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A Novel Genetic Approach to Investigate the Role of Plasma Secretory Phospholipase A2 ($sPLA_2$)-V Isoenzyme in Coronary Heart Disease: A Modified Mendelian Randomization Analysis Using *PLA2G5* Expression Levels

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Abstract

Background—Secretory phospholipase A_2 ($sPLA_2$) enzymes are considered to play a role in atherosclerosis. $sPLA_2$ activity encompasses several $sPLA_2$ isoenzymes, including $sPLA_2$-V. While observational studies show strong association between elevated $sPLA_2$ activity and CHD, no assay to measure $sPLA_2$-V levels exists and the only evidence linking the $sPLA_2$-V isoform to atherosclerosis progression comes from animal studies. In the absence of an assay that directly quantifies $sPLA_2$-V levels, we used *PLA2G5* mRNA levels in a novel, modified Mendelian randomization approach to investigate the hypothesized causal role of $sPLA_2$-V in coronary heart disease (CHD) pathogenesis.

Methods and Results—Using data from the Advanced Study of Aortic Pathology, we identified the single nucleotide polymorphism (SNP) in *PLA2G5* showing strongest association with *PLA2G5* mRNA expression levels, as a proxy for $sPLA_2$-V levels. We tested the association of this SNP with $sPLA_2$ activity and CHD events in four prospective and 14 case-control studies with 27,230 events and 70,500 controls. rs525380C>A showed the strongest association with *PLA2G5* mRNA expression ($P=5.1\times10^{-6}$). There was no association of rs525380C>A with plasma $sPLA_2$ activity (difference in geometric mean of $sPLA_2$ activity per rs525380 A-allele 0.4% (95%CI: −0.9%, 1.6%), P=0.56). In meta-analyses, the odds ratio for CHD per A allele was 1.02 (95% CI: 0.99, 1.04; P=0.20).

Conclusions—This novel approach for SNP selection for this modified Mendelian randomization analysis showed no association between rs525380 (the lead SNP for *PLA2G5* expression, a surrogate for $sPLA_2$-V levels) and CHD events. The evidence does not support a causal role for $sPLA_2$-V in CHD.

Keywords

Mendelian randomization; cardiovascular disease risk factors; DNA polymorphisms; GATA2; sPLA2-V; PLA2G5

The secretory phospholipases ($sPLA_2$s) are a family of enzymes that hydrolyse phospholipids on lipoprotein particles, initially in the plasma, leading to the modification of low density lipoproteins (LDL) to small, dense, pro-atherogenic LDL particles that can transcytose the endothelial layer of the arterial wall.[1] Further modification of these intimal apolipoprotein B (apo B)-containing lipoproteins[2], by $sPLA_2$s in the arterial wall, leads to their accumulation and retention on the proteoglycans within the intima, a pro-atherosclerotic process.[3] Additionally, by hydrolysing lipoprotein phospholipids, $sPLA_2$s generate lysophospholipid and non-esterified free fatty acids (NEFAs), such as arachidonic acid, a precursor of eicosanoids and leukotreines[4,5] that are pro-inflammatory cytokines.

Three $sPLA_2$ isoenzymes have been identified in human atherosclerotic lesions: $sPLA_2$-IIa, $sPLA_2$-V and $sPLA_2$-X.[3] It is thought that $sPLA_2$-V may contribute to the quantitative trait "$sPLA_2$ activity", a composite measure of $sPLA_2$-IIa, -V and –X,[6] although there is no direct biological proof for this. There is converging evidence from both the prospective EPIC-Norfolk study,[6] and GRACE,[7] a study of patients with acute coronary syndrome[15], that $sPLA_2$ activity shows stronger association with cardiovascular risk than $sPLA_2$-IIa levels alone. This provides some indirect evidence that $sPLA_2$ activity might encompass more than just $sPLA_2$-IIa and this identifies sPLA2-V as a potential contributor to CHD risk in humans. While a large body of observational studies support the relationship between higher $sPLA_2$-IIa levels and risk of CHD in humans,[7–11] no such studies exist for $sPLA_2$-V. A specific ELISA assay exists that enables the quantification of $sPLA_2$-IIa levels, but there is currently no assay to specifically measure $sPLA_2$-V levels. Despite the lack of observational studies in man for $sPLA_2$-V, animal studies report a pro-atherogenic role for $sPLA_2$-V as well as -IIa, showing increased susceptibility to atherosclerosis in $sPLA_2$-V (*Pla2g5*)[12,13] and $sPLA_2$-IIa (*Pla2g2a*) transgenic mice.[14,15] Studies of human tissue also indicate that $sPLA_2$-V is expressed in human endothelial cells, macrophages and lipid-loaded macrophages.[16] Suggested mechanisms by which $sPLA_2$-V is thought to increase risk of CHD include increasing the entrapment of LDL in the atherosclerotic plaque, and modification of LDL to encourage generation of foam cells.[16]

The specific catalytic dyad found in $sPLA_2$ enzymes [17] makes them suitable drug targets, and indeed a $sPLA_2$ inhibitor has been developed. The drug varespladib, with a primary target of sPLA2-IIa, also inhibits $sPLA_2$-V.[18] However, since the exact contribution of $sPLA_2$-V to plasma $sPLA_2$ activity is unknown, this provides a challenge for inferring the nature of the relationship between $sPLA_2$-V and CHD events.

Genetics provides a powerful tool to examine whether a relationship between a biomarker and a disease outcome is likely to be causal. This process, called Mendelian randomization (MR), makes use of a genetic variant that associates with the biomarker of interest as a means to investigate whether the biomarker is causally related to disease.[19] There are three steps in traditional MR analysis, often referred to as MR triangulation. The first side of the triangle usually is the starting point of the analysis and arises from observational studies which report the association of the biomarker with CHD, in this case it would be $sPLA_2$-V levels. However, in the absence of measures of plasma $sPLA_2$-V levels, observational studies report the association of elevated levels of the composite measure of $sPLA_2$ activity with CHD risk.[6,7] The second side of the MR triangle validates the association of the genetic variant with the biomarker of interest. In this modified MR study, the absence of a specific assay to quantify $sPLA_2$-V levels motivated us to pursue a novel approach that exploited the availability of vascular tissue mRNA expression of *PLA2G5* (the gene encoding $sPLA_2$-V) as a proxy for $sPLA_2$-V levels and for this we identified a common *PLA2G5* gene variant most strongly associated with *PLA2G5* mRNA expression. We feel this novel approach is justified as a recent study we conducted for $sPLA_2$-IIa found that the SNP showing strongest association with *PLA2G2A* mRNA was in very strong linkage disequilibrium with the SNP that showed strongest association with $sPLA_2$-IIa (a specific assay for $sPLA_2$-IIa).[20] Finally, to validate if the biomarker is causal or not, the MR triangle is completed by examining the

association of the *PLA2G5* variant with CHD risk and comparing this value to the observational estimate for a similar difference in biomarker.

Methods

SNP selection for Mendelian randomization using mRNA expression

We searched publicly available eQTL data sets to identify SNPs in *PLA2G5* associated with eQTL effects at genome-wide significance in circulating cells in blood.[21–24] This did not identify any associations and we therefore focused on mRNA expression in tissue samples in our own dataset. We used the Advanced Study of Aortic Pathology (ASAP) (n=272) as a source of *PLA2G5* mRNA expression. Individuals undergoing valve surgery had tissue biobanked from liver (n=212), mammary artery intima-media (n=89), ascending aorta intima-media (n=138), aorta adventitia (n=133) and heart (n=127), and subsequently mRNA levels extracted. mRNA levels were quantified using Affymetrix Gene Chip Human Exon 1.0 ST expression arrays and DNA was genotyped using Illumina Human 610W-Quad Bead array.[25] We investigated the association between SNPs in and within 200kb of the *PLA2G5* gene with mRNA expression of *PLA2G5* and selected the SNP that showed strongest differential association with *PLA2G5* expression levels. SNPs with a call rate <80% or Hardy-Weinberg Chi-square statistic >3.84 were excluded. The overall call rate per SNP was 99.84%. 12 samples were genotyped in duplicate and the concordance was 99.99%. The rs525380 SNP was in Hardy-Weinberg equilibrium (P=0.54) and had a call rate of 100%.

Association of the gene variant with non-index mRNA expression and $sPLA_2$ activity

In order to investigate the specificity of our genetic variant, we examined the relationship between the SNP with mRNA levels of *PLA2G2A* and *PLA2G10*. To gauge insight into the relative contribution of $sPLA_2$-V to $sPLA_2$ activity, we investigated the per-allele association of the SNP with $sPLA_2$ activity in EPIC-Norfolk (measured by a selective fluorometric assay).[7]

Genotyping of rs525380

The lead SNP in the analysis, rs525380, was present on various GWAs platforms used by the CARDioGRAM studies.[26] For EPIC-Netherlands, Whitehall II and Women's Health Initiative, genotyping was carried out using the IBC CardioChip array (Illumina HumanCVD).[27] For the remaining study (EPIC-Norfolk), the rs525380 SNP was genotyped using TaqMan technology (Applied Biosciences, ABI, Warrington UK) (Supplementary Table 1). In each study, rs525380 was in Hardy Weinberg equilibrium with call rates >97%.

Association of the gene variant with LDL-C levels

We previously reported an association of *PLA2G5* SNPs with LDL-cholesterol levels in a small study of patients with type 2 diabetes.[28] To investigate whether LDL-C may represent a mediator between $sPLA_2$-V and CHD, we looked up the association of rs525380 in a recent large gene-centric analysis of 32 studies including 66,240 individuals of European ancestry.[29]

Association of the gene variant with CHD events

Data from 18 studies were used in the analysis of the association between the *PLA2G5* lead SNP and CHD risk, comprising three nested case-control studies (Women's Health Initiative,[30] EPIC-Norfolk[8] and EPIC-Netherlands[31]), one prospective cohort (Whitehall II[32]) and 14 case-control studies (participants in the CARDIoGRAM GWA meta-analysis of coronary artery disease (CAD)). [26] All studies were approved by their institutional review committees and subjects gave informed consent.

These studies are described in Supplementary Table 1 and the details of the CARDIoGRAM consortium in Supplementary Table 2.

Statistical Analysis

All gene expression values were $\log_2$ transformed prior to analysis as part of the microarray preprocessing algorithm. Association strength between genotype and gene expression levels were calculated using a linear regression model with the gene expression as response variable and the genotype recoded numerically (as 0, 1, and 2) as the explanatory variable. A Bonferroni-adjusted P-value threshold of $P< 8.4\times10^{-5}$ was taken as the level of significance for the association of SNPs with mRNA expression. The mRNA analysis was conducted using R 2.13.0 and Bioconductor.

$sPLA_2$ activity was log(e) transformed prior to analysis due to a skewed distribution. We used an additive model for the genetic association analysis of rs525380 with $sPLA_2$ activity and CHD events. The univariate per-minor A allele estimates for the rs525380 variant with $sPLA_2$ activity and CHD events were estimated using linear and logistic regression, respectively. Study-level estimates (beta coefficients or log odds with their respective standard errors) were pooled using fixed-effects (inverse variance) meta-analysis and heterogeneity was quantified using the I^2 statistic. For the association of rs525380 with $sPLA_2$ activity, summary estimates were exponentiated and converted into a percentage difference in the geometric mean. All analyses, unless otherwise stated, were performed using Stata 12.1 (StataCorp, College Station, Texas USA).

Results

Identification of the SNP showing strongest the association with *PLA2G5* expression

The SNP showing strongest association with *PLA2G5* mRNA expression was rs525380 at $P=5.1\times10^{-6}$ (n=272, Figure 1), which surpassed our Bonferroni-adjusted P-value threshold. *PLA2G5* was most highly expressed in the heart (Figure 2) where it was amongst the top 11% most highly expressed genes and in the top 50% of expression in other investigated tissues (mammary artery, liver, aorta media and adventitia). The rs525380C>A was associated with the strongest differential mRNA expression of *PLA2G5* in the aortic adventitia explaining 14.5% of the *PLA2G5* mRNA variance (n=133, Figure 2, Supplementary Figure 1); the rare A allele was associated with 37.6% higher mRNA levels than the common C allele. Associations of rs525380 with *PLA2G5* mRNA expression were also identified in the aortic media and mammary artery (P<0.001) (Figure 2). The regional plot for rs525380, showing the linkage disequilibrium (LD) with SNPs in the vicinity, is

presented in Supplementary Figure 1. This plot shows that the LD falls off around the lead SNP, rs525380 in *PLA2G5* and shows very little LD with *PLA2G2A* SNPs, with $R^2 \leq 0.2$.

Bioinformatic analysis of rs525380

rs525380 is located ~12.5 kb downstream of the *PLA2G5* transcription start site within a potential enhancer motif, experimentally determined by DNaseI-seq and FAIRE-seq open chromatin marks (liver and vascular cells), and by ChIP-seq for the transcription factor GATA-2 (UCSC Genome Browser GRCh37/hg19)[33] (Supplementary Figure 2), suggesting rs525380 may be functional, potentially playing a distal regulatory role and altering *PLA2G5* expression.

Association of *PLA2G5* SNPs with *PLA2G2A* and *PLA2G10* mRNA expression levels and $sPLA_2$ activity

We next examined the association of *PLA2G5* rs525380 with *PLA2G2A* (lying head to tail with *PLA2G5* on chr1) and *PLA2G10* (chr10) mRNA expression levels. We did not observe an association of rs525380 and *PLA2G2A* mRNA expression in vascular tissues, but we did identify an association of rs525380 with liver *PLA2G2A* mRNA expression (n=212, p=0.001, Supplementary Figure 3). rs525380 showed no association with *PLA2G10* mRNA expression in any tissue (P>0.05 for all associations).

There was no association between the A allele of rs525380 and plasma $sPLA_2$ activity (n=3095, 0.4% difference in geometric mean per A-allele of rs525380; 95% CI: −0.9%, 1.6%; P=0.56).

Association of rs525380 with LDL-cholesterol levels

A look-up in a large meta-analysis across 32 studies[29] yielded a pooled per-A allele estimate of 0.002 mmol/l (95%CI: −0.008, 0.012) difference in LDL-C in 66,240 individuals (P=0.71), thus showing no association between rs525380 and LDL-C levels.

Association of rs525380 with CHD events

The pooled estimate of the association of rs525380 with CHD events in meta-analysis of 18 studies with 27,230 CHD events in 97,730 individuals did not identify any evidence of association. The per-A-allele estimate was OR 1.02 (95%CI: 0.99, 1.04), and the heterogeneity was low (I^2=0%; 95%CI: 0%, 48%) (Figure 3). When we restricted the analysis to only large studies with >1000 CHD events (11 studies with 22,757 cases in 85,494 individuals), the estimate remained unchanged (OR 1.01; 95%CI: 0.99, 1.04).

Discussion

We conducted a modified Mendelian randomization analysis to evaluate whether the relationship between $sPLA_2$-V and CHD events is likely to be causal. In the absence of a suitable assay to directly quantify $sPLA_2$-V levels, we took the novel approach of using vascular mRNA expression levels of the gene encoding $sPLA_2$-V, *PLA2G5,* as a proxy measure. We found *PLA2G5* to be highly expressed in all available tissues, being amongst the top 11% genes expressed in the heart and in the top 50% of expression in other

investigated tissues. We identified a SNP that surpassed the pre-defined Bonferroni-adjusted P-value threshold for association with *PLA2G2A* mRNA expression, explaining 14% of the variance in *PLA2G5* mRNA levels. We took this genetic variant forward to investigate the association with CHD in a large collection of studies. In analysis of 27,230 CHD events across 97,730 total individuals, the SNP was not associated with CHD, suggesting that $sPLA_2$-V may not be causally involved in CHD pathogenesis.

We recently used a similar technique for SNP selection when we investigated the role of $sPLA_2$-IIa in CHD.[20] In the case of $sPLA_2$-IIa, we *did* have access to a trait that directly quantified circulating levels of $sPLA_2$-IIa. We showed that the SNP showing strongest association with circulating $sPLA_2$-IIa levels was in very high linkage disequilibrium with the SNP showing strongest association with *PLA2G2A* mRNA expression. This serves to justify the method we used here: that is we assume that if we could quantify circulating $sPLA_2$-V levels, we would find that the SNP that showed strongest association with $sPLA_2$-V levels would also show strongest association with *PLA2G5* mRNA expression.

mRNA expression is considered a good proxy for its encoded protein, although it might only reflect a proportion of protein expression, since post transcriptional and post translational modifications may further influence protein levels.[34] Thus, the association of rs525380 with *PLA2G5* mRNA in vascular tissue may be a good marker of $sPLA_2$-V expression. This is supported by immunohistochemistry and *in situ* hybridization of $sPLA_2$-V in human atherosclerotic aortas showing that $sPLA_2$-V protein expression was limited to smooth muscle cells and this correlated well with *PLA2G5* mRNA expression.[35]

One of the limitations of our study is the lack of observational data on the association of $sPLA_2$-V levels and risk of CHD, limited by the absence of an available $sPLA_2$-V ELISA. *PLA2G5* mRNA expression measures are limited by availability of datasets with tissue mRNA expression in individuals with and without CHD. The lack of a quantitative trait also means that a formal Mendelian “triangulation” analysis is not possible.[19] However, the genetic analysis that we present is a form of Mendelian randomization as the SNP (rs525380) will, according to Mendel’s second law, be randomized at conception, meaning that individuals grouped by rs525380 genotype should be equal in all respects apart from exposure to differing *PLA2G5* mRNA expression levels. Several animal studies support an atherosclerotic role of $sPLA_2$-V,[12,13,36,37] although we accept that positive findings from animal studies do not always translate into meaningful advances in combatting human disease.[38] Even in the absence of availability of an observational quantification of the association of $sPLA_2$-V (or for that matter *PLA2G5* mRNA) with CHD events in humans, our genetic findings show that if such an associat ion were to exist, it would most likely be attributable to confounding and/or reverse causality rather than a causal relationship. We have made the assumption that vascular expression of $sPLA_2$-V is a likely pro-atherogenic mediator and therefore we have considered vascular *PLA2G5* mRNA as a good proxy for circulating levels of $sPLA_2$-V levels. Our findings do not support those from animal studies and suggest $sPLA_2$-V is not an important cause of CHD in man. The outcome of this study is in part validated by the phase III Vista 16 trial of varespladib (a drug that inhibits $sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-X), prematurely terminated due to lack of efficacy.[39]

Since we had no measure of $sPLA_2$-V levels, we were unable to estimate the effect of the *PLA2G5* SNP on $sPLA_2$-V levels, and without an estimate of the observed association between $sPLA_2$-V and CHD to obtain the expected effect size, we were unable to perform a power calculation. However, for comparison, in our Mendelian randomization analysis of $sPLA_2$-IIA,[20] in studies set in the general population we had a total of 15,534 incident and prevalent cardiovascular events out of a total of 74,683 individuals. In this current study we almost doubled the number of events with 27,230 events in 97,730 individuals. With an OR of 1.02 (95%CI 0.99, 1.04) between rs525380 and CHD, we are able to exclude a large effect of the SNP on CHD. Furthermore, the I^2 value of 0%, indicating low heterogeneity, means that the values reported in the individual studies included in this meta-analysis were very similar (i.e. low between- study heterogeneity), adding further confidence to a "true negative" finding.

Plasma $sPLA_2$ activity is suggested to represent a composite of the activities of the -IIa, -V and -X isoenzymes,[6] however there is currently no experimental evidence supporting this. In a recent Mendelian randomization investigation of $sPLA_2$-IIa,[20] we reported that the SNP showing strongest association with $sPLA_2$-IIa levels explained 31% of *PLA2G2A* mRNA expression and 21% of $sPLA_2$-IIa variance, yet accounted for only 0.5% of the variance of $sPLA_2$ activity. In a similar fashion, the rs525380 SNP, which explained 15% of the variance of *PLA2G5* mRNA may only explain a very small variance of $sPLA_2$ activity (for which we may be underpowered to detect with precision in the current analysis). Thus, $sPLA_2$-V may only make a minor contribution to plasma $sPLA_2$ activity. An alternative explanation is that despite rs525380 showing strongest association with *PLA2G5* mRNA in the aortic adventitia of the vasculature, rs525380 may not represent a suitable proxy for circulating $sPLA_2$-V. The high level of expression of *PLA2G5* mRNA in several relevant atherosclerosis-prone tissues, such as heart, mammary artery intima-media, ascending aorta intima-media and aorta adventitia that we identified) suggest that $sPLA_2$-V may have its greatest biological effect in these tissues and not in the plasma. Thus circulating plasma $sPLA_2$ activity may not reflect tissue levels of $sPLA_2$-V.

PLA2G5 rs525380 showed a weak association with *PLA2G2A* expression in the liver, but not in the other tissues we examined. This association could be a spurious finding since it did not exceed the Bonferoni adjusted P-value threshold. Alternatively, it could represent a real association. Our Bioinformatic analysis suggests that rs525380, 12.5 kb downstream of the *PLA2G5,* disrupts the binding site of the transcription factor GATA-2. GATA-2 is a transcription factor implicated in endothelial inflammatory responses.[40] This might be particularly relevant to *PLA2G5* given that *Pla2g5* knock-out mice show 50% reduction in eicosanoid generation in response to zymosan stimulus, thus suggesting that $sPLA_2$-V plays an important role in innate immunity.[12] The association of the *PLA2G5* SNP rs525380 with *PLA2G2A* expression in the liver suggests that GATA-2 might also act as a transcription factor for the control of expression of *PLA2G2A.* However, while the rare A allele of rs525380 is associated with higher *PLA2G5* expression levels it is the common C allele that is associated with higher *PLA2G2A* expression levels. We previously showed that of the five tissues available in ASAP, *PLA2G2A* was most highly expressed in the liver,[41] a tissue that showed the lowest level of *PLA2G5* expression in this current study. This suggests a

potential complementarity expression of these two transcripts in the liver, possibly under the control of GATA-2.

In conclusion, we identified no association between a SNP strongly linked to *PLA2G5* mRNA tissue levels in atherosclerosis prone tissues and risk of CHD. Although the findings we report are by no means definitive, they do not support the hypothesis that $sPLA_2$-V plays an important role in CHD. The methods we present demonstrate that in the absence of a specific plasma biomarker measure, it may be possible to use mRNA expression levels of the coding gene as a surrogate to examine the potential causal relationship of a biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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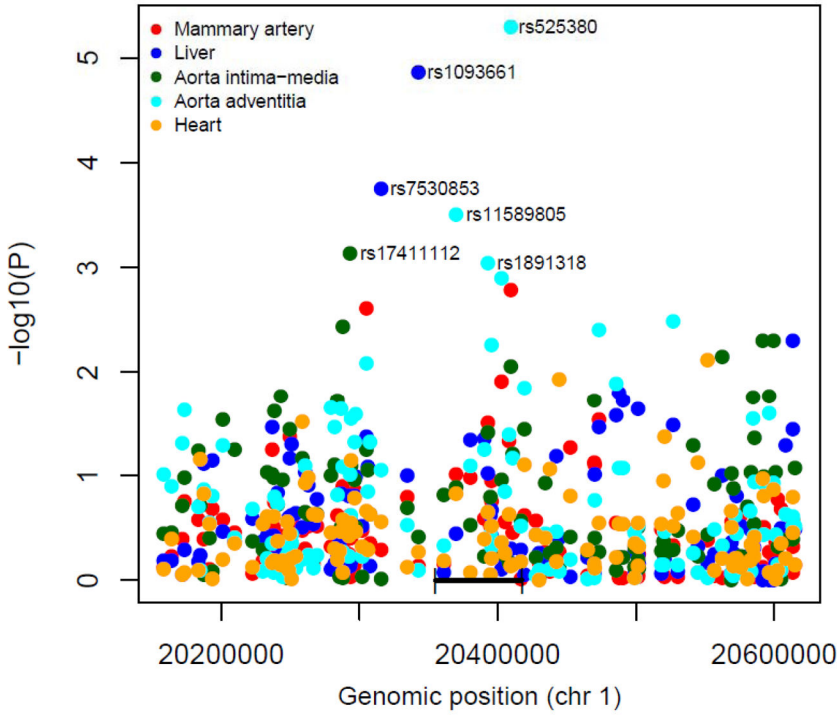

Figure 1. Manhattan plot of the association between SNPs in the PLA2G5 region and PLA2G5 mRNA expression by tissue type

rs525380 A>C showed the strongest association with PLA2G5 mRNA expression in the aorta adventitia ($P=5.05\times10^{-6}$). The black horizontal line above the scale represents the position of PLA2G5. Total number of individuals providing tissue samples for analysis = 272 (samples available for each tissue: Mammary Artery 89, Liver 212, Aorta Med 138, Aorta Adventitia 133, Heart 127).

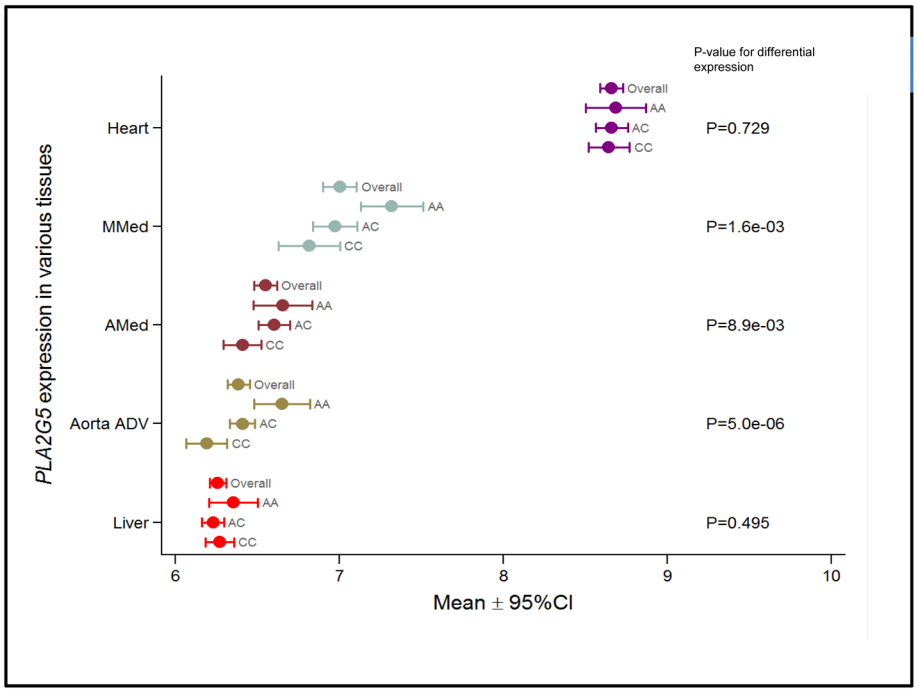

Figure 2. Overall expression of all probe-sets and the differential expression of PLA2G5

rs525380 C>A with PLA2G5 mRNA in the five tissue types. MMed: Mammary artery intima-media; AMed: dilated and non-dilated ascending aorta intima-media; Aorta ADV: aorta adventitia. For CC/AC/AA the sample sizes are as follows: heart 43/68/16, MMed 21/51/17, AMed 44/70/24, Aorta ADV 38/73/22, Liver 59/120/32.

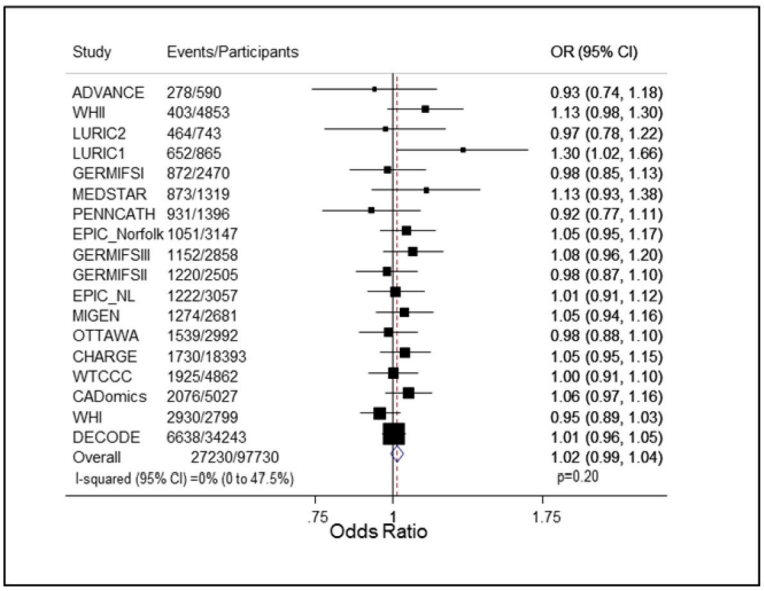

Figure 3. Forest plot of the association of PLA2G5 rs525380 (per A-allele) with CHD in 27,230 cases in a total of 97,730 individuals

When limited to studies with fewer than 1000 CHD events, the OR was 1.04 (95%CI: 0.97, 1.11) with an I^2 of 31% (95%CI: 0% to 71%). For studies with more than 1000 CHD events, the OR was 1.01 (95%CI: 0.99, 1.04) with an I^2 of 0% (95%CI: 0% to 43%).