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Title: Genetic Variants in PPARGC1B and CNTN4 are Associated with Thromboxane A2 Formation and with Cardiovascular Event Free Survival in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT).

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Abstract: Background and aims: Elevated urinary 11-dehydro thromboxane B2 (TxB2), a measure of thromboxane A2 formation in vivo, predicts future atherothrombotic events. To further understand this relationship, the genetic determinants of 11-dehydro TxB2 and their associations with cardiovascular morbidity were investigated in this study.

Methods and Results: Genome-wide and targeted genetic association studies of urinary 11-dehydro TxB2 were conducted in 806 Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) participants. The strongest associations were in PPARGC1B (rs4235745, rs32582, rs10515638) and CNTN4 (rs10510230, rs4684343) - these 5 single nucleotide polymorphisms (SNPs) were independently associated with 11-dehydro TxB2 formation. Haplotypes of 11-dehydro TxB2 increasing alleles for both PPARGC1B and CNTN4 were significantly associated with 11-dehydro TxB2, explaining 5.2% and 4.5% of the variation in the whole cohort, and 8.8% and 7.9% in participants not taking aspirin, respectively. In a second ASCOT population (n=6,199), addition of these 5 SNPs significantly improved the covariate-only cox proportional hazards model for cardiovascular events (chisq=14.7, P=0.01). Two of the risk alleles associated with increased urinary 11-dehydro TxB2 were individually associated with greater incidences of cardiovascular events - rs10515638 (HR =1.31, P=0.01) and rs10510230 (HR=1.25, P=0.007); effect sizes were larger in those not taking aspirin.

Conclusions: PPARGC1B and CNTN4 genotypes are associated with elevated thromboxane A2 formation and with an excess of cardiovascular events. Aspirin appears to blunt these associations.
If specific protection of PPARGC1B and CNTN4 variant carriers by aspirin is confirmed by additional studies, PPARGC1B and CNTN4 genotyping could potentially assist in clinical decision making regarding the use of aspirin in primary prevention.

Highlights

- This paper describes the first genome wide association study of thromboxane A_2 formation.
- It shows that 5 SNPs in two genes, *PPARGC1B* and *CNTN4,* are associated with elevated thromboxane A_2 formation in 806 Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) participants and with an excess of cardiovascular events in an independent ASCOT population (n=6,199).
- Results indicate specific protection of aspirin for *PPARGC1B* and *CNTN4.* If confirmed, *PPARGC1B* and *CNTN4* genotyping could potentially provide guidance in the use of aspirin in primary prevention.

Full Title: Genetic Variants in *PPARGC1B* and *CNTN4* are Associated with Thromboxane A_2 Formation and with Cardiovascular Event Free Survival in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT).

First author's surname and running title: McCarthy; Genetic associations with Thromboxane A_2

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Abstract

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Conclusions: *PPARGC1B* and *CNTN4* genotypes are associated with elevated thromboxane A_2 formation and with an excess of cardiovascular events. Aspirin appears to blunt these associations. If specific protection of *PPARGC1B* and *CNTN4* variant carriers by aspirin is confirmed by additional studies, *PPARGC1B* and *CNTN4* genotyping could potentially assist in clinical decision making regarding the use of aspirin in primary prevention.

Key words: Genome wide association study, thromboxane, thrombosis

Introduction

Thromboxane A_2 (TxA_2) is a potent platelet agonist formed during platelet activation and contributes to the risk of arterial thrombosis [1]. Aspirin exerts its major antithrombotic effect by irreversibly acetylating platelet cyclo-oxygenase-1 (COX-1), inhibiting production of TxA_2. In high risk patients, low-dose aspirin reduces the risk of major cardiovascular events by about 20% [2]. Direct measurement of TxA_2 is not feasible as it is rapidly metabolised in vivo to its stable metabolite, thromboxane B_2 (TxB_2). Measurement of plasma or urinary levels of 11-dehydro TxB_2 by mass spectrometry gives an accurate reflection of in vivo TxA_2 production [3,4]. Increased urinary 11-dehydro TxB_2 concentration is an independent predictor of atherothrombotic events even in aspirin-treated patients [5,6].

Measures of TxA_2, including urinary [7] and plasma [8] 11-dehydro TxB_2 have been shown to be heritable in Caucasian populations off and on aspirin (h^2 = 0.5-0.7 and 0.2-0.4 respectively). Genetic studies have largely focused on the influence of the functional COX-2 single nucleotide polymorphism (SNP) rs20417 (–765G>C) on cardiovascular endpoints; a recent meta-analysis (n=49,232) reported the minor allele was associated with reduced risk of cardiovascular events and lower urinary 11-dehydro TxB_2 [9].

To more comprehensively assess genetic contribution to TxA_2 levels, we conducted genome-wide and targeted genetic association studies of urinary 11-dehydro TxB_2 in a subset of participants from the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). In addition, we investigated whether variants associated with elevated 11-dehydro TxB_2 levels increased the risk of atherothrombotic events in a second subset of ASCOT participants.

Methods

The ASCOT trial was a randomized, multicentre trial comparing the long-term effects of two antihypertensive regimens on myocardial infarction [10]. The population characteristics, genotyping, and imputation of the subsets of ASCOT participants investigated in this study are described in full in supplementary material online. All patients gave written informed consent. Written informed consent and approval by local research ethics committees and/or institutional review boards were obtained for ASCOT, the ASCOT DNA Repository, and the Hypertension Associated Cardiovascular Disease (HACVD) sub-study.

Genotypes Associated with Elevated Thromboxane A2:

Fasting urinary 11-dehydro TxB_2 was measured by mass spectrometry in 1,006 participants of the HACVD sub-study [11] of the ASCOT trial, and expressed as pg 11-dehydro TxB_2 /mg creatinine to normalize for urinary output. Genotyping was performed using the genome-wide Illumina HumanCNV370-Duo array (CNV370) and/or the Illumina HumanCVD BeadChip (CVD50k, targeting >2,000 genic regions related to cardiovascular disease (CVD)), and quality control exclusions applied as described previously [12, 13]. After further excluding all copy number variant (CNV) markers and SNPs with minor allele frequency (MAF) <5%, 272,166 genotyped SNPs (2,031,499 including imputed SNPs) on the CNV370 chip and 31,570 SNPs on the CVD50K chip were analysed in n=777 and n=544 individuals with 11-dehydro TxB_2 measurements, respectively (Supplementary Figure 1).

For each SNP, a linear regression was performed of genotype (assuming an additive genetic model) on urinary 11-dehydro TxB_2 level (a continuous trait) in PLINK v1.07 [14]. Unless otherwise stated, analyses were adjusted for the covariates: age, sex, smoking habit (current smokers vs. never & ex-smokers), presence of type 2 diabetes, systolic blood pressure (SBP), body mass index (BMI), high density lipoprotein (HDL), low density lipoprotein (LDL), randomized anti-hypertensive regimen,

reported aspirin use, study location (UK/Ireland or Scandinavia), and the first ten vectors from ancestry principal component analysis to avoid confounding due to population stratification. The 11-dehydro TxB_2 measurement was log transformed to an approximately normal distribution prior to analysis and quantile-quantile plots did not indicate any inflation of the test statistics (Supplementary methods and Supplementary Figures 2 and 3).

Haplotypes were imputed in PLINK using the standard E-M algorithm. Bonferroni threshold for the CNV370 chip was P=1.8E-07 (0.05/272,166 genotyped SNPs) to account for multiple testing with a 5% false positive rate. As many SNPs on the dense CVD50K chip are correlated, correction for the 21,180 effective tests on the chip is appropriate [13], resulting in a Bonferroni threshold of P=2.4E-06 (0.05/21,180). SNPs associated at P<1E-03 were considered suggestive of association on both chips.

Effect of PPARGC1B and CNTN4 genotypes on Cardiovascular Event Free Survival:

All ASCOT participants with DNA (n=9,063) were considered for inclusion in the survival analysis. The following exclusions were applied: study participants with prior cardiovascular event at baseline (n=1,382), other non- ischemic/haemorrhagic event during the study (n=342), self-declared non-European ancestry (n=370), missing phenotype data or DNA (n=770) (Supplementary Figure 1). The 5 SNPs independently associated with 11-dehydro TxB_2 were successfully genotyped in 6,199 participants (n=3,369 from Scandinavian centres and n=2,851 from UK/Ireland centres). The primary endpoint was a composite endpoint including all ischaemic cardiovascular events and procedures, defined as any of: fatal and non-fatal myocardial infarction (MI), fatal and non-fatal heart failure, fatal and non-fatal ischaemic stroke and transient ischaemic attack, angina (stable and unstable), peripheral arterial disease, revascularization procedures, and retinal vascular thrombosis. Where participants had multiple events, the earliest qualifying event was used. The study was stopped after 5.5 patient-years of follow-up because of benefits of the amlodipine-based regimen on all-cause

mortality and stroke outcomes. Study participants with no qualifying event were censored at the earlier of the date of withdrawal from the study or the date of study termination (median, range: 5.7 [1.1-7.1] years).

Survival analyses were conducted using the R packages 'survival' version 3.2.0 and 'survivalROC' version 1.0.3 [15]. The proportional hazards assumption was met for all variables (Supplementary Table 1). Multivariate Cox proportional hazards models were used to collectively analyse all clinical covariates which were associated with the endpoint of cardiovascular event free survival, plus all genotypes. Chi square comparison of the log likelihoods from the covariates-only and full (covariates plus SNPs) model was used to test the alternative hypothesis that the addition of thromboxane-associated SNPs would significantly improve the risk model for CV events. We hypothesized that genotypes associated with increased urinary 11-dehydro TxB_2 in the association study would be inversely correlated with cardiovascular event-free survival, therefore *P* values and confidence intervals reported for individual SNPs are based on a 1-tailed test.

Results

Genotypes Associated with Elevated Thromboxane A_2.

Baseline characteristics of the cohort are shown in Table 1. Approximately half of the patients reported taking aspirin at the time of urinary 11-dehydro TxB_2 measurement. As expected, 11-dehydro TxB_2 levels were significantly lower in those on aspirin.

Manhattan plots (Figure 1) show that none of the SNPs was individually associated with 11-dehydro TxB_2 level beyond Bonferroni threshold. There were no associations between COX-1 (*PTGS1*) and COX-2 (*PTGS2*) SNPs and 11-dehydro TxB_2 in this study, even at the suggestive level (*P*<1E-03, Supplementary Table 2).

Analysis of CVD50K chip: a *PPARGC1B* haplotype is significantly associated with 11-dehydro TxB_2

The strongest association signal from the CVD50K chip was in the *PPARGC1B* (peroxisome proliferator-activated receptor gamma, coactivator 1 beta) gene (Figure 1), where 14 SNPs were suggestive of association with 11-dehydro TxB_2 (Supplementary Table 3). The most significant SNP at this locus was rs4235745 (*P*=4.3E-06, Table 2). Conditioning on rs4235745 genotype, two SNPs (rs32582 and rs10515638) remained associated with 11-dehydro TxB_2 and were not correlated with each other (r^2=0.15, Supplementary Table 4, Supplementary Figure 4). Although rs4235745 and rs32582 were not genotyped on the CNV370 chip, other variants in the gene did show some evidence of association with 11-dehydro TxB_2 (Supplementary Table 5 and Figure 1).

The haplotype of 11-dehydro TxB_2-increasing alleles of the three independently associated SNPs (TTA, frequency 6%) was significantly associated with an increase of 382pg/mg creatinine 11-dehydro TxB_2, surpassing Bonferroni threshold (P=2.0E-06, Table 2). This haplotype explained 5.2% of the variation in 11-dehydro TxB_2 in all subjects, 8.8% in subjects not on aspirin, and 1.8% in those on aspirin (multiple R^2 values calculated in R). The 11-dehydro TxB_2-decreasing alleles haplotype (GCC, frequency 66%) was associated with a decrease of 146pg/mg creatinine (Supplementary Table 6).

Analysis of CNV370 chip: a *CNTN4* haplotype is significantly associated with 11-dehydro TxB_2

The strongest association signal from the CNV370 analysis was in the *CNTN4* (contactin 4) gene (Figure 1), where 13 SNPs were suggestive of association with 11-dehydro TxB_2 (Supplementary Table 7). The most significant genotyped SNP at this locus was rs10510230 (P=5.1E-07, Table 2) and conditioning on this genotype revealed one other SNP, rs4684343, independently associated with 11-dehydro TxB_2 and not correlated with rs10510230 (r^2 =0.09, Supplementary Table 8 and Supplementary Figure 5). Only one SNP in the *CNTN4* gene was genotyped on the CVD50k chip

(rs1171387), and this SNP is not very close to either of the top hit *CNTN4* SNPs (rs10510230 or rs4684343). Hence there was very little signal at the *CNTN4* gene on the CVD50K chip (Supplementary Table 5 and Figure 1).

The haplotype of both 11-dehydro TxB_2–increasing alleles of rs10510230 and rs4684343 (CG, frequency 13%) was significantly associated with increased 11-dehydro TxB_2, surpassing Bonferroni threshold (*P*=2E-09, Table 2). This haplotype explained 4.5% of the variation in 11-dehydro TxB_2 in all subjects, 7.9% for subjects not on aspirin, and 1.5% for those on aspirin. The 11-dehydro TxB_2-decreasing alleles haplotype (AA, frequency 44%) was associated with a decrease of 128pg/mg creatinine (Supplementary Table 9).

Interaction with aspirin

The five independently associated PPARGC1B and CNTN4 SNPs showed evidence for interaction with aspirin use in a model including the main SNP and aspirin effects and all covariates, with a greater association between genotype and 11-dehydro TxB_2 in those not taking aspirin (Figure 1, Table 2, Supplementary Tables 10 and 11). The interaction was significant for three of these five SNPs after correction for multiple testing (P value threshold <0.01; interaction P-values for PPARGC1B SNPs: rs4235745 (P=3.3E-04), rs32582 (P= 3.0E-04), rs10515638 (P=0.02) and for CNTN4 SNPs: rs4684343 (P=0.02), rs10515638 (P= 6.6E-05); Supplementary Tables 10 and 11.

Function of 11-dehydro TxB_2 – associated *CNTN4* and *PPARGC1B* SNPs

None of the independently associated SNPs nor their proxies were in exonic or predicted functional regions using the ensemble variant effect predictor (http://www.ensembl.org/Tools/VEP), nor were they associated with expression of any genes in any tissues in the GTEx portal (http://www.gtexportal.org/home/), (Supplementary Table 12).

Effect of PPARGC1B and CNTN4 genotypes on Cardiovascular Event Free Survival.

Demographic and clinical characteristics of the survival analyses population are shown in Table 1.

Addition of the 11-dehydro TxB_2-associated genotypes improves survival model.

The three *PPARGC1B* and two *CNTN4* independently associated SNPs which comprised the haplotypes significantly associated with 11-dehydro TxB_2 were included in the survival analysis. The addition of these five SNPs significantly improved the covariates-only Cox proportional hazards model of cardiovascular event free survival (chisq comparison of the log likelihoods *P*=0.01, Table 3).

None of the SNPs were associated with any of the cardiovascular risk factors with the exception of a slightly increased risk of type 2 diabetes in rs32582 and rs10510230 carriers (Supplementary Table 13). Results of the univariate survival analysis (Supplementary Table 14) were consistent with those reported in the multivariate analysis. There was a small sample overlap between the genetic association study and the survival analysis; when these individuals were excluded from the survival sample in a sensitivity analysis, it did not alter the overall results (Supplementary Table 15).

Two SNPs independently associated with risk of event

After Bonferroni correction for the 5 SNPs tested (P value threshold <0.01), rs10515638 (*PPARGC1B)* and rs10510230 (*CNTN4*) were associated with increased risk of event in multivariate (P=0.01 and 6.9E-03 respectively) and univariate (P=0.03 and P=8.5E-03 respectively) survival models. The magnitudes of effect on survival associated with carriage of each minor allele of rs10515638 (*PPARGC1B, HR=1.31*) and rs10510230 (CNTN4, HR=1.25) were similar to or greater than those associated with increments of 10 years age, 20 mmHg systolic BP, 1 mmol LDL-cholesterol, a diagnosis of diabetes mellitus, or use of amlodipine-based antihypertensive therapy in this cohort (Table 3).

Stratification by aspirin use

Kaplan Meier plots of survival analysis stratified by aspirin use for the two significant SNPs show that effect sizes were larger in subjects not taking aspirin compared to those on aspirin (Figure 2 and Supplementary Table 16) and survival receiver operating characteristic (ROC) curves showed that addition of the SNPs improved the area under the curve (AUC) for the all subjects and subjects not on aspirin samples, but not for those on aspirin (Supplementary Figure 5). However, when Aspirin:SNP interaction terms were added to the multivariate survival analysis, none were significant beyond correction for multiple testing (Supplementary Table 17).

Stratification by type of event

Comparing the separate stroke and coronary event analyses, the PPARGC1B SNP (rs10515638) had a larger effect size for coronary events (HR 1.41, P=0.002, 433 events) than for stroke events. By contrast, despite much lower power in the stroke analysis, the CNTN4 SNP (rs10510230) had a larger effect size for stroke (1.31, P=0.17, 57 events) than for coronary events, especially in those not on aspirin (HR 2.71, P=0.003, 26 events), Supplementary Table 18.

The 11-dehydro TxB_2-associated haplotypes are associated with cardiovascular event free survival.

Survival analysis of the PPARGC1B and CNTN4 haplotypes which were associated with the highest 11-dehydro TxB_2 levels (Supplementary Table 19 and Supplementary Figure 6) showed that carriership of one copy of the PPARGC1B haplotype (HR= 1.21, P=0.05) and of the CNTN4 haplotype (HR=1.33, P=0.005), had similar effect sizes on survival to those of the highest effect size SNPs in the haplotypes (rs10515638 (HR=1.31, P=0.01) and rs10510230 (HR=1.25, P=0.007) respectively, Table 3).

Discussion

Association analysis of genome-wide and targeted, CVD-based genotype data revealed that a 3-SNP haplotype of *PPARGC1B* and a 2-SNP haplotype of *CNTN4* are significantly associated with urinary levels of 11-dehydro TxB_2, a surrogate measure for thromboxane A2 in the HACVD cohort. The 5 constituent *PPARGC1B*/*CNTN4* SNPs were genotyped in a second ASCOT cohort, and addition of these genotypes to clinical and demographic covariates significantly improved the Cox proportional hazards model of cardiovascular event-free survival, with 11-dehydro TxB_2-increasing alleles associated with reduced event-free survival. In both the genetic association and the survival analyses, stratification by aspirin use showed that most of the genetic associations were more prominent in those not on aspirin. This may suggest that the genetic variants are primarily associated with COX1-synthesized thromboxane A_2.

Unfortunately, suitable data were not available to formally replicate the genetic associations with 11-dehydro TxB_2 in an independent cohort. However, our finding that the 11-dehydro TxB_2-increasing alleles of the same genetic variants increase the risk of cardiovascular events in an independent population does support our finding that these variants are associated with increased 11-dehydro TxB_2, (an established cardiovascular risk factor). In addition, Mendel's observation that inheritance of one trait should be independent of the inheritance of other traits asserts that it is unlikely the same SNPs would be associated with both 11-dehydro TxB_2 and CHD unless the phenotypes are causally linked. Therefore our finding that the same SNPs are associated with increased 11-dehydro TxB_2 and increased risk of cardiovascular event (unconfounded by association with other measured cardiovascular risk factors) supports a causal relationship between 11-dehydro TxB_2 and CHD and strengthens the finding by Eickelboom *et al* that urinary 11-dehydro TxB_2 is an independent risk factor for atherothrombotic events. Whether the atherosclerosis causes the elevated 11-dehydro TxB_2 or vice versa, however, cannot be clearly ascertained from these data.

All five 11-dehydro TxB_2-associated SNPs and their proxies are intronic. They are not in strong LD with neighbouring genes and it appears likely therefore that these tagging SNPs implicate *PPARGC1B* and *CNTN4*, however substantial further work is required to ascertain the true causal variants behind the association signals.

The *PPARGC1B* gene is a member of the peroxisome proliferator-activated receptor gamma coactivator-1 (*PGC-1*) family, along with its homologue, *PPARGC1A*, and more distant relative PGC-1–related coactivator (*PRC*). *PPARGC1B* and *PPARGC1A* appear to have overlapping function and they exhibit shared, wide tissue expression, especially in heart, skeletal muscle, brain and brown adipose tissue [16]. A variety of metabolic programs are regulated by PGC-1s, including in the heart where PGC-1s help maintain energy homeostasis and activate a broad program of angiogenic factors [17]. PGC-1s can co-activate a large range of transcription factors, including the nuclear receptor PPAR γ (Supplementary Figure 7) which plays an important role in metabolism, is anti-atherosclerotic and anti-inflammatory, and regulates the expression of many genes involved in atherosclerosis including COX-2 [18, 19]. Regulation of COX-2 expression by PPAR γ signalling may be the mechanism whereby polymorphisms in *PPARGC1B* influence thromboxane A_2 levels. However, the larger effect size in subjects not on aspirin suggest the effect is more likely mediated by the primary aspirin target, COX-1, or alternatively through influencing substrate availability or enzymes downstream of COX-1 in the thromboxane A_2 synthetic pathway. It is not known whether PGC-1/ PPAR γ signalling effects expression of COX-1.

Contactin 4 is a member of the immunoglobulin superfamily and is a known neuronal membrane cell adhesion molecule. *CNTN4* expression has been shown in many tissue types other than neuronal, however its role(s) there are not known [20].

We conducted a search of the term 'cardiovascular in the NHGRI-EBI GWAS catalogue- (https://www.ebi.ac.uk/gwas/search?query=cardiovascular, accessed 25/10/17, Supplementary Table 20) in order to put our findings in the context of the vast GWAS data now available. The *CNTN4* gene has been associated with serum uric acid levels in previous genome-wide association and linkage studies, [21, 22], which is itself correlated with a variety of CVD risk factors. There is some evidence for cross talk between COX and uric acid pathways - uric acid has been shown to stimulate COX-2 synthesis [23], for example. Although PPARGC1B does not feature in the GWAS catalogue results, genetic polymorphisms in the PPARGC1B genes have previously been putatively associated with type 2 diabetes [24, 25].

A search of the five 11-dehydro TxB_2 SNPs reported in this study in GWAS central, (http://www.gwascentral.org/ accessed 25/10/17), showed that none of the five SNPs have been included in GWAS of MI in this catalogue (Supplementary Table 21). Some of the SNPs have shown previous nominal associations ($P<0.05$) with other cardiovascular phenotypes, including associations between rs32583 and ischemic stroke (Supplementary Table 21). It should be noted that the five 11-dehydro TxB_2 -associated SNPs in this study were not present on most of the commonly used GWAS chips; rs4235745 for example is only on the IBC (CVD50K) chip (Supplementary Table 5). In addition, the thromboxane pathway represents one of many risk factors for a cardiovascular disease; therefore, we would not necessarily expect the SNPs reported here to be among the top GWAS hits for CVD.

Identification of the genetic determinants of thromboxane A_2 levels will help to improve our understanding of the physiology of this important prothrombotic agent. They may also prove to be useful biomarkers for assessing thromboxane levels without the need for urinary thromboxane measurement. If specific protection of *PPARGC1B* and *CNTN4* variant carriers by aspirin is confirmed

by additional studies, *PPARGC1B* and *CNTN4* genotyping could potentially assist in clinical decision making regarding the use of aspirin in primary prevention.

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Disclosures:

TJ is a full time employee of GlaxoSmithKline and owns company stock. NMcC and AVS are named inventors on an RCSI filed patent application entitled “Identification of thrombosis or bleeding risk in an individual with and without antiplatelet therapy”, Patent application number 11166142.7 – 2402. All other authors: none declared.

Table 1. Baseline characteristics

Baseline characteristics of the genetic association cohort (n=806).	**All Subjects (n=806)**	**Not On Aspirin (n=415)**	**On Aspirin (n=391)**	***P***
11-dehydro TxB_2, pg/mg creatinine (median,IQR)	507(305-818)	716(480-1072)	331(224-552)	2.00E-16
Age, years (mean±sd)	64.7±8.1	64.2±8.1	65.2±8.09	0.09
Male (n,%)	626(78%)	336(81%)	290(74%)	0.02
Current smoker (n,%)	186(23%)	94(23%)	92(23%)	0.77
Current diabetes (n,%)	150(19%)	55(13%)	95(24%)	6.00E-05
SBP, mmHg (mean±sd)	135.2±12.0	133.1±12.5	137.4±12.5	2.00E-07
BMI, kg/m^2 (mean±sd)	29.2±4.7	29.1±4.7	29.3±4.8	0.53
HDL, mmol/L (mean±sd)	1.3±0.3	1.3 ± 0.4	1.3±0.3	0.16
LDL, mmol/L (mean±sd)	3.0±0.9	3.0 ± 0.9	3.0±0.8	0.17
Amlodipine treatment group (n,%)	407(50%)	215(52%)	192(49%)	0.44
Baseline characteristics of the survival cohort (n=6,199)	**All subjects (n=6,199)**	**Not on aspirin (n=4,355)**	**On aspirin (n=1,844)**	***P***
Age, years (mean±sd)	61.8±8.4	61.2±8.4	63.0±8.1	2.40E-15
Male (n,%)	4,996(80.6)	3,470(79.7)	1,526(82.7)	5.70E-03
Current smoker (n,%)	2,007(32.4)	1,525(35.0)	482(26.1)	1.00E-11
Diabetes, type 2 (n,%)	1,404(22.6)	894(20.5)	510(27.6)	1.10E-09
SBP, mmHg (mean±sd)	152.7±14.7	153.3±14.9	151.2±14.2	5.40E-07
BMI, kg/m^2 (mean±sd)	28.9±4.6	28.8±4.6	29.2±4.6	9.90E-04
HDL, mmol/L (mean±sd)	1.28±0.34	1.30±0.34	1.25±0.32	1.60E-06
LDL, mmol/L (mean±sd)	3.79±0.91	3.78±0.89	3.81±0.95	0.4
UK/Ireland cohort (n,%)	2,830(45.6)	1,534(35.2)	1,296(70.3)	<2.2E-16
Amlodipine treatment group (n,%)	3,153(50.9)	2,231(51.2)	922(50.0)	0.39

Follow-up, years (median, IQR)	5.6(5.1-6.0)	5.7(5.2-6.1)	5.5(5.0-6.0)	6.60E-15
n events (composite endpoint)	477(7.7)	189(4.3)	288(15.6)	<2.2E-16
n events per 100 person-years (95%CI)	1.40(1.28-1.53)	0.77(0.67-0.90)	2.98(2.64-3.34)	<2.2E-16
-Coronary revascularisation procedure (n)	149	55	94	<2.2E-16
-Non-fatal MI (incl. silent) + fatal CHD (n)	271	108	163	<2.2E-16
-Ischemic stroke (n)	44	15	29	9.20E-08
-Other (n)	13	11	2	0.46

Amlodipine treatment group: membership of amlodipine (vs atenolol) regime. SBP: Systolic blood pressure; BMI: Body mass index; HDL: High density lipoprotein; LDL: Low-density lipoprotein. UK/Ireland: UK/Ireland (vs Scandinavian) centres. Composite endpoint: total ischaemic cardiovascular events/procedures as described in methods. MI: myocardial infarction. CHD: coronary heart disease. IQR: Inter quartile range; SD: Standard deviation; 95% CIs for events were calculated using an exact Poisson test. *P*: comparison of on-aspirin and not-on-aspirin groups (Student's t-test or Mann-Whitney U test for normally and non-normally distributed variables respectively, or Pearson's chi-squared test of proportions).

Table 2. *PPARGC1B* and *CNTN4* SNPs independently associated with 11-dehydro TxB_2.

PPARGC1B							**all subjects (n=544)**			**subjects not on aspirin (n=276)**		**subjects on aspirin (n=268)**	
Chr	**SNP**	**Position**	**MAF**	**A1/2**	**r^2**	**D'**	**beta**	***P***	***P ^{SNP*Asp}***	**beta**	***P***	**beta**	***P***
5	rs10515638	intron 1	0.08	T/G	0.07	0.58	233.1	7.30E-04	0.02	391.9	1.90E-03	74.7	0.12
5	rs4235745	intron 1	0.3	T/C	NA	NA	166.7	4.30E-06	3.30E-04	284.9	2.20E-05	50.5	0.06
5	rs32582	intron 2	0.18	A/C	0.42	0.93	201.1	9.10E-06	3.00E-04	353.1	2.10E-05	45.4	0.18
3-SNP minor allele haplotype			0.06	TTA			382.4	2.10E-06		658.7	9.50E-06	105.5	8.00E-02
CNTN4							**all subjects (n=772)**			**subjects not on aspirin (n=396)**		**subjects on aspirin (n=376)**	
Chr	**SNP**	**Position**	**MAF**	**A1/2**	**r^2**	**D'**	**beta**	***P***	***P ^{SNP*Asp}***	**beta**	***P***	**beta**	***P***
3	rs10510230	intron 1	0.15	C/A	NA	NA	189.8	5.10E-07	6.60E-05	329.5	1.50E-07	37.6	0.34
3	rs4684343	intron 2	0.45	A/G	0.1	0.77	-133.4	8.80E-07	1.90E-02	-194.6	2.50E-05	-65.2	0.02
2-SNP minor/major allele haplotype			0.13	CG			249	2.00E-09		456.2	1.30E-10	48.4	0.24

CHR: chromosome, BP: base pair position, MAF: minor allele frequency, beta: change in 11-dehydro TxB_2 associated with one copy of the minor allele / haplotype. Position: of the SNP in the *PPARGC1B* gene, r^2: squared correlation coefficient between the genotype of the SNP and the top hit SNP in the gene

(rs4235745 for PPARGC1B and rs10510230 for CNTN4). D': the normalised coefficient of linkage disequilibrium between the genotype of the SNP and the top hit SNP in the gene. A1: major allele, A2: minor allele.

Table 3. Multivariate Cox proportional hazards analysis all significant clinical covariates plus the five 11-dehydro TxB_2-associated SNPs with a composite cardiovascular endpoint of all ischaemic cardiovascular events and procedures.

All subjects (n=6,199, 477 ischaemic cardiovascular events)			
	HR	**95% CI**	***P***
Age (yrs)	1.02	1.01-1.04	7.70E-05
Male sex	1.54	1.17-2.02	2.00E-03
Current smoker	1.43	1.17-1.74	0.03
Type 2 diabetes	1.12	0.92-1.37	5.30E-04
SBP (mmHg)	1.01	1.00-1.02	0.06
BMI (kg/m^2)	0.98	0.95-1.00	0.25
HDL (mmol/L)	0.44	0.31-0.62	8.90E-05
LDL (mmol/L)	1.21	1.10-1.34	1.80E-06
Amlodipine treatment	0.82	0.68-0.98	1.40E-04
Aspirin use	4.68	3.84-5.71	< 2e-16
UK/Ireland cohort	0.56	0.45-0.68	2.10E-08
rs4235745 (per minor (T) allele)	0.84	0.71-0.99	0.96
rs32582 (per minor (A) allele)	1.00	0.82-1.21	0.48
rs10515638 (per minor (T) allele)	1.31	1.08-1.58	0.01
rs10510230 (per minor (C) allele)	1.25	1.08-1.46	6.90E-03
rs4684343 (per minor (G) allele)	1.04	0.93-1.17	0.28
LRT comparison with covariates-only model: chisq 14.7 on 5 df, p= 0.01			

The primary endpoint was a composite endpoint including all ischaemic cardiovascular events and procedures, defined as any of: fatal and non-fatal MI, fatal and non-fatal heart failure, fatal and non-fatal ischaemic stroke and transient ischaemic attack, angina (stable and unstable), peripheral arterial disease, revascularization procedures, and retinal vascular thrombosis. The Cox proportional

hazards model included all SNP genotypes and all covariates. Df: degrees of freedom. SNP statistics are based a one-tailed test; where the observed direction of effect is opposite to that predicted, 1-*P* is reported and 95% CI excludes the lower 5% of the distribution. For all SNPs, minor alleles were associated with increased 11-dehydro TxB_2. SBP: systolic blood pressure. BMI: body mass index. HDL: high density lipoprotein cholesterol. LDL: low density lipoprotein cholesterol. Amlodipine treatment group: membership of amlodipine (vs atenolol) regime. UK/Ireland: UK/Ireland: UK/Ireland (vs Scandinavian) centres participants at UK/Ireland centres vs Scandinavian participants. LRT: likelihood ratio test, comprising the chisq (chi squared) comparison of the log likelihoods of the covariates-only and covariates-plus-genotypes models.

Figures

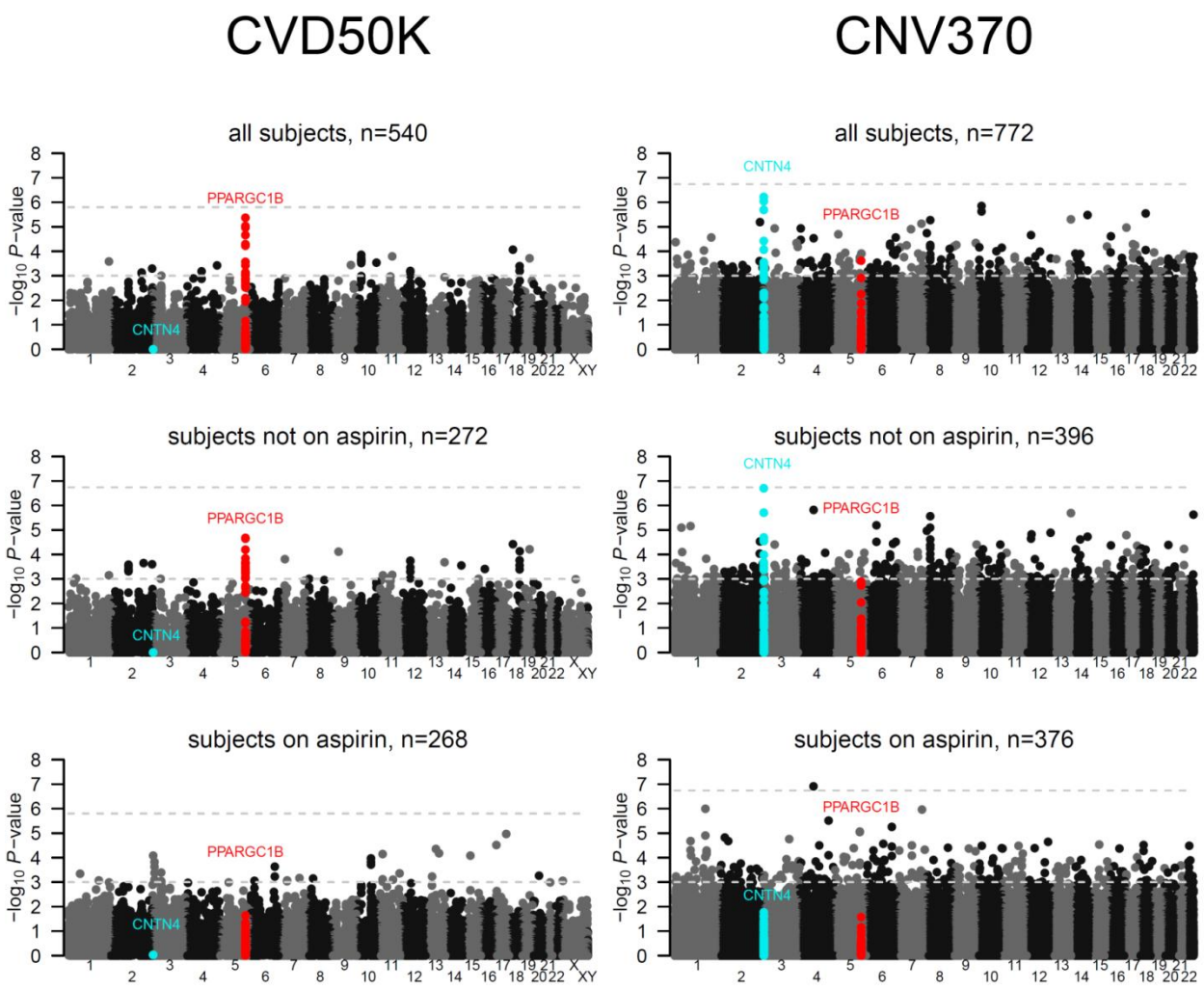


Figure 1. Association with 11-dehydro TxB_2 for the 31,570 SNPs (MAF≥0.05) on the CVD50K chip (A) and 2,031,499 SNPs (MAF≥0.05) on the CNV370 chip (B). X-axis: chromosomal location. All SNP associations were adjusted for age, sex, smoking habit, diabetes, systolic blood pressure, BMI, HDL, LDL, aspirin and anti-hypertensive regimen. The lower dashed lines are the threshold for 'suggestive' association (P<1E-03), and the upper dashed lines are the Bonferroni threshold for significance (P<2.4E-06 for the CNV50K chip and P=1.8E-07 for the CNV370K chip). The most strongly associated locus in all subjects on the CVD50K chip, *PPARGC1B*, is coloured in red and on the CNV370 chip, *CNTN4*, is coloured in blue.

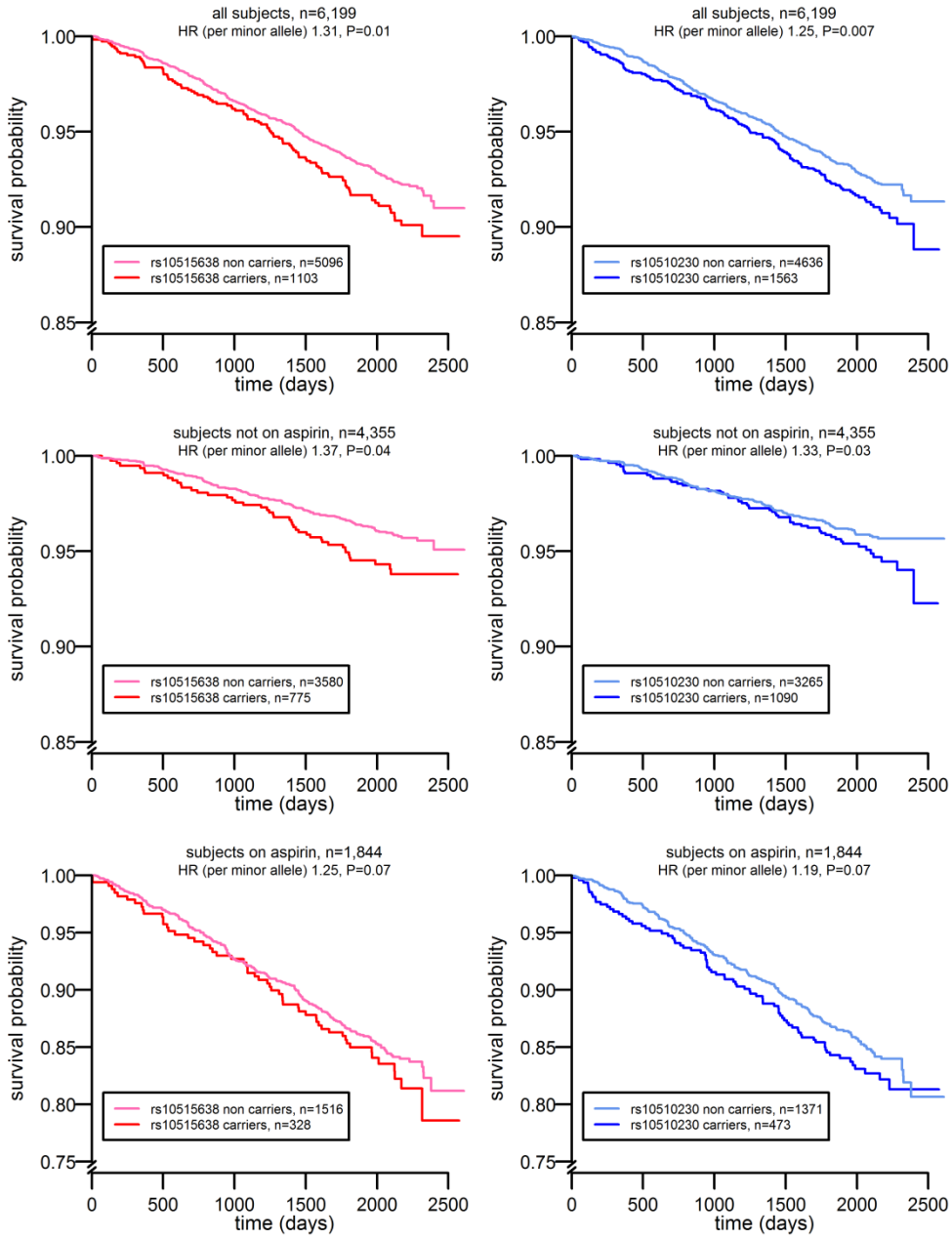


Figure 2. Kaplan Meier plot of survival by genotype for the two SNPs independently associated with cardiovascular event-free survival, rs10515638 (PPARGC1B) and rs10510230 (CNTN4).

'Carriers': carriers of one or more minor allele. 'Non carriers': major allele homozygotes. HR: Hazard ratio for the SNP in the multivariate Cox proportional hazards analysis ~~,~~ and corresponding *P* value (Table 3).

References

1. Davi G and Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357:2482-94.

2. Antithrombotic Trialists' (ATT) Collaboration. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. 2009;373:1849-60.

3. Catella F, Healy D, Lawson JA and FitzGerald GA. 11-Dehydrothromboxane B2: a quantitative index of thromboxane A2 formation in the human circulation. *Proc Natl Acad Sci U S A*. 1986;83:5861-5.

4. Ciabattoni G, Pugliese F, Davi G, Pierucci A, Simonetti BM and Patrono C. Fractional conversion of thromboxane B2 to urinary 11-dehydrothromboxane B2 in man. *Biochim Biophys Acta*. 1989;992:66-70.

5. Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q and Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation*. 2002;105:1650-5.

6. Eikelboom JW, Hankey GJ, Thom J, Bhatt DL, Steg PG, Montalescot G, et al. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on cardiovascular risk. *Circulation*. 2008;118:1705-12.

7. Faraday N, Yanek LR, Mathias R, Herrera-Galeano JE, Vaidya D, Moy TF, et al. Heritability of platelet responsiveness to aspirin in activation pathways directly and indirectly related to cyclooxygenase-1. *Circulation*. 2007;115:2490-6.

8. Vila L, Martinez-Perez A, Camacho M, Buil A, Alcolea S, Pujol-Moix N, et al. Heritability of thromboxane A2 and prostaglandin E2 biosynthetic machinery in a Spanish population. *Arterioscler Thromb Vasc Biol*. 2010;30:128-34.

9. Ross S, Eikelboom J, Anand SS, Eriksson N, Gerstein HC, Mehta S, et al. Association of cyclooxygenase-2 genetic variant with cardiovascular disease. *Eur Heart J*. 2014;35:2242-8a.

10. Dahlof B, Sever PS, Poulter NR, Wedel H, Beevers DG, Caulfield M, et al. Prevention of cardiovascular events with an antihypertensive regimen of amlodipine adding perindopril as required versus atenolol adding bendroflumethiazide as required, in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA): a multicentre randomised controlled trial. *Lancet*. 2005;366:895-906.

11. Stanton A, Fitzgerald D, Hughes A, Mayet J, O'Brien E, Poulter NR, et al. An intensive phenotyping study to enable the future examination of genetic influences on hypertension-associated cardiovascular disease. *J Hum Hypertens*. 2001;15 Suppl 1:S13-8.

12. Deshmukh HA, Colhoun HM, Johnson T, McKeigue PM, Betteridge DJ, Durrington PN, et al. Genome-wide association study of genetic determinants of LDL-c response to atorvastatin therapy: importance of Lp(a). *J Lipid Res*. 2012;53:1000-11.

13. Johnson T, Gaunt TR, Newhouse SJ, Padmanabhan S, Tomaszewski M, Kumari M, et al. Blood pressure loci identified with a gene-centric array. *Am J Hum Genet*. 2011;89:688-700.

14. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81:559-75.

15. R Core Team. *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria, 2010.

16. Meirhaeghe A, Crowley V, Lenaghan C, Lelliott C, Green K, Stewart A, et al. Characterization of the human, mouse and rat PGC1 beta (peroxisome-proliferator-activated receptor-gamma co-activator 1 beta) gene in vitro and in vivo. *Biochem J*. 2003;373:155-65.

17. Rowe GC, Jiang A and Arany Z. PGC-1 coactivators in cardiac development and disease. *Circ Res*. 2010;107:825-38.

18. Inoue H, Tanabe T and Umesono K. Feedback control of cyclooxygenase-2 expression through PPARgamma. *J Biol Chem*. 2000;275:28028-32.

19. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPARgamma signaling and metabolism: the good, the bad and the future. *Nat Med*. 2013;19:557-66.

20. Hansford LM, Smith SA, Haber M, Norris MD, Cheung B and Marshall GM. Cloning and characterization of the human neural cell adhesion molecule, CNTN4 (alias BIG-2). *Cytogenet Genome Res*. 2003;101:17-23.

21. Voruganti VS, Nath SD, Cole SA, Thameem F, Jowett JB, Bauer R, et al. Genetics of variation in serum uric acid and cardiovascular risk factors in Mexican Americans. *J Clin Endocrinol Metab*. 2009;94:632-8.

22. Voruganti VS, Kent JW, Jr., Debnath S, et al. Genome-wide association analysis confirms and extends the association of SLC2A9 with serum uric acid levels to Mexican Americans. Frontiers in genetics 2013;4:279.

23. Kanellis J, Watanabe S, Li JH, Kang DH, Li P, Nakagawa T, et al. Uric acid stimulates monocyte chemoattractant protein-1 production in vascular smooth muscle cells via mitogen-activated protein kinase and cyclooxygenase-2. *Hypertension*. 2003;41:1287-93.

24. Villegas R, Williams SM, Gao YT, Long J, Shi J, Cai H, et al. Genetic variation in the peroxisome proliferator-activated receptor (PPAR) and peroxisome proliferator-activated receptor gamma co-activator 1 (PGC1) gene families and type 2 diabetes. *Ann Hum Genet*. 2014;78:23-32.

25. Park KS, Shin HD, Park BL, Cheong HS, Cho YM, Lee HK, et al. Putative association of peroxisome proliferator-activated receptor gamma co-activator 1beta (PPARGC1B) polymorphism with Type 2 diabetes mellitus. *Diabet Med*. 2006;23:635-42.

***Point-by-Point Response**

Dear Editor,

Thank you for considering our manuscript for publication in Atherosclerosis. We appreciate the reviewers' time, positive remarks and helpful suggestions. We have responded to individual comments below.

We have used text formatting for clarity as follows:

Blue upright font = our responses

Black upright font = reviewers' comments

Black italic font = unchanged manuscript text

Red italic font = revised manuscript text

Reviewer #1:

Comment 1: This is an interesting pharmacogenomic exploration. However, replication in an independent population must be performed to enable interpretation of the results. A larger population will give opportunities for a more convincing Mendelian randomization approach for 11-dehydro TxB2 and also allow more formal multiple way analyses of variance in treatment subgroups.

We agree that an independent replication in a large cohort is desirable. Unfortunately, 11-dehydro TxB_2 is not an easily measured phenotype, and we have not been able to identify a suitable replication population. Although not a formal replication of the association between the genetic variants and 11-dehydro TxB_2, the finding that the same variants increase the risk of cardiovascular events in an independent population does back up our finding that these genetic variants increase 11-dehydro TxB_2 levels.

We have amended the following paragraph in the discussion section:

'Unfortunately, suitable data were not available to formally replicate the genetic associations with 11-dehydro TxB_2 in an independent cohort. However, our finding that the 11-dehydro TxB_2-increasing alleles of the same genetic variants increase the risk of cardiovascular events in an independent population does support our finding that these variants are associated with increased 11-dehydro TxB_2, (an established cardiovascular risk factor). In addition, Mendel's observation that inheritance of one trait should be independent of the inheritance of other traits asserts that it is unlikely the same

SNPs would be associated with both 11-dehydro TxB_2 and CHD unless the phenotypes are causally linked. Therefore our finding that the same SNPs are associated with increased 11-dehydro TxB_2 and increased risk of cardiovascular event (unconfounded by association with other measured cardiovascular risk factors) supports a causal relationship between 11-dehydro TxB_2 and CHD and strengthens the finding by Eickelboom et al that urinary 11-dehydro TxB_2 is an independent risk factor for atherothrombotic events. Whether the atherosclerosis causes the elevated 11-dehydro TxB_2 or vice versa, however, cannot be clearly ascertained from these data.'

Comment 2: Moreover, the findings about the relationship with cardiovascular events must be discussed in context of the previous large GWA studies.

We have added the following text to the discussion section, plus supplementary tables 5, 20 and 21, which we hope addresses this valid comment.

'We conducted a search of the term 'cardiovascular in the NHGRI-EBI GWAS catalogue- (https://www.ebi.ac.uk/gwas/search?query=cardiovascular, accessed 25/10/17, Supplementary Table 20) in order to put our findings in the context of the vast GWAS data now available. The CNTN4 gene has been associated with serum uric acid levels in previous genome-wide association and linkage studies, [21, 22], which is itself correlated with a variety of CVD risk factors. There is some evidence for cross talk between COX and uric acid pathways - uric acid has been shown to stimulate COX-2 synthesis [23], for example. Although PPARGC1B does not feature in the GWAS catalogue results, genetic polymorphisms in the PPARGC1B genes have previously been putatively associated with type 2 diabetes [24, 25].

A search of the five 11-dehydro TxB_2 SNPs reported in this study in GWAS central, (http://www.gwascentral.org/ accessed 25/10/17), showed that none of the five SNPs have been included in GWAS of MI in this catalogue (Supplementary Table 21). Some of the SNPs have shown previous nominal associations ($P<0.05$) with other cardiovascular phenotypes, including associations between rs32583 and ischemic stroke (Supplementary Table 21). It should be noted that the five 11-

dehydro TxB_2 -associated SNPs in this study were not present on most of the commonly used GWAS chips; rs4235745 for example is only on the IBC (CVD50K) chip (Supplementary Table 5). In addition, the thromboxane pathway represents one of many risk factors for a cardiovascular disease; therefore, we would not necessarily expect the SNPs reported here to be among the top GWAS hits for CVD.'

Reviewer #2:

I find the post-GWAS study and the related manuscript by McCarthy and colleagues potentially interesting. Although the design of the study is a bit unusual, it is clear. Also the manuscript is nicely written.

Thank you for your positive comments.

Comments:

Comment 1: The concept of selecting best hits from two separate ChIPs is intriguing. However, the question arises, why the overlap between the results from those two SNPs is limited? Or there are some additional hits showing the overlap between the chips? Theoretically, at least some top-scoring results should overlap, even considering methodological differences (selection of the SNPs) between the SNPs.

The two chips were quite different in term of their SNPs content, as noted in supplementary information section "quality control and imputation":

'merging of the two datasets would have incurred substantial loss of information - 5,328 SNPs were unique to the CVD50K chip following quality control – and they were therefore analyzed separately'.

We have highlighted SNPs in both the CNTN4 and PPARGC1B genes on both chips in the amended Manhattan plots (Figure 1). We have also added Supplementary Table 3 which shows the results of CNTN4 and PPARGC1B SNPs on both chips, and amended the results section as follows:

'Analysis of CNV370 chip: a CNTN4 haplotype is significantly associated with 11-dehydro TxB_2

The strongest association signal from the CNV370 analysis was in the CNTN4 (contactin 4) gene

(Figure 1), where 13 SNPs were suggestive of association with 11-dehydro TxB_2 (Supplementary Table 7). The most significant genotyped SNP at this locus was rs10510230 (P=5.1E-07, Table 2) and conditioning on this genotype revealed one other SNP, rs4684343, independently associated with 11-dehydro TxB_2 and not correlated with rs10510230 (r^2 =0.09, Supplementary Table 8 and Supplementary Figure 5). Only one SNP in the CNTN4 gene was genotyped on the CVD50k chip (rs1171387), and this SNP is not very close to either of the top hit CNTN4 SNPs (rs10510230 or rs4684343). Hence there was very little signal at the CNTN4 gene on the CVD50K chip (Supplementary Table 5 and Figure 1).'

Comment 2: If no SNP reached genome-wide significance after correction for multiple testing, why only one region per each chip was selected for further analysis of haplotypes? Looking at the Manhattan plots, there are some other hits with or without stratification for aspirin (especially in subjects on aspirin).

Our a priori analysis plan was to take only the top association peaks from each chip forward for survival analysis to avoid a high burden of multiple testing. In addition, although the individual SNPs were not genome-wide significant, both the PPARGC1B and the CNTN4 haplotypes using the 5 independently associated SNPs were, so we took forward the SNPs which comprised these genome-wide significant haplotypes. We will include the 11-dehydro TxB_2 association results in their entirety as supplementary information so that future studies will hopefully explore the other interesting associations.

Comment 3: The Authors consider their survival analysis as a replication of the discovery associations (page 10, lines 52-57). I would not go that far. If those results are reported as an extension/expansion of the discovery results, it is enough.

We agree with this comment, and hence we have changed the wording of the relevant paragraph in the discussion section to read:

'Unfortunately, suitable data were not available to formally replicate the genetic associations with 11-dehydro TxB_2 in an independent cohort. However, our finding that the 11-dehydro TxB_2-increasing alleles of the same genetic variants increase the risk of cardiovascular events in an independent

population does support our finding that these variants are associated with increased 11-dehydro TxB_2, (an established cardiovascular risk factor). In addition, Mendel's observation that inheritance of one trait should be independent of the inheritance of other traits asserts that it is unlikely the same SNPs would be associated with both 11-dehydro TxB_2 and CHD unless the phenotypes are causally linked. Therefore our finding that the same SNPs are associated with increased 11-dehydro TxB_2 and increased risk of cardiovascular event (unconfounded by association with other measured cardiovascular risk factors) supports a causal relationship between 11-dehydro TxB_2 and CHD and strengthens the finding by Eickelboom et al that urinary 11-dehydro TxB_2 is an independent risk factor for atherothrombotic events. Whether the atherosclerosis causes the elevated 11-dehydro TxB_2 or vice versa, however, cannot be clearly ascertained from these data.'

Comment 4: By this occasion, if the major findings of the discovery phase were obtained for haplotypes, why the survival analysis is conducted for single SNPs (especially if you consider survival analysis a replication)?

Our main finding from the survival analysis was that the addition of all 5 SNPs contributing to the two haplotypes together improved the survival model over covariates alone, rather than the individual associations, which were just provided for completeness. However, we have added a formal analysis of the haplotypes to the supplementary data (Supplementary Table 19 and Supplementary Figure 6), and added a section to the manuscript results:

'The 11-dehydro TxB_2-associated haplotypes are associated with cardiovascular event free survival.

Survival analysis of the PPARGC1B and CNTN4 haplotypes which were associated with the highest 11-dehydro TxB_2 levels (Supplementary Table 19 and Supplementary Figure 6) showed that carriership of one copy of the PPARGC1B haplotype (HR= 1.21, P=0.05) and of the CNTN4 haplotype (HR=1.33, P=0.005), had similar effect sizes on survival to those of the highest effect size SNPs in the haplotypes (rs10515638 (HR=1.31, P=0.01) and rs10510230 (HR=1.25, P=0.007) respectively, Table 3).'

Comment 5: Page 9, lines 4-10. I would suggest some additional analysis here. Even if the 5 major

SNPs were not (in silico) functional, their LD-proxies might have been. LD-proxies of those SNPs should be determined (using e.g. SNAP software; https://www.broadinstitute.org/mpg/snap/ldsearch.php) and check for their experimental (literature) and/or in silico function as described elsewhere (https://www.ncbi.nlm.nih.gov/pubmed/22909159, https://www.ncbi.nlm.nih.gov/pubmed/24866380, https://www.ncbi.nlm.nih.gov/pubmed/26473826, https://www.ncbi.nlm.nih.gov/pubmed/24930997).

We have added a table (Supplementary Table 12) showing all of the proxy SNPs and their annotations (from ensembl and GTEx). None of the LD proxies were exonic, or predicted to have high functional consequence. We have changed the relevant paragraph to read:

'Function of 11-dehydro TxB_2 – associated CNTN4 and PPARGC1B SNPs

None of the independently associated SNPs nor their proxies were in exonic or predicted functional regions using the ensemble variant effect predictor (http://www.ensembl.org/Tools/VEP), nor were they associated with expression of any genes in any tissues in the GTEx portal (http://www.gtexportal.org/home/), (Supplementary Table 12).'

Comment 6: Figure 2. How can you describe the magnitude of the observed effects? Is it large or small?

The magnitude of the effect of the lead SNPs on cardiovascular free survival (Figure 2) could be described as 'moderate' – they are similar in magnitude to other CV risk factors (Table 3) and to the hazard ratios for the risk haplotypes (see above response). The relevant text in the paper is below:

'Two SNPs independently associated with risk of event

After Bonferroni correction for the 5 SNPs tested (P value threshold <0.01), rs10515638 (PPARGC1B) and rs10510230 (CNTN4) were associated with increased risk of event in multivariate (P=0.01 and 6.9E-03 respectively) and univariate (P=0.03 and P=8.5E-03 respectively) survival models. The magnitudes of effect on survival associated with carriage of each minor allele of rs10515638 (PPARGC1B, HR=1.31) and rs10510230 (CNTN4, HR=1.25) were similar to or greater than those associated with increments of 10 years age, 20 mmHg systolic BP, 1 mmol LDL-cholesterol, a

diagnosis of diabetes mellitus, or use of amlodipine-based antihypertensive therapy in this cohort (Table 3).'

Comment 7: If haplotype analysis is used, it would be good to show in Table 2 also D' between the major SNPs. Also the structure of the particular haplotypes with frequencies should be given somewhere.

We agree with this comment and therefore have made the following changes:

D' has been added to Table 2.

The lower panel of Supplementary Table 6 shows all haplotypes of the three SNPs in *PPARGC1B* independently associated with 11-dehydro TxB_2 and their frequencies. Similarly, the lower panel of Supplementary Table 9 shows all haplotypes of the two *CNTN4* SNPs independently associated with 11-dehydro TxB_2.and their frequencies.

Other comments:

1. Page 10, line 2. "Table 5"? Or "Table 3"? -

We thank the reviewer for identification of this error, and have now corrected the text to "Table 3"

2. Page 9, lines 37-38. "(…) was consistent the multivariate (…)"? Or "(…) was consistent with the multivariate (…)"?

We thank the reviewer for identification of this lack of clarity and have reworded the 2 sentences to read:

'None of the SNPs were associated with any of the cardiovascular risk factors with the exception of a slightly increased risk of type 2 diabetes in rs32582 and rs10510230 carriers (Supplementary Table 13). Results of the univariate survival analysis (Supplementary Table 14) were consistent with those reported in the multivariate analysis. There was a small sample overlap between the genetic association study and the survival analysis; when these individuals were excluded from the survival sample in a sensitivity analysis, it did not alter the overall results (Supplementary Table 15).'

3. Page 9, lines 49-55. What do you mean here? What does "range" correspond to (i.e. what is between e.g. 21% and 27%)?

We thank the reviewer for identification of this lack of clarity and have changed the text to read:

'The magnitudes of effect on survival associated with carriage of each minor allele of rs10515638 (PPARGC1B, HR=1.31) and rs10510230 (CNTN4, HR=1.25) were similar to or greater than those associated with increments of 10 years age, 20 mmHg systolic BP, 1 mmol LDL-cholesterol, a diagnosis of diabetes mellitus, or use of amlodipine-based antihypertensive therapy in this cohort (Table 3).

4. The title. I would remove "elevated" and "reduced" as long as alleles are not mentioned.

We also agree with this point and have therefore changed the title to:

'Genetic Variants in PPARGC1B and CNTN4 are Associated with Thromboxane A_2 Formation and with Cardiovascular Event Free Survival in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT).'

5. Page 12, lines 30-36. I would remove this sentence since it is overstatement. I would also (especially) remove it from the abstract (it is identical there).

We do understand this point, and while we certainly do not want to make any unwarranted claim, we also believe that it is important to outline the *potential* clinical implications of this work. Hence we have reworded the sentence to try to make it clear that more work will need to be done before these findings are clinically useful:

'If specific protection of PPARGC1B and CNTN4 variant carriers by aspirin is confirmed by additional studies, PPARGC1B and CNTN4 genotyping could potentially assist in clinical decision making regarding the use of aspirin in primary prevention.'

Reviewer #3: Manuscript Number: ATH-D-17-00958

Genetic Variants in PPARGC1B and CNTN4 are Associated with Elevated Thromboxane A2 Formation and with Reduced Cardiovascular Event Free Survival in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)

This study sought to identify genetic determinants of urinary 11-dehydro thromboxane B2 (TxB2) and their associations with cardiovascular morbidity. Genome-wide association study between urinary 11-dehydro TxB2 and SNP genotypes showed suggestive associations with 3 variants in

PPARGC1B and with 2 variants in CNTN4 using 806 Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) study subjects. Two of the variants previously identified were also associated with increased risk of cardiovascular events in an independent sample from ASCOT (n=6199), and it appears that these effects were blunted by aspirin intake.

Although the study is well done, presentation of the results is not always clear. To make this paper easier to for a reader to understand, following points should be addressed:

Comment 1: It is not clear why significance level for CNV370 was set to P<1.8E-7 (0.05/272,166) and not conventionally used P<5-8. According to Methods section, there were 2,031,499 genotyped/imputed SNPs after QC. Also, threshold for suggestive association should be indicated for separately for CNV370 and CVD50K separately in the methods section.

We thank the reviewer for identification of this lack of clarity.

The CNV370K is an older chip with less genotyped SNPs than more modern chips which have much higher coverage and for which the more conventional P<5-8 is used (estimated to cover the whole genome at Bonferroni correction after LD has been accounted for).

Only 272,166 SNPs were genotyped on the CNV370 chip, hence Bonferroni being set at 0.05/272,166 (as the imputed SNPs are based on the information from the genotyped SNPs, no additional correction is necessary).

We have changed the relevant text in the methods section to make these points more clearly:

'Genotypes Associated with Elevated Thromboxane A2:

……. After further excluding all copy number variant (CNV) markers and SNPs with minor allele frequency (MAF) <5%, 272,166 genotyped SNPs (2,031,499 including imputed SNPs) on the CNV370 chip and 31,570 SNPs on the CVD50K chip were analysed in n=777 and n=544 individuals with 11-dehydro TxB_2 measurements, respectively (Supplementary Figure 1)….

…… Haplotypes were imputed in PLINK using the standard E-M algorithm. Bonferroni threshold for the CNV370 chip was P=1.8E-07 (0.05/272,166 genotyped SNPs) to account for multiple testing with a 5% false positive rate. As many SNPs on the dense CVD50K chip are correlated, correction for the 21,180 effective tests on the chip is appropriate [13], resulting in a Bonferroni threshold of P=2.4E-06 (0.05/21,180). SNPs associated at P<1E-03 were considered suggestive of association on both chips.'

Comment 2: Significant and suggestive cut offs should be also added to footnotes of each table/figure. It would be helpful if suggestive threshold was also added to manhattan plots.

We thank the reviewer for this suggestion. A suggestive threshold has been added to the Manhattan plots (Figure 1), along with the following text:

'The lower dashed line is the threshold for 'suggestive' association (P<1E-03), and the upper dashed line is the Bonferroni threshold for significance (P<2.4E-06 for the CNV50K chip and P=1.8E-07 for the CNV370K chip).'

Moreover, when testing 5 SNPs for interaction, significance level should be P<0.05/5=0.01, instead of P<0.05.

We agree with this comment. This error has been corrected in the revised version, with the interactions described in a new paragraph:

'Interaction with aspirin

The five independently associated PPARGC1B and CNTN4 SNPs showed evidence for interaction with aspirin use in a model including the main SNP and aspirin effects and all covariates, with a greater association between genotype and 11-dehydro TxB_2 in those not taking aspirin (Figure 1, Table 2, Supplementary Tables 10 and 11). The interaction was significant for three of these five SNPs after correction for multiple testing (P value threshold <0.01; interaction P-values for PPARGC1B SNPs: rs4235745 (P=3.3E-04), rs32582 (P= 3.0E-04), rs10515638 (P=0.02) and for CNTN4 SNPs: rs4684343 (P=0.02), rs10515638 (P= 6.6E-05); Supplementary Tables 10 and 11.'

Comment 3: Reported associations should always include p-values throughout the manuscript, rather than stating that an association was significant because it was less than a specific cut off.

We agree with this suggestion, and have amended accordingly throughout in the revised manuscript.

Comment 4: Cox Proportion Hazards Analysis (Table 3): This table needs major revision.

- First, outcome name should be included the table header (e.g. composite cardiovascular

evets) and footnotes should have a definition for the outcome (including fatal and non-fatal MI, ischemic stroke etc.)

The title of table 3 has been revised accordingly as follows:

'Table 3. Multivariate Cox proportional hazards analysis all significant clinical covariates plus the five 11-dehydro TxB_2-associated SNPs with a composite cardiovascular endpoint of all ischaemic cardiovascular events and procedures.'

- It is not necessary to show the effect of individual covariates. Instead, a final model should be build and individual SNP Hazard Ratios for each SNP should be reported (covariates + SNP1, covariates + SNP2 etc.). If so desired, final analysis can include covariates + all 5 SNPs. Example below.

Outcome

SNP	Event rate	HR	95% CI	p-value (LRT)
SNP1				
SNP2				
SNP3				
SNP4				
SNP5				
SNP1-5				

As genomic context is important, we decided to make our primary analysis a complete model including all SNPs and all covariates. However, univariate analysis of SNPs individually is included in Supplementary Table 14. We have removed the covariates-only analysis from Table 3, but reported the effects of the covariates in the full model as it is useful to see the magnitude of the hazard ratios of the covariates in comparison to those of the SNPs.

Comment 5: It would be interesting to see if any of the individual outcomes forming the composite outcome is driving the associations. You may not have enough power to see significant associations if the composite outcome is broken down, but it may give us additional information Example below.

	SNP1				SNP2			
	Event Rate	HR	95% CI	p-value (LRT)	Event Rate	HR	95% CI	p-value (LRT)
ETC.								

Outcome1

Outcome2

Outcome3

Outcome4

Outcome5

Outcome6

Outcome7

Outcome8

ALL Combined

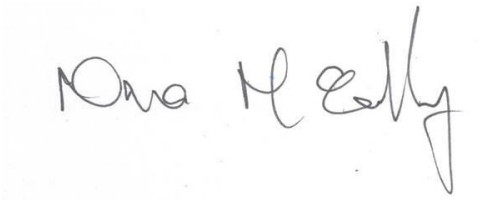
Although, as you say, we do not have particularly good power to look at individual outcomes, we have looked at stroke and coronary events separately and included these analyses in Supplementary Table 18 and in an additional results paragraph in the main text:

'Stratification by type of event

Comparing the separate stroke and coronary event analyses, the PPARGC1B SNP (rs10515638) had a larger effect size for coronary events (HR 1.41, P=0.002, 433 events) than for stroke events. By contrast, despite much lower power in the stroke analysis, the CNTN4 SNP (rs10510230) had a larger effect size for stroke (1.31, P=0.17, 57 events) than for coronary events, especially in those not on aspirin (HR 2.71, P=0.003, 26 events), Supplementary Table 18.'

***Statement of Originality**

The work described represents original work carried out by the authors and has not been published previously, except in the form of an abstract. The article is not under consideration for publication elsewhere. Publication of the article is approved by all authors and the responsible authorities where the work was carried out. If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.



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Nina McCarthy, 25th August 2017, on behalf of all authors.

Editor-in-Chief Professor Arnold von Eckardstein
Institute of Clinical Chemistry
University Hospital and University of Zurich
Rämistrasse 100, Zurich
CH-8091
Switzerland

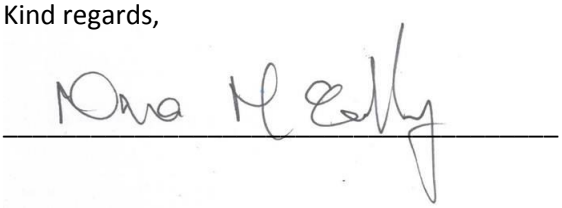
25th August 2017

Dear Professor von Eckardstein

Thank you for considering our paper, *'Genetic Variants in PPARGC1B and CNTN4 are Associated with Elevated Thromboxane A_2 Formation and with Reduced Cardiovascular Event Free Survival in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)'* for publication in *Atherosclerosis.*

This paper describes the first genome wide association study of thromboxane A_2 formation, which showed that 5 SNPs in two genes, *PPARGC1B* and *CNTN4,* are associated with elevated thromboxane A_2 formation in 806 Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) participants and with an excess of cardiovascular events in an independent ASCOT population (n=6,199). Aspirin appears to blunt these associations. If specific protection of *PPARGC1B* and *CNTN4* variant carriers by aspirin is confirmed, *PPARGC1B* and *CNTN4* genotyping could potentially provide guidance in the use of aspirin in primary prevention.

Kind regards,



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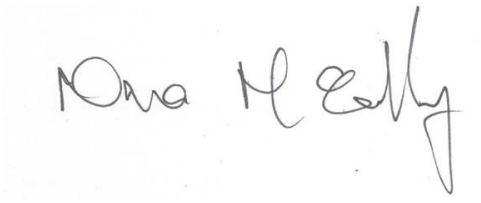
***Conflict of interest form**

Conflict of Interest Disclosures:

Toby Johnson is a full time employee of GlaxoSmithKline and owns company stock. Nina McCarthy and Alice Stanton are named inventors on an RCSI filed patent application entitled “Identification of thrombosis or bleeding risk in an individual with and without antiplatelet therapy”, Patent application number 11166142.7 – 2402. All other authors: none declared.



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Nina McCarthy, 25th August 2017, on behalf of all authors.

Supplementary Material for online publication only

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- Introduction
- Materials and methods (or Patients and methods)
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- Conflict of interest (mandatory)
- Financial support (if applicable)
- Author contributions (mandatory)
- Acknowledgements (if applicable)
- References
- Figures and Tables (with legends in the suitable style)

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Are abbreviations defined when first used in the text? **Yes**

Use of abbreviations should be kept at a minimum.

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Are gene names italicized? **Yes**

Gene names should be italicized; protein products of the loci are not italicized.

For murine models, the gene and protein names are lowercase except for the first letter.
(e.g., gene: *Abcb4;* protein: Abcb4)

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Mouse strains and cell lines

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(e.g. *ob/ob* , $p53^{+/+}$, $p53^{-/-}$)

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Are *p* values consistently formatted according to the below style throughout the manuscript (including figures and tables)? **Yes**

p <X
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