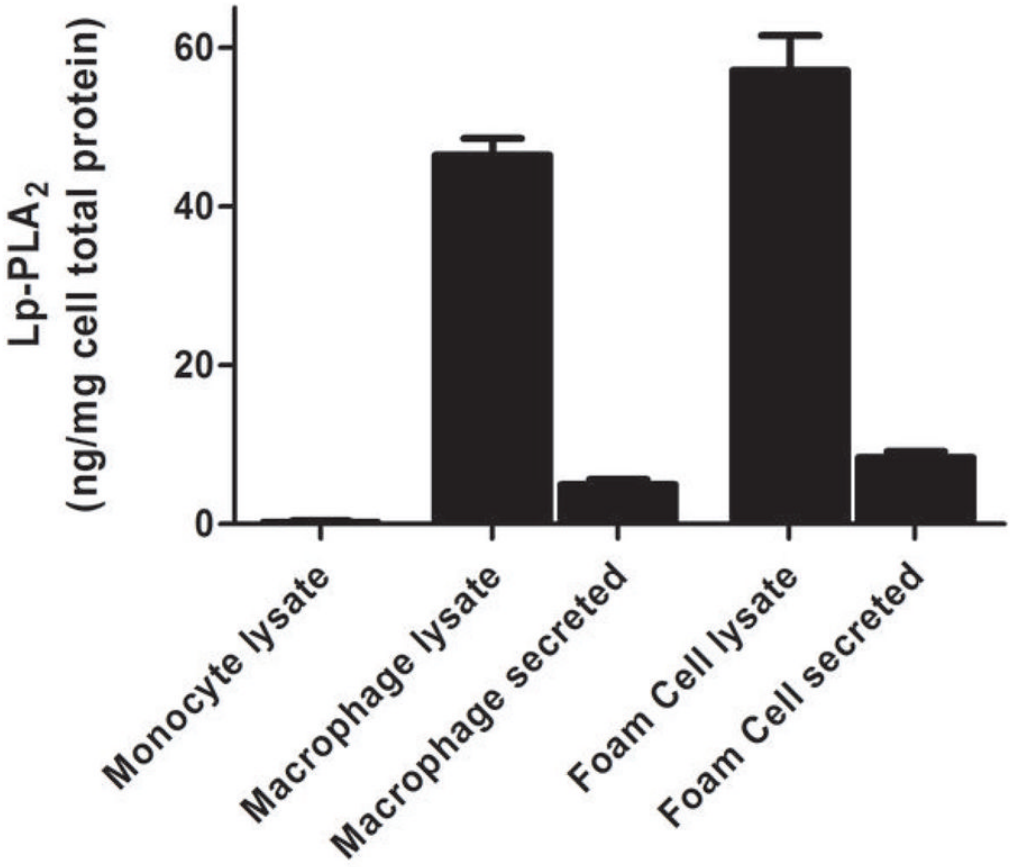


Figure 3. Lp-PLA$_2$ mRNA and protein are upregulated in human foam-like cells *in vitro*

Lp-PLA$_2$ mRNA was significantly greater (P<0.01) in foam cells than in mature macrophages (A). Similarly, cell-associated (P=0.05) and secreted (P=0.008) Lp-PLA2 protein levels were higher in foam cells than in macrophages † (B). (ANOVA and Bonferroni post-hoc tests). † As monocytes were grown in suspension, protein levels were measured in monocyte cell lysates but could not be measured in media.

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Table 1

(A) Lack of association between SNPs in *PLA2G7* and circulating Lp-PLA2 protein mass or activity but (B) significant relation of multiple SNPs in *CRP* with circulating CRP levels*.

(A)			Lp-PLA$_2$ Mass Meta-analysis (n=1723)		Lp-PLA$_2$ Activity Meta-analysis (n=1683)	
†***PLA2G7*** **SNP (Allele: Minor/Major)**	**Type of SNP**	**MAF**	**Z**	**P**	**Z**	**P**
rs1805017 (A/G)	Non-synonymous Coding	0.26	2.34	0.02	0.23	0.82
rs2216465 (C/G)	Intronic	0.34	1.54	0.12	0.66	0.51
rs1421378 (G/A)	Upstream	0.41	1.37	0.17	0.37	0.71
rs17288905 (G/A)	Intronic	0.08	1.74	0.08	0.79	0.43
rs10948300 (T/A)	Intronic	0.21	1.77	0.08	0.31	0.76
rs12195701 (A/G)	Intronic	0.21	1.46	0.14	0.77	0.44
rs1051931 (A/G)	Non-synonymous Coding	0.21	1.53	0.13	0.07	0.95
rs7756935 (C/A)	Intronic	0.21	1.53	0.13	0.07	0.95
rs12528807 (C/A)	Intronic	0.08	−0.36	0.72	0.44	0.66
rs1362931 (A/C)	Intronic	0.20	1.15	0.25	0.06	0.96
rs1421372 (A/G)	Intronic	0.20	1.15	0.25	0.06	0.96
rs3799861 (A/G)	Intronic	0.20	1.15	0.25	0.06	0.96
rs9472830 (A/G)	Intronic	0.20	1.07	0.29	0.08	0.94
rs16874962 (A/G)	Intronic	0.07	−1.12	0.26	−0.46	0.64
rs3799863 (A/T)	Intronic	0.05	−0.71	0.48	1.01	0.31
rs1421368 (G/A)	Intronic	0.10	0.42	0.68	0.02	0.99
rs9349373 (A/G)	Upstream	0.20	0.23	0.82	−0.49	0.63
rs16874967 (A/G)	Intronic	0.06	−0.34	0.74	0.68	0.49
rs1805018 (G/A)	Non-synonymous Coding	0.06	−0.34	0.74	0.68	0.49

(B)			Meta-analysis (n=2026)	
†***CRP*** **SNP (Allele: Minor/Major)**	**Type of SNP**	**MAF**	**Z**	**P**
rs1205 (A/G)	3′UTR	0.34	−5.63	1.8E-08
rs1800947 (G/C)	Synonymous coding	0.07	−5.41	6.4E-08
rs3091244 (A/G)	5′ Upstream	0.37	4.40	1.1E-05
rs12084589 (A/C)	5′ Upstream	0.07	3.23	1.0E-03
rs12068753 (A/T)	5′ Upstream	0.07	3.18	1.0E-03

(B)			Meta-analysis (n=2026)	
†*CRP* SNP (Allele: Minor/Major)	Type of SNP	MAF	Z	P
rs3093059 (G/A)	5′ Upstream	0.07	3.14	2.0E-03
rs3093068 (G/C)	3′ Downstream	0.06	2.94	3.0E-03
rs2794521 (G/A)	5′ Upstream	0.25	0.29	0.77
rs3093066 (A/C)	3′UTR	0.001	1.99	0.05
rs2808634 (A/G)	5′ Upstream	0.27	0.39	0.69
rs1130864 (A/G)	3′UTR	0.30	2.94	0.003
rs2808631 (G/A)	3′ Downstream	0.002	−1.49	0.14
rs3093070 (C/A)	3′ Downstream	0.02	−0.82	0.41
rs3093069 (C/A)	3′ Downstream	0.001	1.37	0.17
rs3093071 (A/C)	3′ Downstream	0.01	1.23	0.22

*Meta-analysis of data from the Study of Inherited Risk of Coronary Atherosclerosis (SIRCA) and the Penn Diabetes Heart Study (PDHS).

†For all analyses, the effect allele corresponds to the minor allele. MAF=minor allele frequency. The 19 SNPs in *PLA2G7* fall into 10 LD blocks (r^2>0.8), therefore we used Bonferroni multiple testing correction for 10 effective tests, giving a *P* value threshold of significance of 0.005. The 15 SNPs in *CRP* fall into 15 LD blocks (r^2>0.8), therefore we used Bonferroni multiple testing correction for 15 effective tests, giving a *P* value threshold of significance of 0.0033.

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Table 3

Association of SNPs in (A) *PLA2G7* but not in (B) *CRP* with Coronary Artery Calcification.

(A)			*SIRCA&PAMSYN (n=1279)		*PDHS (n=782)		Meta-analysis (n=2061)	
†*PLA2G7* SNP (Allele: Minor/Major)	Type of SNP	MAF	Beta	P	Beta	P	Z	P
rs1421378 (G/A)	Upstream	0.41	0.31	9.8E-05	0.05	0.65	3.35	8.1E-04
rs2216465 (C/G)	Intronic	0.34	0.29	3.9E-04	0.05	0.67	3.06	0.002
rs12528807 (C/A)	Intronic	0.08	0.39	0.004	0.40	0.07	3.36	7.7E-04
rs9349373 (A/G)	Upstream	0.20	0.26	0.008	0.22	0.13	3.04	0.002
rs10948300 (T/A)	Intronic	0.21	0.24	0.013	0.32	0.02	3.36	7.8E-04
rs1805017 (A/G)	Non-synonymous Coding	0.26	0.22	0.014	0.09	0.49	2.37	0.018
rs3799863 (A/T)	Intronic	0.05	0.46	0.016	−0.23	0.39	1.37	0.17
rs16874967 (A/G)	Intronic	0.06	0.39	0.02	−0.14	0.56	1.41	0.16
rs1805018 (G/A)	Non-synonymous Coding	0.06	0.39	0.02	−0.14	0.56	1.41	0.16
rs12195701 (A/G)	Intronic	0.21	0.21	0.03	0.26	0.07	2.85	0.004
rs1421368 (G/A)	Intronic	0.10	−0.27	0.04	−0.17	0.37	−2.16	0.03
rs9472830 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.26	0.02
rs1362931 (A/C)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs1421372 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs3799861 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs7756935 (C/A)	Intronic	0.21	−0.16	0.11	−0.15	0.25	−1.98	0.05
rs1051931 (A/G)	Non-synonymous Coding	0.21	−0.16	0.11	−0.16	0.23	−2.01	0.04
rs16874962 (A/G)	Intronic	0.07	0.12	0.44	0.15	0.46	1.06	0.29
rs17288905 (G/A)	Intronic	0.084	−0.04	0.76	−0.02	0.94	−0.29	0.77

(B)			* SIRCA&PAMSyN (n=1279)		* PDHS (n=782)		Meta-analysis (n=2061)	
†*CRP* SNP (Allele: Minor/Major)	Type of SNP	MAF	Beta	P	Beta	P	Z	P
rs3093068 (G/C)	3′ Downstream	0.06	−0.32	0.04	−0.09	0.70	−1.82	0.07
rs3093059 (G/A)	5′ Upstream	0.07	−0.31	0.06	−0.11	0.65	−1.78	0.07
rs12068753 (A/T)	5′ Upstream	0.07	−0.31	0.06	−0.08	0.75	−1.70	0.09
rs12084589 (A/C)	5′ Upstream	0.07	−0.31	0.06	−0.05	0.83	−1.63	0.10
rs2808631 (G/A)	3′ Downstream	0.002	−1.52	0.07	0.52	0.64	−1.16	0.25
rs1800947 (G/C)	Synonymous coding	0.07	0.19	0.23	0.29	0.18	1.77	0.08

(B)			* SIRCA&PAMSyN (n=1279)		* PDHS (n=782)		Meta-analysis (n=2061)	
†*CRP* SNP (Allele: Minor/Major)	Type of SNP	MAF	Beta	P	Beta	P	Z	P
rs2794521 (G/A)	5′ Upstream	0.25	−0.11	0.23	0.01	0.93	−0.90	0.37
rs1130864 (A/G)	3′UTR	0.30	0.10	0.27	−0.15	0.21	0.10	0.92
rs2808634 (A/G)	5′ Upstream	0.27	−0.09	0.30	0.00	0.98	−0.84	0.40
rs3093071 (A/C)	3′ Downstream	0.01	−0.23	0.54	−0.21	0.67	−0.75	0.45
rs3093066 (A/C)	3′UTR	0.001	−0.57	0.57	−1.52	0.33	−1.04	0.30
rs3093069 (C/A)	3′UTR	0.001	−0.57	0.57	1.54	0.49	−0.01	0.99
rs1205 (A/G)	3′ Downstream	0.34	0.04	0.65	0.16	0.17	1.20	0.23
rs3093070 (C/A)	5′ Upstream	0.02	−0.13	0.69	−0.67	0.56	−0.22	0.50
rs3091244 (A/G)	3′ Downstream	0.37	−0.89	0.99	0.00	0.15	−0.17	0.37

*PAMSyN=the Philadelphia Area Metabolic Syndrome Network; SIRCA=the Study of Inherited Risk of Coronary Atherosclerosis; PDHS=the Penn Diabetes Heart Study. MAF=minor allele frequency.

†For all analyses, the effect allele corresponds to the minor allele. MAF=minor allele frequency.