Genome-wide association study of coronary and aortic calcification implicates risk loci for coronary artery disease and myocardial infarction

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ABSTRACT

Arterial calcification is a well-known risk factor for coronary artery disease (CAD) and myocardial infarction (MI). We performed a genome-wide association study on coronary artery calcification and aortic calcification as intermediate traits for CAD/MI. We tested ~2.5 million SNPs for association with coronary artery calcification and aortic calcification in 2620 male individuals of the NELSON trial, who underwent chest CT scans. All participants were current or former heavy smokers. No SNPs were associated with aortic calcification on a genome-wide scale. The 9p21 locus was significantly associated with coronary artery calcification (rs1537370, $P = 2.3 \times 10^{-11}$). Since this locus corresponds to the strongest SNP association for CAD/MI, we tested 24 published and validated CAD/MI risk SNPs for association with arterial calcification. Besides the CAD/MI SNP at 9p21 (rs4977574, $P = 3.1 \times 10^{-10}$), two additional loci at ADAMTS7 (rs3825807, $P = 6.5 \times 10^{-6}$) and at PHACTR1 (rs12526453, $P = 1.0 \times 10^{-3}$) show a nominally significant association with coronary artery calcification with MI/CAD risk alleles increasing the degree of arterial calcification. The 9p21 locus was also nominally associated with aortic calcification ($P = 3.2 \times 10^{-4}$). These findings indicate that these CAD and MI risk loci are likely involved in arterial calcification.

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1. Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the world. Calcification of coronary arteries and the

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aorta is a major risk factor for coronary artery disease (CAD) and myocardial infarction (MI), independent of well-known risk factors, including cholesterol levels, body mass index, smoking behavior, age, gender, and family history [1,2]. Recent genome-wide association studies have identified 25 genomic regions reproducibly associated with CAD/MI risk [3]. Of these 25 loci, eight are clearly linked to genes that modulate lipid levels in the circulation, including LDL-cholesterol and triglycerides [4]. The functional basis of the remaining 17 loci associated with CAD/MI is poorly understood, suggesting a role for mechanisms other than lipid metabolism.

In this study, we used quantitative computed tomography (CT) analysis to measure calcification levels in coronary arteries and aorta in a cohort of heavy smokers. Heritability estimates of coronary artery and aortic calcification are approximately 50% [5,6], making them suitable intermediate traits. We performed a genome-wide study of these continuous traits, testing 2.5 million common SNPs across the genome. We demonstrate that three previously found CAD/MI risk loci are associated with arterial calcification.

2. Methods

2.1. Cohort description

The Dutch and Belgian Lung Cancer Screening Trial (NELSON trial) [7,8] was designed to study the early detection of lung cancer in an at-risk population. In total, 15,523 participants were enrolled at two different locations: the University Medical Center Groningen and the University Medical Center Utrecht. The study was approved by the Ministry of Health of the Netherlands and written informed consent was obtained from all participants. Screening trial participants were current or former (<10 year) heavy smokers (predominantly males) between 50 and 75 years of age with a smoking history of at least 16 cigarettes/day for 25 years or at least 11 cigarettes/day for 30 years (i.e. >16.5 pack years), with an average smoking history of 40 pack years. Exclusion criteria were a self-reported moderate or bad health with inability to climb 2 flights of stairs, a recent chest CT, current or past cancer, and a body weight ≥140 kg.

2.2. CT-based imaging

Low-dose, non-ECG synchronized, non-contrast enhanced baseline chest CTs were available for all participants. CTs were acquired on a 16 detector-row scanner (Mx8000 IDT, Somatom Sensation 16, or Brilliance 16P, Philips Medical Systems, Cleveland, OH, USA) in spiral mode with 16×0.75 mm collimation. Axial images of 1.0 mm thickness at 0.7 mm increment were reconstructed with a moderately soft kernel (Philips "B"). The peak voltage was 120–140 kVp depending on patient weight, with a tube current of 30 mAs. A detailed description of the inclusion criteria and the scanning protocol is provided by van Klaveren et al. [8] To perform calcium scoring with the usual section thickness, CTs were subsampled to 3.1 mm thick sections with a total increment of 1.4 mm [9].

We developed computer-aided systems for automatic detection and quantification of aortic and coronary calcifications [10,11]. A threshold of 130 Hounsfield units was used to identify potential calcifications, which were extracted by three-dimensional connected component labeling. Coronary calcifications were detected using a system based on a previously described algorithm [10]. First, a probabilistic coronary calcium map was designed, which provided an *a priori* probability for spatial appearance of coronary calcifications in a chest CT scan. This was followed by a supervised pattern recognition system which detected calcifications based on spatial and texture information. Aortic calcifications were detected using a multi-atlas-based segmentation of the aorta, followed by a supervised pattern recognition system which detected aortic calcifications based on the spatial, size and texture features [11]. After manual inspection by up to four experienced observers, calcifications in the aorta were quantified in terms of total volume calcium score (mm^3), while coronary artery calcifications were measured in terms of the Agatston score [12].

2.3. Genotyping and quality control

As part of the COPACETIC study, genome-wide SNP genotype data was collected in 3082 randomly sampled participants from the NELSON trial (Table 1). DNA samples were obtained from blood using a high salt method [13], and hybridized on Illumina Quad610 BeadChip containing 620,901 markers providing good coverage of common SNP variation. Genotypes were called with the GenomeStudio software by Illumina. Quality control was performed on sample and SNP level using PLINK [14].

A flowchart of the sample selection and quality control is shown in Figure S1. We removed 264 samples with a rate of missing genotypes >2%. We selected high quality SNPs by applying the following filters: missing rate <1%, Hardy-Weinberg $P > 0.001$, minor allele frequency >10%, and LD-pruning (leaving no pairs with $R^2 > 0.05$), resulting in 64,902 SNPs. Using these SNPs, we removed 56 samples with heterozygosity >2 standard deviations from the mean of all samples, and 49 samples of non-European ancestry, on the basis of principal component analysis using ten HapMap 3 populations (CEU, YRI, JPT + CHB, ASW, CHD, GIH, LWK, MEX, MKK, and TSI) as reference. We also removed 39 related samples (proportion of IBD >0.2), keeping only the sample with the lowest missingness. Since we only had data available on 14 females, we excluded those samples from all analyses.

Before genome-wide imputation, we removed SNPs if the missing rate was >2%, minor allele frequency <0.05, Hardy–Weinberg $P < 0.001$, missing genotyping rate greater than the minor allele frequency, or allele frequency difference to HapMap-CEU > |0.15|.

After these quality control steps, we repeated principal component analysis without HapMap samples, based on the same high quality SNPs as described above, to estimate the first 20 principal components. We tested each of the principal components for association with coronary artery and aortic calcification (using linear regression with age and smoking history as covariates), and detected that 13 of 20 components were nominally significant ($P < 0.05$). We included these 13 principal components as covariates in all association analyses. We also performed genome-wide association for each individual principal component (as a quantitative trait), but we found no evidence for global inflation.

2.4. Imputation

Imputation of untyped SNPs was performed in batches of 300 randomly drawn individuals. Using Beagle 3.0.4 [15], we performed imputation with 120 phased haplotypes from CEU HapMap phase 2

Table 1
NELSON/COPACETIC study information.

Number of samples after QC	2620
Groningen[a]/Utrecht[b]	1484/1136
Median aorta score	498 (IQR 90-1842) mm^3
Median Agatston score	127 (IQR 6-736) HU
Average pack years	40.4 (sd 17.1)
Average age	59.9 (sd 5.5)

Abbreviations: IQR: interquartile range, HU: Hounsfield Units, sd: standard deviation.
[a] Samples collected at the University Medical Center Groningen.
[b] Samples collected at the University Medical Center Utrecht.

data (UCSC hg18/NCBI 36) [16] encompassing 2,543,807 SNPs. Of the 25 SNPs previously associated with CAD/MI, 23 were successfully imputed. Two SNPs (rs17465637 and rs3798220) could not be imputed because they were absent from HapMap-CEU. One (rs17465637) had a good proxy (rs17011681, $R^2 = 1$ in 1000 Genomes pilot 1) that could be included in the analysis. Another SNP (rs3798220) had no good proxies and was therefore excluded from the analysis.

2.5. Association analysis

We performed a genome-wide association study on Agatston scores and calcium volume as measures for coronary artery calcification and aortic calcification, respectively, in 2620 individuals from the NELSON trial. Both phenotypes were $\log_e$ transformed (ln(score +1)) to obtain a normally distributed range of calcium scores (Figure S2). We used a linear regression model with age, smoking history (pack years), and principal components (to correct for population structure) [17] as covariates. Smoking history and age were independently correlated with calcium scores ($P < 0.01$ for both coronary artery and aortic calcification).

Genomic inflation (λ_{GC}) [18] was low before and after imputation ($\lambda \leq 1.024$ for coronary artery and aortic calcification, Figure S3), suggesting the influence of population structure and other technical biases was minimal. Since low frequency SNPs and SNPs with low imputation-quality scores showed little genomic inflation ($\lambda = 0.98$ for minor allele frequency < 0.05, $\lambda = 1.05$ for imputation-quality < 0.25), we decided not to remove these SNPs. Genome-wide significance for novel associations is set at $P < 5 \times 10^{-8}$.

2.6. CAD/MI lookup

Twenty-four previously described risk SNPs for CAD and MI [3] were tested for associations with aortic and coronary artery calcification. We set statistical significance at $P < 0.05/24 = 0.0021$ using Bonferroni correction, reflecting an elevated prior probability of being associated on the basis of evidence from GWAS of CAD/MI. Using this threshold, we have sufficient power ($>80\%$) to detect associations with SNPs that account for at least 0.6% of genetic variance. We calculated a genetic risk score (GRS) for each sample, defined by the total number of risk alleles they carry weighted by the ln(OR) reported by the CARDIoGRAM study. We tested this GRS for association with aortic and coronary artery calcification using linear regression with age, smoking history and principal components as covariates.

3. Results

3.1. Genome-wide association analysis

After testing 2.5 million SNPs for association with coronary artery and aortic calcification levels measured in 2620 NELSON participants, we found no genome-wide significant associations for aortic calcification but observed one region significantly associated with coronary artery calcification (Table S1 and Figure S4). This region is located on chromosome 9p21 near CDKN2A/CDKN2B with 37 SNPs that exceed genome-wide significance ($P < 5 \times 10^{-8}$). SNP rs1537370 is the most significant SNP with the T allele increasing the Agatston score ($P = 2.3 \times 10^{-11}$). This SNP is highly correlated (pairwise $r^2 > 0.9$ in European populations) to the SNP rs4977574, reported by the CARDIoGRAM study [3] as being associated with CAD/MI. Conditional analysis on these two SNPs shows that they are statistically indistinguishable, consistent with the high degree of LD between the two SNPs.

3.2. CAD/MI look-up

Since the 9p21 locus has been previously associated with CAD/MI, we decided to test all known CAD/MI risk SNPs for association with aortic and coronary artery calcification. Results for 24 SNPs are shown in Table 2. Three SNPs are significantly associated with coronary artery calcification (Figure S5): rs3825807 (ADAMTS7, $P = 6.5 \times 10^{-6}$), rs12526453 (PHACTR1, $P = 1.0 \times 10^{-3}$) and rs4977574 (CDKN2A/CDKN2B, $P = 3.1 \times 10^{-10}$). SNP rs4977574 at 9p21 is also significantly associated with calcification in the aorta ($P = 3.2 \times 10^{-4}$). All these associations were consistent with the expected direction of effect: CAD/MI risk alleles increased calcification levels. After adjusting for known CAD/MI loci, the resulting distribution of SNP associations across the genome is consistent with chance expectation (Figure S6).

Approximately ten percent of our study population ($n = 473$) had no detectable calcification in their coronary arteries (Figure S2). We removed all individuals with zero Agatston scores, repeated the association analysis and found that qualitatively all results remain unchanged. At 9p21, PHACTR1, and ADAMTS7, all SNPs are still significantly associated with coronary artery calcification ($P = 5.0 \times 10^{-5}$ for rs4977574 at 9p21, $P = 1.4 \times 10^{-4}$ for rs3825807 at ADAMTS7, $P = 0.003$ for rs12526453 at PHACTR1). When we split our population in one group with zero Agatston scores (as "cases"), and another group with non-zero scores (as "controls"), two out of three SNPs are associated at the nominal level ($P = 1.2 \times 10^{-6}$ for rs4977574 at 9p21, $P = 0.002$ for rs3825807 at ADAMTS7, $P = 0.12$ for rs12526453 at PHACTR1).

3.3. Genetic risk score

We tested the collective impact of 24 CAD/MI SNPs on the degree of calcification in the coronary arteries and aorta. For each sample we derived a GRS by counting in each individual the number of risk alleles, multiplied by the ln(OR) as reported by the CARDIoGRAM study. Fig. 1 shows the relation between the GRS (divided in eight equal groups) and the observed degree of calcification.

Using linear regression adjusting for age, smoking history, and principal components, we observed a statistically significant association between the GRS and coronary artery calcification ($P = 1.1 \times 10^{-14}$). The GRS explains 2.4% of the variance of the Agatston score in our cohort. There is a significant difference in Agatston scores between the lower and upper GRS bins (Fig. 1a), with on average almost ten times greater Agatston scores in individuals in the highest GRS bin compared to individuals in the lowest GRS bin. Although the relation between the GRS and aortic calcification was nominally significant ($P = 9.0 \times 10^{-3}$), the trend across GRS bins is much less pronounced, with only 0.2% variance explained of aortic calcification.

4. Discussion

In a large cohort of heavy smokers we quantified calcifications in the aorta and coronary arteries on chest CT scans using automated CT imaging analysis and tested 2.5 million common SNPs for association with these two continuous traits. We found a significant association signal at the CDKN2A/CDKN2B locus as well as nominally significant signals at ADAMTS7 and PHACTR1. Overall, this provides a potential biological mechanism that links vascular calcification to CAD and MI.

The 9p21 locus is significantly associated with coronary artery calcification, consistent with previously published results [19]. To our knowledge, we are the first to describe a significant association between 9p21 and aortic calcification. In addition, this locus has

Table 2
Association results for 24 CAD/MI risk SNPs.

SNP info						CARDIoGRAM	Agatston score			Aortic calcification		
rsID	Locus	Gene	Coded allele	Non-coded allele	Frequency	Odds ratio	Beta	SE	P-value	Beta	SE	P-value
rs599839	1p13.3	SORT1	A	G	0.78	1.11	0.072	0.093	0.437	0.096	0.082	0.241
rs17114036	1p32.2	PPAP2B	A	G	0.91	1.17	0.175	0.141	0.216	0.196	0.125	0.117
rs11206510	1p32.3	PCSK9	T	C	0.82	1.08	−0.003	0.103	0.975	0.053	0.091	0.563
rs17011681	1q41	MIA3	C	G	0.74	1.14	0.296	0.118	0.012	0.114	0.104	0.277
rs6725887	2q33.1	WDR12	C	T	0.15	1.14	−0.061	0.115	0.594	−0.009	0.102	0.931
rs2306374	3q22.3	MRAS	C	T	0.18	1.12	−0.025	0.105	0.817	0.039	0.093	0.675
rs17609940	6p21.31	ANKS1A	G	C	0.75	1.07	−0.022	0.096	0.819	−0.056	0.085	0.508
rs12190287	6p23.2	TCF21	C	G	0.62	1.08	0.108	0.103	0.292	0.015	0.091	0.875
rs12526453	6p24.1	PHACTR1	C	G	0.67	1.10	0.287	0.084	0.001	0.032	0.075	0.664
rs11556924	7q32.2	ZC3HC1	C	T	0.62	1.09	0.112	0.093	0.229	0.009	0.083	0.914
rs4977574	9p21.3	CDKN2A/CDKN2B	G	A	0.46	1.29	0.497	0.079	3.14E-10	0.253	0.070	3.21E-04
rs579459	9q34.2	ABO	C	T	0.21	1.10	0.100	0.096	0.299	−0.121	0.085	0.155
rs1746048	10q11.21	CXCL12	C	T	0.87	1.09	0.161	0.117	0.168	−0.064	0.103	0.535
rs12413409	10q24.32	CYP17A1/CNNM2/NT5C2	G	A	0.89	1.12	−0.034	0.147	0.813	−0.054	0.129	0.675
rs964184	11q23.3	ZNF259/APOA5-A4-C3-A1	G	C	0.13	1.13	0.267	0.118	0.024	−0.007	0.105	0.943
rs3184504	12q24.12	SH2B3	T	C	0.44	1.07	0.114	0.080	0.154	0.012	0.071	0.870
rs4773144	13q34	COL4A1/COL4A2	G	A	0.44	1.07	−0.160	0.169	0.341	−0.062	0.149	0.677
rs2895811	14q32.2	HHIPL1	C	T	0.43	1.07	0.029	0.079	0.716	0.110	0.070	0.117
rs3825807	15q25.1	ADAMTS7	A	G	0.57	1.08	0.363	0.080	6.50E-06	−0.070	0.071	0.327
rs12936587	17p11.2	RASD1/SMCR3/PEMT	G	A	0.56	1.07	0.163	0.083	0.050	0.111	0.074	0.130
rs216172	17p13.3	SMG6/SRR	C	G	0.37	1.07	0.099	0.081	0.223	0.079	0.072	0.272
rs46522	17q21.32	UBE2Z/GIP/ATP5G1/SNF8	T	C	0.53	1.06	0.024	0.078	0.757	0.006	0.069	0.932
rs1122608	19p13.2	LDLR	G	T	0.77	1.14	−0.031	0.091	0.737	−0.010	0.081	0.901
rs9982601	21q22.11	MRPS6	T	C	0.15	1.18	0.143	0.114	0.210	−0.121	0.101	0.233

Twenty-four CAD/MI risk SNPs were tested for association with coronary artery calcification and aortic calcification. Odds ratio from CARDIoGRAM taken from Ref. [3]. The frequency, odds ratio, effect estimates (beta) and standard error (SE) are given for the coded allele.

been previously associated with a number of traits including abdominal aortic aneurysms, intracranial aneurysms, stroke, peripheral artery disease, type 2 diabetes, and several cancers, reinforcing the idea that this locus has pleiotropic effects [20]. The locus contains a large non-coding RNA gene named ANRIL. Two cyclin-dependent kinases inhibitors, CDKN2A and CDKN2B are located about 100 kb away from the top signal at rs4977574. Expression of these three genes has been associated with a number of atherosclerosis-related phenotypes [21]. It has been shown that mice lacking an orthologous 70-kb region have a reduced

expression of CDKN2A and CDKN2B [22]. Deletion of this genomic region in human mutant aortic smooth muscle cells in culture leads to a doubling of proliferative capacity, which is a cellular phenotype relevant to atherosclerosis [22]. In addition, Motterle et al. showed that the CAD risk allele of SNP rs1333049 (pairwise $R^2 = 0.97$ with rs4977574 in 1000 Genomes pilot 1) was associated with a decreased expression of CDKN2A, CDKN2B, and ANRIL, and with an increased proliferation of vascular smooth muscle cells [23]. This locus should therefore be investigated for its role in calcifications in the vascular wall and how it confers risk to CAD/MI.
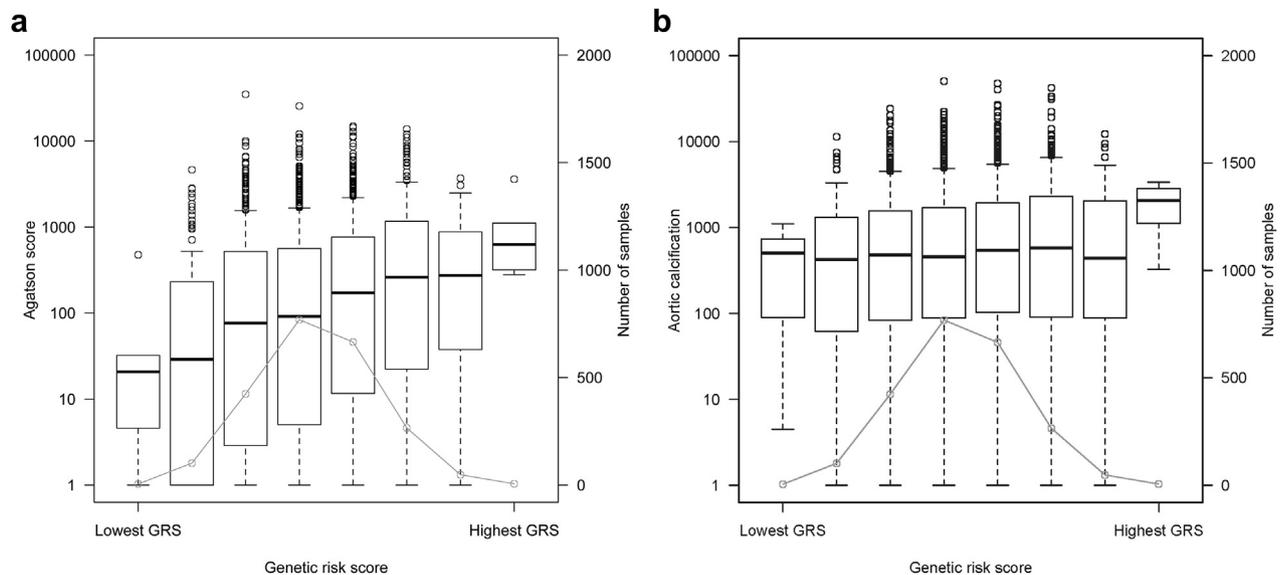

Fig. 1. Correlation between genetic risk score and arterial calcification. Genetic risk scores are based on the number of risk alleles for 24 previously described CAD/MI risk loci, weighted by their reported effect sizes. Scores are correlated with coronary artery calcification measured in terms of the Agatston score (a) and aortic calcification quantified in terms of total volume calcium score (b). Low levels of coronary artery calcification are evident in individuals with a lower genetic risk score. The correlation is less pronounced for aortic calcification, but still statistically significant.

Our second significant SNP rs3825807 encodes a missense change (Pro214Ser) in *ADAMTS7*, which is a member of the *ADAMTS* (a disintegrin and metalloproteinase with thrombospondin motifs) family. This amino acid substitution is predicted to be "benign" according to PolyPhen-2 [24], leaving it unresolved as to whether this SNP is the causal variant affecting calcification. *ADAMTS7* plays a role in vascular smooth muscle cell migration and intimal thickening after vascular injury, and it has been shown that this process is at least partially mediated by *ADAMTS7*-dependent degradation of the extracellular matrix protein COMP (cartilage oligomeric matric protein) [25]. Two hundred kb downstream of *ADAMTS7*, a SNP rs1051730 is associated with smoking [26], and is in LD with our index SNP rs3825807 (pairwise $R^2 = 0.43$ in 1000 Genomes pilot 1). Adjusting for rs1051730, we still observed residual association at rs3825807 ($P = 0.0024$), suggesting that our signal is independent from the smoking risk locus.

The third SNP significantly associated with coronary artery calcification is located in an intron of *PHACTR1* (phosphatase and actin regulator 1), the only gene within 200 kb of this SNP. The *PHACTR1* gene product is thought to regulate protein phosphatase 1 (PP1), an enzyme that dephosphorylates serine and threonine residues on a range of proteins [27]. Furthermore, Jarray et al. suggest that this gene is important in tube formation and endothelial cell functioning [28].

Interestingly, Reilly et al. argued that these three loci are all associated with angiographic CAD, but not with MI in angiographic CAD positive patients, leading them to suggest that these genes play only an indirect role in MI [29]. Possibly these three genes might exert their effects on arterial calcification, thus contributing only indirectly to MI.

Our results are consistent with recent results presented by O'Donnell and colleagues [19]. They found two loci (*CDKN2A*/*CDKN2B* and *PHACTR1*) associated with coronary artery calcification at genome-wide significance, with additional evidence for four CAD/MI SNPs, including *SORT1*, *MRAS*, *COL4A1*/*COL4A2*, and *ADAMTS7*. Hence, our study provides independent replication evidence for *CDKN2A*/*CDKN2B*, *PHACTR1* and *ADAMTS7*, and observes also consistent effects in the same direction for the remaining three loci.

We tested for the robustness of our results by removing individuals with zero Agatston scores, and by dichotomizing the Agatston score distribution in a group with zero scores and a group with non-zero scores. These results of these association tests are consistent with a model where the associated SNPs at 9p21, *PHACTR1* and *ADAMTS7* influence levels of calcification in all individuals and where individuals with zero Agatston scores are particularly enriched for protective alleles that lower calcification.

When we combine the 24 CAD/MI SNPs into a GRS, we observe that the prevalence of coronary artery calcification, and in lesser extent aortic calcification, increases with greater GRS. This may be clinically relevant because low amounts of coronary calcifications are generally associated with good prognosis and may be used to reclassify subjects to low cardiovascular risk [30].

Since the NELSON trial was set up to screen for lung cancer in a heavy smoking population, information on cardiovascular risk factors such as diabetes and hypertension was not collected. It is unclear to what extent genes associated with these risk factors (and with lung cancer) have influenced the study results, but the consistency of the associations observed for coronary calcification and CAD/MI is certainly compelling and makes it rather unlikely that the present results are due to confounding. Furthermore, we are unaware of an etiological link between lung cancer and coronary calcification. Over the whole NELSON trial, the number of incident lung cancer cases is very low and unlikely to influence (or explain) our results. Nevertheless, a potential link cannot be excluded, since coronary artery calcification is strongly correlated with all-cause mortality, even if other risk factors are taken into account [30].

The principal limitation of our study is the sample size and power to detect novel associations. Although the effect of 9p21 appears to be shared between coronary artery and aortic calcification, separate mechanisms may still be involved, even if there is weak correlation between calcification in the aorta and coronaries ($r^2 = 0.13$ in our data). Larger studies will be needed to study the genetic basis of calcification in different parts of the vasculature. The exact causal variants that drive the observed associations are as yet unknown, and future fine-mapping studies should shed light on their identity and function. Lastly, the present data are not sufficient to demonstrate the order of events related to vascular calcification, CAD and MI. Future studies are necessary to test the hypothesis if vascular calcification leads to atherosclerosis, or if atherosclerosis ultimately causes calcification of the vessel wall.

In a study comprising 2620 current and former heavy smoking males, our results show that at least three genes that have previously been associated with CAD and MI are possibly involved in arterial calcification. These findings provide a better understanding of the biological mechanisms behind vascular calcification and cardiovascular diseases.

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Disclosures

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.02.039.

References

[1] Arad Y, Goodman KJ, Roth M, Newstein D, Guerci AD. Coronary calcification, coronary disease risk factors, C-reactive protein, and atherosclerotic cardiovascular disease events: the St. Francis Heart Study. J Am Coll Cardiol 2005;46: 158–65.

[2] Wilson PW, Kauppila LI, O'Donnell CJ, et al. Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. Circulation 2001;103:1529–34.

[3] Schunkert H, Konig IR, Kathiresan S, et al. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. Nat Genet 2011;43:333—8.

[4] Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 2010;466:707—13.

[5] Fischer M, Broeckel U, Holmer S, et al. Distinct heritable patterns of angiographic coronary artery disease in families with myocardial infarction. Circulation 2005;111:855—62.

[6] O'Donnell CJ, Chazaro I, Wilson PW, et al. Evidence for heritability of abdominal aortic calcific deposits in the Framingham Heart Study. Circulation 2002;106:337—41.

[7] van Iersel CA, de Koning HJ, Draisma G, et al. Risk-based selection from the general population in a screening trial: selection criteria, recruitment and power for the Dutch-Belgian randomised lung cancer multi-slice CT screening trial (NELSON). Int J Cancer 2007;120:868—74.

[8] van Klaveren RJ, Oudkerk M, Prokop M, et al. Management of lung nodules detected by volume CT scanning. N Engl J Med 2009;361:2221—9.

[9] Jacobs PC, Prokop M, van der Graaf Y, et al. Comparing coronary artery calcium and thoracic aorta calcium for prediction of all-cause mortality and cardiovascular events on low-dose non-gated computed tomography in a high-risk population of heavy smokers. Atherosclerosis 2010;209:455—62.

[10] Isgum I, Prokop M, Niemeijer M, Viergever M, van Ginneken B. Automatic coronary calcium scoring in low-dose chest computed tomography. IEEE Trans Med Imaging 2012. http://dx.doi.org/10.1109/TMI.2012.2216889. Epub ahead of print, Sep 4, 2012.

[11] Isgum I, Rutten A, Prokop M, et al. Automated aortic calcium scoring on low-dose chest computed tomography. Med Phys 2010;37:714—23.

[12] Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte Jr M, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol 1990;15:827—32.

[13] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

[14] Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559—75.

[15] Browning BL, Browning SR. A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals. Am J Hum Genet 2009;84:210—23.

[16] Frazer KA, Ballinger DG, Cox DR, et al. A second generation human haplotype map of over 3.1 million SNPs. Nature 2007;449:851—61.

[17] Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006;38:904—9.

[18] Devlin B, Roeder K. Genomic control for association studies. Biometrics 1999;55:997—1004.

[19] O'Donnell CJ, Kavousi M, Smith AV, et al. Genome-wide association study for coronary artery calcification with follow-up in myocardial infarction. Circulation 2011;124:2855—64.

[20] Holdt LM, Teupser D. Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations. Arterioscler Thromb Vasc Biol 2012;32:196—206.

[21] Congrains A, Kamide K, Oguro R, et al. Genetic variants at the 9p21 locus contribute to atherosclerosis through modulation of ANRIL and CDKN2A/B. Atherosclerosis 2011;220:449—55.

[22] Visel A, Zhu Y, May D, et al. Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice. Nature 2010;464:409—12.

[23] Motterle A, Pu X, Wood H, et al. Functional analyses of coronary artery disease associated variation on chromosome 9p21 in vascular smooth muscle cells. Hum Mol Genet 2012;21:4021—9.

[24] Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248—9.

[25] Wang L, Zheng J, Bai X, et al. ADAMTS-7 mediates vascular smooth muscle cell migration and neointima formation in balloon-injured rat arteries. Circ Res 2009;104:688—98.

[26] Genome-wide meta-analyses identify multiple loci associated with smoking behavior. Nat Genet 2010;42:441—7.

[27] Allen PB, Greenfield AT, Svenningsson P, Haspeslagh DC, Greengard P. Phactrs 1-4: a family of protein phosphatase 1 and actin regulatory proteins. Proc Natl Acad Sci U S A 2004;101:7187—92.

[28] Jarray R, Allain B, Borriello L, et al. Depletion of the novel protein PHACTR-1 from human endothelial cells abolishes tube formation and induces cell death receptor apoptosis. Biochimie 2011;93:1668—75.

[29] Reilly MP, Li M, He J, et al. Identification of ADAMTS7 as a novel locus for coronary atherosclerosis and association of ABO with myocardial infarction in the presence of coronary atherosclerosis: two genome-wide association studies. Lancet 2011;377:383—92.

[30] Nasir K, Rubin J, Blaha MJ, et al. Interplay of coronary artery calcification and traditional risk factors for the prediction of all-cause mortality in asymptomatic individuals. Circ Cardiovasc Imaging 2012;5:467—73.