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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)

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ABSTRACT

Background and aims: Elevated urinary 11-dehydro thromboxane B_2 (TxB_2), a measure of thromboxane A_2 formation *in vivo*, predicts future atherothrombotic events. To further understand this relationship, the genetic determinants of 11-dehydro TxB_2 and their associations with cardiovascular morbidity were investigated in this study.

Methods: Genome-wide and targeted genetic association studies of urinary 11-dehydro TxB_2 were conducted in 806 Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) participants.

Results: 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 TxB_2 formation. Haplotypes of 11-dehydro TxB_2 increasing alleles for both *PPARGC1B* and *CNTN4* were significantly associated with 11-dehydro TxB_2, 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 = 6199), 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 TxB_2 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 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.

1. 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

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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.

2. Materials and 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.

2.1. Genotypes associated with elevated thromboxane A2

Fasting urinary 11-dehydro TxB_2 was measured by mass spectrometry in 1006 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 >2000 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 Fig. 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 Figs. 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.

2.2. Effect of PPARGC1B and CNTN4 genotypes on cardiovascular event free survival

All ASCOT participants with DNA (n = 9063) were considered for inclusion in the survival analysis. The following exclusions were applied: study participants with prior cardiovascular event at baseline (n = 1382), 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 Fig. 1). The 5 SNPs independently associated with 11-dehydro TxB_2 were successfully genotyped in 6199 participants (n = 3369 from Scandinavian centres and n = 2851 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.

3. Results

3.1. 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 (Fig. 1) show that none of the SNPs was