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Genetic variants in cardiac calcification in Northern Sweden

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Abstract

Extensive coronary calcification without significant stenosis, described as calcific coronary artery disease (CCAD) may cause abnormal myocardial perfusion and hence generalized ischemia. There is a discrepancy in the expression pattern of CCAD compared to the well-known atherosclerotic disease which raises questions about the exact pathophysiology of coronary calcification and whether there is a genetic etiology for it.

In this pilot study we studied 3 candidate genes, ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP1*), ATP Binding Cassette Subfamily C Member 6 (*ABCC6*), and 5'-Nucleotidase Ecto (*NT5E*) involved in pyrophosphate (PP_i) and inorganic phosphate (P_i) metabolism, which may predispose to coronary arterial or valvular calcification. We studied 70 patients with calcific cardiac disease; 65 with CCAD (age 43–83 years) and 5 with calcific aortic valve disease (CAVD) (age 76–82 years).

Five DNA variants potentially affecting protein function were found in 6 patients. One variant is a known disease-causing mutation in the *ABCC6* gene. Our findings support that disturbances in the PP_i and P_i metabolism might influence the development of CCAD and CAVD. However, segregation in the families must first be performed to ascertain any damaging effect of these variants we have found.

We report 4 new genetic variants potentially related to coronary calcification, through the disturbed P_i and PP_i metabolism. The search for direct causative genetic variants in coronary artery and aortic valve calcification must be broadened with other genes particularly those involved with P_i and PP_i metabolism.

Abbreviations: ABCC6 = ATP Binding Cassette Subfamily C Member 6, BGI = Beijing Genetics Institute, CAC = coronary artery calcification, CAVD = calcific aortic valve disease, CCAD = calcific coronary artery disease, ENPP1 = ecto-nucleotide pyrophosphatase/phosphodiesterase, ESP = NHLBI Exome Sequencing Project, ExAC = Exome Aggregation Consortium, NT5E = 5'-Nucleotidase Ecto, PolyPhen2 = Polymorphism Phenotyping v2, SIFT = Sorting Intolerant From Tolerant, SNP = single nucleotide polymorphism.

Keywords: arterial calcification, coronary artery disease, gene

1. Introduction

Chest pain typical/atypical is a frequent symptom in cardiac patients. While the most common cause for chest pain is coronary stenosis, many patients show smooth arteries or only minor irregularities on convention angiography,^[1] suggesting an element of arterial stiffness. Coronary artery calcification (CAC) is a well-recognized cause of arterial stiffness and develops with age, with its extent correlates with the severity of luminal stenosis in symptomatic patients.^[2,3] Low-grade stenosis and extensive calcification in calcific coronary artery disease (CCAD) may also cause limiting symptoms due to

compromised coronary flow reserve causing abnormal myocardial perfusion and hence generalized ischemia.^[4,5] Such discrepancy in the expression pattern of coronary disease raises questions about the exact pathophysiology of coronary stiffness, particularly that caused by calcification, and whether there is a genetic etiology for it.

The potential role of cellular, molecular, and genetic influences in the pathophysiology of arterial calcification have attracted researcher's interest for years, and results showed that genetic determinants of calcification appear to be partially independent of those involved in atherogenesis, accounting for almost 40% of risk variability. This is supported by the significant difference in atheromatous burden in blacks, in the absence of calcification, compared to whites, thus suggesting ethnic influence on the calcification pathology. Two specific chromosomal loci harboring genes have been found associated with peripheral arterial calcification, as well as other polymorphisms including apolipoprotein E, E-selectin, matrix metalloproteinase 3, matrix GLA protein and CC Chemokine receptor 2, but the individual role of each appears to be modest.^[6,7] This suggests that a genetic variant with a potentially stronger association with arterial calcification may yet be found. Mutations in the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP1*) and ATP Binding Cassette Subfamily C Member 6 (*ABCC6*) genes have also been described in generalized arterial calcification in infancy and mutations in the 5'-Nucleotidase Ecto (*NT5E*) gene have been associated with peripheral vascular calcification.^[8] Furthermore, common polymorphisms causing the amino acid changes p.Thr376Ala (c.1126A>G, rs2229523) and p.Met379Thr (c.1136T>C,

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Table 1**Characteristics of CAVD and CCAD patients.**

Disease	Sex	n	Age	BMI	Calcium score	DM, n	HT, n	Cholesterol, n	Hereditary, n	Smoker, n
CAVD	Female	3	78 (76–79)	28.7 ± 1.1	423 ± 374	1	2	3	1	0 (1 former)
CAVD	Male	2	81 (80–82)	28.6 ± 3.5	1574 ± 1245	0	2	2	0	0 (2 former)
CCAD	Female	37	65 (48–82)	27.4 ± 4.8	150 ± 370	8	27	28	27	2 (15 former)
CCAD	Male	28	65 (43–83)	27.5 ± 3.8	313 ± 423	2	18	19	18	3 (17 former)

BMI and calcium score presented as mean ± standard deviation. BMI = body mass index, CAVD = calcific aortic valve disease, CCAD = calcific coronary artery disease, DM = diabetes mellitus, HT = hypertension, n = number.

rs2229524) in the *NT5E* gene have been reported in aortic valve calcification.^[9]

In the present pilot study, we attempted to identify the key genetic variants (in these candidate genes) which may predispose to coronary arterial stiffness, mostly caused by calcification pathology and whether there is any impact of gender on this potential relationship.

2. Patients and methods

We studied 70 patients with calcific cardiac disease; 65 with CCAD (age 43–83 years, 37 female/28 male) and 5 with calcific aortic valve disease (CAVD) (age 76–82 years, 3 female/2 male) (Table 1).

The CCAD patients were collected from the CAC study established in Umeå University, Sweden, in 2008, which involved patients who presented with exertional angina, typical/atypical, and who had a positive exercise ECG, ≥1 mm ST shift at peak exercise, but did not have any evidence for obstructive coronary artery disease on conventional coronary angiography. All CAVD patients had symptomatic severe aortic stenosis diagnosed by Doppler echocardiography, with a mean trans-aortic valve gradient >40 mmHg in the presence of maintained LV size and function, ejection fraction ≥60%. All CAVD patients were planned to have aortic valve surgery which was preceded by conventional coronary angiography which excluded any obstructive disease. Study exclusion criteria were, prior coronary intervention, angioplasty, or bypass graft surgery, heart failure, chronic kidney, thyroid, or parathyroid disease.

The CCAD and CAVD studies were separately approved by The Regional Ethics Review Board in Umeå and the investigations were carried out according to the Declaration of Helsinki of 1975, revised in 2008. All subjects gave informed consent for inclusion before they participated in the study.

In the 2 cohorts, patients were interviewed by a research nurse who explained the study protocol, obtained the informed consent and a detailed account on lifestyle and dietary habits. Information on conventional atherosclerosis risk factors was also obtained, including hypertension, diabetes, dyslipidemia, smoking, obesity, and family history for coronary artery disease according to standard guidelines.^[19] Blood samples were also taken and separated for genetic, biochemical, and metabolomics analysis. The CCAD and CAVD patients underwent coronary calcium scoring investigation using a multidetector CT 64 scanner (MDCT) (GE, Netherlands). The CCAD patients underwent the scan as part of their outpatient visit while the CAVD group had it during the hospital admission for aortic valve replacement surgery. Coronary calcification conventional protocol was used for image acquisition and coronary and valve calcification scores were calculated, off-line, using the Agatston scoring system based on the Hounsfield units.^[10]

The 70 recruited patients were classified into the following subgroups:

Group A: 5 patients with severe CAV stenosis, assessed by Doppler echocardiography.

Group B: 11 patients with severe CCAD (CAC score >400).

Group C: 15 patients with moderate CCAD (CAC score 100–400).

Group D: 39 patients with mild CCAD (CAC score <100).

2.1. DNA extraction

Blood was withdrawn from a venepuncture from the cubital fossa and genomic DNA was extracted from peripheral white blood cells using the DNeasy blood and tissue DNA extraction kit (Qiagen, MD).

2.2. Genetic investigations of CAVD and CCAD patients

Genetic investigations of CAVD and CCAD were undertaken at Beijing Genetics Institute (BGI). DNA samples from patients were sent to BGI and the genes *NT5E*, *ENPP1*, and *ABCC6* (~46 kb) were sequenced by Illumina HiSeq 2000 and bioinformatics analysis was performed to detect genetic variants.

2.3. Target region sequencing performed at BGI

Qualified genomic DNA samples from the patients were randomly fragmented and adapters ligated to both ends of the DNA fragments. The adapter-ligated templates were purified by the Agencourt AMPure SPRI beads and amplified by ligation-mediated PCR (LM-PCR). The amplified fragments were then hybridized to the SureSelect Biotinylated RNA Library (BAITS) for enrichment. Each target region enriched library was loaded on HiSeq2000 platform and high-throughput sequencing was performed for each captured library to ensure that each sample met the desired average sequencing depth. Raw image files were processed by Illumina base-calling Software 1.7 for base calling with default parameters and the sequences of each individual are generated as 90/100 bp pair-end reads.

2.4. Bioinformatics analysis

Bioinformatics analysis was undertaken at BGI. The original image data was transferred into sequence data via base calling and saved as FASTQ files. Before data analysis, the adapter sequence and low-quality reads were removed. Then the Burrows–Wheeler Aligner (BWA) was used to align the clean read to human genome build37 (hg19) references and the output of the alignment was presented in Sequence Alignment/Map (SAM) format. The combined alignment results were compressed to 1 BAM format file.

SOAPsnv was used to assemble a consensus sequence for the genome of a sequenced individual, based on the alignment of the clean reads onto the known reference. The single nucleotide

Table 2

Five potentially damaging genetic variants found in the 3 investigated genes according to frequency and bioinformatically determined effect on protein function.

Gene	Variant type	Transcript	Codon	Patients, %	SweGen, %	ExAC, %	ESP, %	IGSR, %	SIFT	PolyPhen2	MA	Condel
NT5E	MISSENSE	NM_002526:p.Phe421Tyr	tTt/tAt	1.4	–	0,0003461	0,0001538	0,000	0.02*	0.346	2.6*	0.804*
ENPP1	MISSENSE	NM_006208:p.Ala19Ser	Gct/Tct	1.4	–	–	–	–	0.02*	0.001	0	0.430
ABCC6	NONSENSE	NM_0011171:p.Arg1141*	Cga/Tga	2.8	0.0045	0,001395	0,002155	0,001	–	–	–	–
ABCC6	MISSENSE	NM_0011171:p.Pro1257His	cCc/cAc	1.4	0.0005	0,00006729	–	0,000	0.13	0.006	1.79	0.518*
ABCC6	MISSENSE	NM_0011171:p.Arg1357Gln	cGg/cAg	1.4	–	0,0001584	–	–	0.00*	1*	2.89*	0.863*
									<0.05	>0.909	>1,938	>0.469

* Probably damaging.

ABCC6 = ATP Binding Cassette Subfamily C Member 6, Condel = consensus deleteriousness score, ENPP1 = ecto-nucleotide pyrophosphatase/phosphodiesterase, ESP = NHLBI Exome Sequencing Project, ExAC = Exome Aggregation Consortium, IGSR = the 1000 Genomes Project Phase 3, MA = Mutation assessor, NT5E = 5'-Nucleotidase Ecto, PolyPhen2 = Polymorphism Phenotyping v2, SIFT = Sorting Intolerant from Tolerant, SweGen = 1000 Swedish genomes variant frequency dataset.

polymorphisms (SNPs) were then identified on the consensus sequence through the comparison with the reference. SNPs were annotated and categorized according to databases such as dbSNP (v131), the 1000 Genomes Project, and the NHLBI Exome Sequencing Project (GO-ESP). All variants where minimum reads of alteration reached to 4 were excluded.

Sorting Intolerant from Tolerant (SIFT) was used to predict whether an amino acid substitution affects protein function so substitutions could be prioritized for further study. Polymorphism Phenotyping v2 (Polyphen-2) was used to predict possible impact of an amino acid substitution on the structure and function of the proteins. Mutation assessor (MA) was used to identify evolutionary conserved positions that contribute to protein function and estimate the functional impact of a missense variant. Pair-end reads for gap alignment were used to detect small insertions and deletions (InDel) with the software GATK. InDels in target regions were further annotated using BGI in-house software AnnoDB to database refGene. The InDels were also categorized according to databases such as dbSNP (v131), The 1000 Genomes Project and The GO-ESP.

2.5. Manual assessments of gene variants

All genetic variants found in the genes *NT5E*, *ENPP1*, and *ABCC6* by BGI were manually controlled using Integrative Genomics Viewer (IGV v2.3, Broad Institute, MA).^[11,12] The Bam files were used to visualize aligned reads in IGV.

2.6. Frequency analysis of genetic variants

As an additional step, in order to estimate the potential pathogenicity of genetic variants found in the study with high or moderate impact on protein function, their relative frequencies

in the normal population were assessed. We used genetic data from the SweGen Project for 1000 healthy individuals from Sweden.^[13] We also used data from 3 published genome sequencing projects, the 1000 Genomes Project Phase 3 (IGSR, <http://www.internationalgenome.org/>), the NHLBI GO Exome Sequencing Project (ESP, <http://evs.gs.washington.edu/EVS/>) and the Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org/>), including a total of 72359 individual samples. Frequencies above 1 percent were considered benign polymorphisms.

3. Results

The coronary calcium score was higher in male compared to female patients, $P = .016$ (Table 1). The reason for this difference was that females of age 50 to 70 years showed mostly zero calcium score (Fig. 1). The calcium score was higher in men below the age of 70 ($P = .011$) but not in patients over the age 70 ($P = .44$). The calcium score also correlated with age only in males, $P = 0.04$. Differences between genders were compared using independent-samples Mann–Whitney U test. Correlation was calculated using Pearson Correlation (2-tailed).

After bioinformatic filtering, 5 DNA variants were found in 6 patients, the analysis of which showed that they could be considered to potentially affect protein function (Tables 2 and 3).

3.1. The Phe421Tyr variation in the NT5E gene

Amino acid (aa) 421 in the 5-nucleotidase (5NTD) protein lies in the C-terminal 5'-nucleotidase domain. The variant affects an 8 aa long beta strand. Both phenylalanine and tyrosine are aromatic aa with hydrophobic side chains. The only difference is an additional hydroxyl group in tyrosine. Despite the small difference between them, the aa substitution is known to affect

Table 3

Characteristics of the 6 patients with genetic variations possibly associated with calcification.

Study ID	Gene	Gene variant	Age	Sex	BMI	Calcium score	DM	HT	Colesterol	Hereditary	Smoker
CAVD	<i>NT5E</i>	NM_002526:p.Phe421Tyr	80	M	26.1	694	border	yes	yes	no	former
CCAD-1	<i>ENPP1</i>	NM_006208:p.Ala19Ser	67	F	26	0	no	no	no	no	no
CCAD-2	<i>ABCC6</i>	NM_0011171:p.Arg1141*	53	F	28.3	0	no	yes	yes	yes	former
CCAD-3	<i>ABCC6</i>	NM_0011171:p.Arg1141*	63	F	32.6	0	no	yes	yes	yes	no
CCAD-4	<i>ABCC6</i>	NM_0011171:p.Pro1257His	73	M	30.9	116	no	yes	no	no	former
CCAD-5	<i>ABCC6</i>	NM_0011171:p.Arg1357Gln	63	M	25.8	367	no	yes	yes	no	yes

* Causing a truncated protein.

ABCC6 = ATP Binding Cassette Subfamily C Member 6, BMI = body mass index, CAVD = calcific aortic valve disease, CCAD = calcific coronary artery disease, DM = diabetes mellitus, ENPP1 = ecto-nucleotide pyrophosphatase/phosphodiesterase, HT = hypertension, NT5E = 5'-Nucleotidase Ecto.

the function of other proteins.^[14–16] This variant in the *NT5E* gene has not been reported before.

3.2. The Ala19Ser variation in the *ENPP1* gene

Aa 19 in the Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (NPP1) protein lies in a domain with unknown function. Alanine is an aliphatic aa with a hydrophobic side chain. Serine has a polar uncharged side chain. This variant in the *ENPP1* gene has not been previously reported but the aa substitution is known to affect the function of other proteins.^[17,18]

3.3. The Arg1141* variation in the *ABCC6* gene

Nonsense variant at aa position 1141 in *ABCC6* produce a stop codon in the Multidrug resistance-associated protein 6 causing a truncated protein with a loss of 24% of the protein. Part of the transmembrane domain (aa 946–1251) and the whole of the triphosphate hydrolase and ATPase domains are lost. This variant in the *ABCC6* gene has previously been shown to associate with the risk of coronary artery disease and pseudoxanthoma elasticum.^[19–21]

3.4. The Pro1257His variation in the *ABCC6* gene

Aa 1257 in the Multidrug resistance-associated protein 6 lies in the beginning of the triphosphate hydrolase and ATPase domain. Proline is a hydrophobic aa and histidine is a polar aa. This variant in the *ABCC6* gene has not been reported but the aa substitution is known to affect the function of other proteins.^[22,23]

3.5. The Arg1357Gln variation in the *ABCC6* gene

Aa 1357 in the Multidrug resistance-associated protein 6 lies in the triphosphate hydrolase and ATPase domain. Arginine is a charged aa and glutamine is a polar aa. This variant in the *ABCC6* gene is also not reported but the aa substitution is known to affect the function of other proteins.^[24,25]

Of note, the frequencies of all variants were less than 1% in the 4 population databases we used, thus suggesting rare variants with possible effect on protein function.

The 2 previously published *NT5E* polymorphisms studied in aortic valve calcification were also found in our patients. c.1126A>G was found in 91.5% (n=65) of the patients and c.1136T>C was found in 16.9% (n=12) of the patients. All patients with c.1136T>C also carried c.1126A>G. The allele frequencies of SNP c.1126 were A=0.338 and G=0.662 in patients and A=0.345 and G=0.665 in the Swedish SweGen Project cohort we used in this study as controls. There was no significant difference in frequencies between patients and controls ($P=.866$). The allele frequencies of SNP c.1136 were T=0.915 and C=0.085 in patients and T=0.954 and C=0.047 in the Swedish SweGen Project cohort. There was significant difference in frequencies between patients and Swedish controls ($P=.043$) (Fig. 2), but no difference between patients and the European (non-Finnish) controls (ExAC) (c.1126 $P=.62$, c.1136 $P=.28$). Chi-squared test was used to determine if there was a significant difference between SNP frequencies in the patients and the control population.

Due to the few findings of possible disease-causing mutations in the CCAD patients we could not determine any relationship between genetics findings and coronary calcium score measured by the Agatston method, neither with conventional risk factors

for atherosclerosis; hypertension, diabetes, hypercholesterolemia, obesity, and smoking.

4. Discussion

4.1. Findings

The aim of this study was to determine genetic factors in arterial stiffness caused by wall calcification based on candidate gene approach. We identified both an already reported mutation associated with coronary artery disease and 4 previously unreported genetic variants possibly affecting protein function. We were also able to confirm a higher frequency of a polymorphism in Swedish patients, previously reported in Polish patients. The identified genes were as follows.

4.1.1. The Arg1141* variant in the *ABCC6* gene. This known mutation was found in 2 of our patients who were females and with several risk factors for coronary artery disease, including positive family history. They were below the expected age for conventional measurable calcification and had a CAC score of zero. Heterozygosity of this mutation has been shown to be associated with coronary artery disease in 2 previous studies, 1 in premature coronary heart disease patients. In these studies, conventional diagnostic criteria were used but the presence of coronary calcification was not studied.^[19] This specific mutation is associated with a wide range of phenotypes including pseudoxanthoma elasticum and is a known risk factor for coronary heart disease, possibly calcification rather than typical plaque formation.

4.1.2. The *NT5E*, *ENPP1*, and *ABCC6* genes. In these 3 genes we found 4 previously unpublished missense variants. They were found in 4 of our patients, 3 men and 1 woman with a wide age range. The men had moderately raised coronary calcium scores, but interestingly the woman had zero calcium, as in the case with the 2 female *ABCC6* Arg1141* carriers. The clinical relevance of these variants is difficult to assess since they were not previously reported, but bioinformatics analysis supports their role as possibly affecting protein function.

4.1.3. *NT5E* polymorphisms in aortic valve calcification. The 2 previously published *NT5E* polymorphisms studied in aortic valve calcification were also found in our patients. The c.1136T>C variant differed significantly between patients and Swedish controls but not between patients and the European (non-Finnish) controls. We are inclined to take this possible association as a positive finding which warrants further investigations.

4.2. Data interpretation

The 3 identified genes were previously found to be related to peripheral arterial calcification and atherosclerotic coronary artery disease.^[7] Indeed, our objective was to find potential links between these genes and coronary artery stiffness caused mostly by calcification. In most of the patients we failed to find any possible damaging variants and our results did not demonstrate a clear association between polymorphisms in the identified genes and coronary calcification as currently assessed by MDCT protocols. Also, it seems there is a gender impact particularly females of age 50 to 70 years who were shown to consistently have zero calcium score according to the conventional Agatston method (Fig. 2). This potential associa-

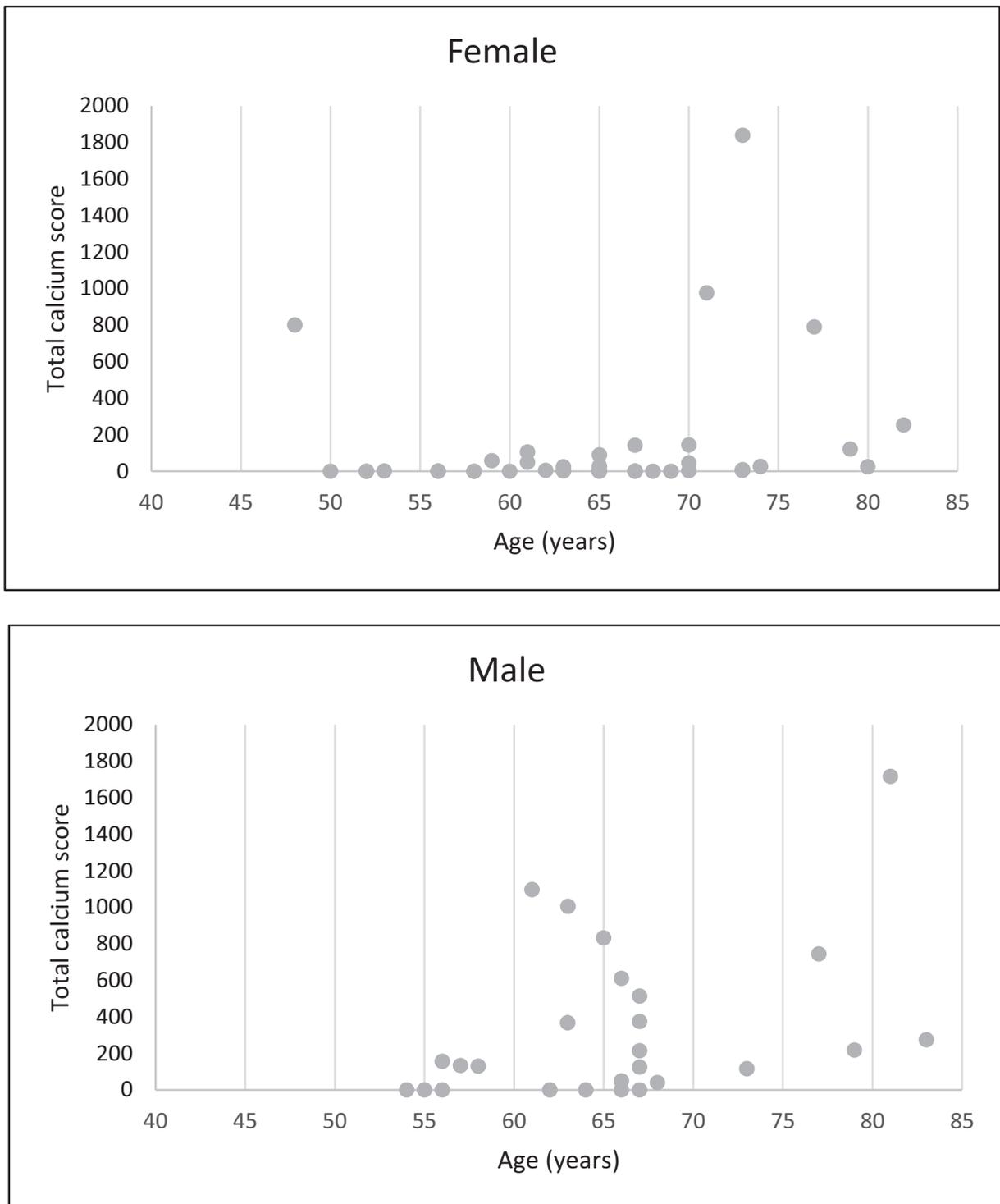


Figure 1. Total calcium score in patients according to gender and age. Women between 50 and 70 years of age have low calcium score compared to men.

tion might be explained on the basis of coronary stiffness caused by a form of microcalcification which cannot be measured using conventional MDCT protocol and applying Agatston scoring system.

The 3 genes investigated all code for proteins involved in pyrophosphate (PP_i) and inorganic phosphate (P_i) metabolism. These proteins are part of a network that balance promotion and

suppression of arterial calcification.^[26] Our findings of 1 known and 4 previously unknown variants of these genes in our patients support that disturbances in the PP_i and P_i metabolism might influence the development of arterial stiffness caused by calcification as is the case with CAVD. However, segregation in the families must first be performed to ascertain any damaging effect of these variants we have found.

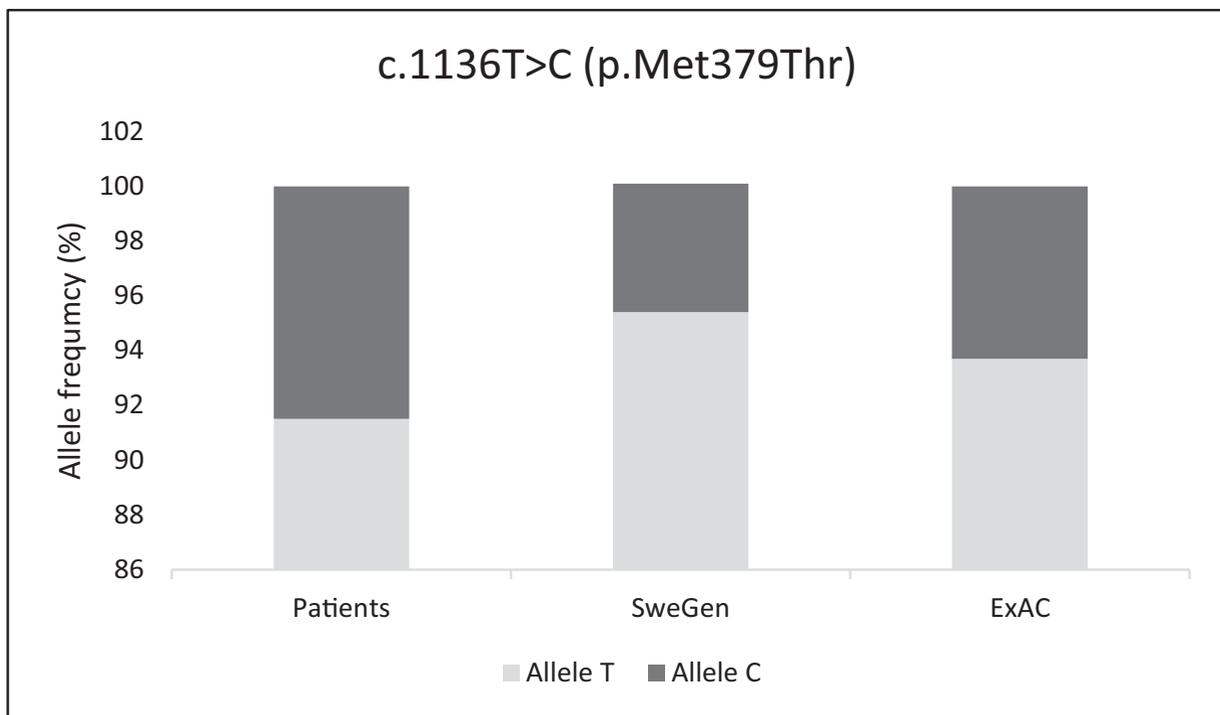


Figure 2. Frequency of allele T and C in c.1136T>C. Swedish CAC patients were compared to Swedish population controls (SweGen, 1000 Swedish genomes variant frequency dataset) and European (non-finnish) controls (ExAC, Exome Aggregation Consortium). The frequency of the C allele was significantly higher in the patients compared to Swedish controls ($P = .043$) but not compared to the European controls ($P = .28$). Chi-squared test was used to test significance.

4.3. Study limitations

Our study has clear limitations. First, we studied a small cohort of patients of various CAC severities and some with no measurable calcium score according to conventional protocols, in an attempt to relate them to the genetic findings. Second, although we suggested microcalcification as a potential explanation for the lack of relationship with genetic findings, we did not have any means for confirming such suggestion, the only currently available method to confirm microcalcification is histopathological investigations. Third, some of the variants we identified were previously found in peripheral calcification and coronary calcification was not studied in those families. Finally, long term follow up might shed light on the clinical relevance of our findings.

5. Conclusion

We hereby report 4 new genetic variants potentially related to coronary stiffness caused mainly by calcification. The search for direct causative genetic variants in coronary artery and aortic valve calcification must be broadened with other genes particularly those involved with P_i and PP_i metabolism.

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