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COL4A1 Is Associated With Arterial Stiffness By Genome Wide Association Scan

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Abstract

Background—Pulse wave velocity (PWV), a non-invasive index of central arterial stiffness, is a potent predictor of cardiovascular mortality and morbidity. Heritability and linkage studies have pointed toward a genetic component affecting PWV. We conducted a genome-wide association study to identify single nucleotide polymorphisms (SNPs) associated with PWV.

Methods and Results—The study cohort included participants from the SardiNIA study for whom PWV measures were available. Genotyping was performed in 4,221 individuals, using either the Affymetrix 500K or the Affymetrix 10K mapping array sets (with imputation of the missing genotypes). Associations with PWV were evaluated using an additive genetic model that included age, age^2 and sex as covariates. The findings were tested for replication in an independent internal

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Sardinian cohort of 1,828 individuals, using a custom-chip designed to include the top 43 non-redundant SNPs associated with PWV. Of the loci that were tested for association with PWV, the nonsynonymous SNP rs3742207 in the *COL4A1* gene on chromosome 13 and SNP rs1495448 in the *MAGI1* gene on chromosome 3 were successfully replicated ($p=7.08\times10^{-7}$ and $p=1.06\times10^{-5}$ respectively for the combined analyses). The association between rs3742207 and PWV was also successfully replicated (p=0.02) in an independent population, the Old Order Amish, leading to an overall $p=5.16\times10^{-8}$.

Conclusions—A genome-wide association study identified a SNP in the *COL4A1* gene that was significantly associated with PWV in two populations. Collagen type 4 is the major structural component of basement membranes, suggesting that previously unrecognized cell-matrix interactions may exert an important role in regulating arterial stiffness.

Keywords

Arterial Stiffness; Pulse Wave Velocity; Genome-Wide Association Study; *COL4A1*; *MAGI1*; genetics

Introduction

Central arterial stiffening is one of the hallmarks of arterial aging. Carotid-femoral pulse wave velocity (PWV) is the preferred non-invasive measure of central arterial stiffness[1]. PWV is increased in patients with cardiovascular conditions such as hypertension, diabetes, metabolic syndrome, and atherosclerosis2. Furthermore, PWV is an independent predictor of hypertension[3] and of coronary heart disease and stroke in healthy subjects[4] and an independent predictor of mortality in the general population[5], in hypertensive subjects[6], in older community dwelling individuals[7], and in patients with end-stage renal disease[8]. Thus, understanding the mechanisms of central arterial stiffening may lead to effective interventions that could improve PWV and favorably impact cardiovascular morbidity and mortality.

Increasing central arterial stiffness has traditionally been thought to result from the breakdown of elastin in central arterial walls, due to the repeated cycles of distension and recoil of the central aorta, and from the deposition and cross-linking of collagen. There is increasing recognition that arterial stiffening is influenced by lifestyle (e.g. dietary salt consumption, exercise), and is regulated by several signaling pathways (e.g. nitric oxide)[2]. Furthermore, gene expression studies9, heritability[10],11 and linkage[11] analyses, and a genome wide association study (GWAS) performed in 644 subjects from the Framingham Heart Study[12] are all consistent with the likely involvement of genetic factors in modulating the variability in PWV.

To look for these factors, we performed a GWAS in a large founder population (see supplementary materials) from Sardinia. Furthermore, to confirm the validity of our findings we tested the association of the detected loci with PWV both in a second Sardinian cohort and in a separate founder population, the Old Older Amish.

Methods

Study Sample

The SardiNIA study recruited 6,148 men and women aged 14–102 years (62.5% of the eligible population)10, from a cluster of four towns in the Lanusei valley on the island of Sardinia. The cohort includes 34,469 relative pairs, 4,933 sibling pairs, 180 half-sibling pairs, 4,014 first cousins, 4,256 parent–child pairs, 675 grandparent–grandchild pairs, and 6,400 avuncular pairs in addition to other more distant relatives. Most subjects (95%) had all 4 grand-parents born in Sardinia10, and environmental factors have remained relatively homogeneous. To achieve

the accrual goal for the study, the project was advertised through provincial, religious, and municipal authorities; in local television, newspaper, and radio messages; through local physicians and by mailings and phone calls. All subjects underwent extensive phenotyping, which included assessment of traditional cardiovascular risk factors (e.g. blood pressure, cholesterol fractions, etc.) with standard methodologies, as well as the assessment of arterial stiffness by PWV. Blood draws yielded lymphocytes for subsequent DNA extraction. All subjects provided a written informed consent for participation in the study that was approved by both the Sardinian and the National Institute on Aging's Institutional Review Boards.

PWV measurement

PWV was measured in triplicate using nondirectional transcutaneous Doppler probes (model 810A, 9 to 10-Mhz probes, Parks Medical Electronics, Inc., Aloha, OR) as previously described[13]: A minimum of 10 arterial flow waves from the right common carotid artery and the right femoral artery were simultaneously recorded and averaged. PWV was calculated as distance divided by time: The distance traveled by the flow wave was measured with an external tape measure over the body surface, and was calculated as the distance between the manubrium and the femoral sampling site, minus the distance between the manubrium and the carotid sampling site; The time traveled by the flow wave was measured as the time delay between the feet of simultaneously recorded carotid and femoral arterial waveforms. The waveforms were simultaneously collected by 2 sonographers, one recording at the carotid site and one recording at the femoral site. All data were subsequently analyzed by a single investigator (AS) who was blinded to the clinical characteristics of the subjects. Details of a reproducibility study for PWV are provided in the supplementary material. Forty four individuals did not contribute PWV data to the analysis because of poor quality waveforms (N=21) or because of atrial fibrillation (N=23).

Genotyping

We took advantage of the relatedness among individuals in our sample to substantially reduce study costs[14]. Specifically, because our sample includes many large families, we reasoned that genotyping a relatively small number of markers in all individuals would allow us to identify shared haplotype stretches within each family. We could then genotype a subset of the individuals in each family at higher density to characterize the haplotypes in each stretch and impute missing genotypes in other individuals in the family[14].

Genotyping was performed with the Affymetrix 10K and 500K Mapping Arrays in 3,329 and 1,412 individuals respectively (436 subjects were genotyped with both chips). Individuals typed with the 500K array were specifically selected because they represented the largest families in our sample, not based on their phenotype. Furthermore, for the larger sibships in our cohort, both parents and one child were selected, whereas in the smaller sibships, only the two parents were selected. The lower density arrays were used to genotype everyone else. Except when parents and offsprings were genotyped in the same family, we tried to ensure that individuals genotyped with the high-density array were only distantly related to one another. In the 2,893 individuals typed with only the 10K panel, we took advantage of the overlapping dataset and of the relatedness of the population to estimate the missing genotypes based on stretches of shared haplotypes, using a modified Lander-Green algorithm.[15, 16] This approach for estimating missing genotypes is implemented in MERLIN (http://www.sph.umich.edu/csg/abecasis/MERLIN/) and is described in detail elsewhere [16, 17] and in the supplementary materials.

Prior the imputation process, low quality markers that could affect the accuracy of dosage estimates were removed. In particular, markers that met any of the following criteria were discarded: call rate ≤ 90%, minor allele frequency ≤ 5%, excess of Mendelian inconsistencies

or departure from Hardy– Weinberg equilibrium. From the 10K and the 500K chips we were able to analyze 7,407 and 356,359 markers respectively, resulting in a total of 362,129 markers after accounting for overlapping polymorphisms[18]. The genotype completeness rates exceeded 98%.

After completing this initial phase, we devised a custom chip to test for internal replication of our major findings and to eliminate possible genotyping errors. This chip consisted of 11,617 SNPs and included, for each of the quantitative traits in the SardiNIA study[10], the top SNPs that were associated with these traits in GWAS. Forty-three unique SNPs associated with PWV were included on this chip. This custom chip was used to type the remaining 1,857 subjects recruited into the SardiNIA study who had not been typed with the 500K or 10K chips (denoted as SardiNIA stage 2). These individuals were not related to the individuals typed with the 500K chip (kinship coefficient =0) (see supplementary materials), and thus were a suitable cohort to serve as an internal replication sample. We considered a SNP to be internally replicated if the direction of effect was in the same direction as the initial study with $p < 0.05$.

Statistical analyses

To ensure adequate control of type I error rates, an inverse normal transformation was applied to PWV prior to analysis, to reduce the impact of outliers and minimize deviations from normality (Figure S1). This transformation involves ranking all available PWV values, transforming these ranks into quantiles and finally converting the resulting quantiles into normal deviates. To perform the genome wide association analysis, a simple regression model was fitted and a variance component approach that modeled background polygenic effects was used to account for correlation between different observed phenotypes within each family17. The association analysis was performed using an additive genetic model, and was adjusted for age, age^2 and sex. An initial Q/Q plot suggested that the genomic control parameter was inflated (lambda = 1.14), likely reflecting residual relatedness in this founder population. Therefore, the p-values were adjusted according to the genomic control method[19] (Figure S2). The p-values derived from the initial GWAS and from the association tests on the individuals genotyped with the custom chip were then combined using the z-scores meta-analysis methods, and taking into account the number of subjects analyzed in each set and the direction and magnitude of the estimated effect.

External replication

The top findings from our study were tested in 813 subjects from a genetically distant second founder population, the Old Order Amish of Lancaster, PA. These included healthy Amish subjects enrolled in two studies, the Heredity and Phenotype Intervention (HAPI) Heart Study and the Amish Longevity Study (ALS). The HAPI Heart Study was initiated in 2002 to measure the cardiovascular response to 4 short-term interventions affecting cardiovascular risk factors and to identify the genetic and environmental determinants of these responses.20 The ALS was initiated in 2000 to identify the genetic factors associated with living to an old age, and recruited Amish individuals living to age 92 years or older, their offspring, and the offspring spouses21. PWV was assessed with the Complior®SP device (Artech Medical, Pantin, France) before the interventions were administered, and genotyping was performed with an Affymetrix 500K chip. GWAS in the Old Order Amish were adjusted for family structure, and the data transformation and statistical analyses were performed in an analogous manner to those in the SardiNIA study.

Results

After excluding subjects with atrial fibrillation or poor quality PWV tracings, a total of 4,221 and 1,828 Sardinians were considered for the initial GWA analysis and for the internal

replication (SardiNIA stage 2), respectively. The demographic and clinical characteristics of the study cohort are shown in Table 1. The mean age was 43.7±17.6 years (range 14–102 years); 58% were women, 20% reported a history of smoking, 29% were hypertensive, 5% were diabetics, and only 1% reported a clinical history of myocardial infarction. As expected[2], PWV increased with advancing age in a quadratic fashion (Figure 1).

We first conducted GWAS to survey the genome for common variants associated with PWV. Figure 2 graphically summarizes the associations of PWV with the >329,129 SNPs with a minor allele frequency >5% that passed quality control checks and transmission disequilibrium testing. The top 100 hits are shown in Table S1. Promising findings were noted on chromosomes 1, 2, 4, 12–14, 17, 20, 21, encompassing 18 SNPs with $p<2\times10^{-5}$.

To further evaluate these initial results, a secondary custom chip from Affymetrix was devised based on the top findings from the GWAS. For PWV, the top 85 SNPs were considered; of these, 43 were not in strong linkage disequilibrium and were included in the custom chip. The 1,828 individuals typed with this custom chip were genetically independent from those genotyped in the initial analysis. Thus, the custom chip helped validate the initial findings, and provided an "internal" replication within the Sardinian cohort. Of the 43 SNPs that were included in the custom designed chip, two SNPs, rs3742207 and rs1495448 in the *COL4A1* and *MAGI1* genes respectively, were significantly associated with PWV with the same directionality of effect as in the GWAS (Table 2). Furthermore, when the results of the initial GWAS study group and the Stage 2 study group were combined in a meta-analysis (Table 2), the association of the C allele of rs3742207 with increased PWV was strengthened ($p=7.08\times10^{-7}$), whereas the association of the T allele of rs1495448 with increased PWV was weakened ($p=1.07\times10^{-5}$).

We repeated the association analyses using models that adjusted for mean arterial pressure, creatinine and the use of blood-pressure lowering medications, which are important covariates of arterial stiffness, in addition to age, age^2 and sex which were adjusted for in the base models. The association of rs3742207 with PWV was slightly strengthened, whereby the p-value decreased from 5.94×10^{-5} to 1.78×10^{-5}. Next, we excluded subjects on antihypertensive medications (N=544) and subjects on dialysis (N=10), and repeated the analyses adjusting for age, age^2, sex, mean arterial pressure and creatinine. The p-value for the association of rs3742207 with PWV was 2.48×10^{-5}. In this last model, the p-value was 3.19×10^{-5} when diabetic individuals (N=50) were excluded. However, the genomic control parameters for these 3 additional models were higher than the one for the base model (lambda =1.16, 1.17, 1.17 respectively, compared to 1.14 of the initial model). Conversely, the association of PWV with rs1495448 (in the MAGI1 gene) was weakened when the analyses were repeated using these three sets of additional adjustments (p=0.0014, p=0.0038, p=0.0081 respectively).

The associations of the two loci rs3742207 and rs1495448 with PWV were further evaluated in the Amish population, a genetically distant founder population of European ancestry. The HAPI Heart Study and the ALS study participants underwent both assessment of PWV and genotyping using the Affymetrix 500K chip. We confirmed that allele C of the SNP rs3742207 (the more frequent allele in the Amish population) was associated with PWV (p=0.02) with a comparable effect size (Table 2A), whereas rs1495448 was not (p=0.49, Table 2B). Combining SardiNIA, SardiNIA stage 2, and the Old Order Amish yielded an overall $p=5.16\times10^{-8}$ for the association between rs3742207 and PWV.

The heritability of PWV in the SardiNIA study is 0.226 (adjusted for age, sex and age by sex interaction[10]. The proportion of variance in PWV (which is equivalent to the proportion of the heritable fraction) that is explained by rs3742207 is 0.87%. The overall effect size, is 18.9 cm/

s (calculated as a weighted average of the effect observed in each of the 3 study cohorts, where the weights correspond to those used in the meta-analysis).

The replicated SNP rs3742207 is a common nonsynonymous coding polymorphism located in exon 45 of the *Col4A1* gene. This polymorphism involves a substitution of adenine by cytosine resulting in an amino acid change from Glycine to Histidine at position 1334, which is located in a central region of the protein that consists of multiple triple-helix repeat domains. Nonetheless, further studies are necessary to determine whether the true causal variant is this SNP or another one, which, according to linkage disequilibrium structure (Figure 3)[12, 22, 23], appears to lie in exon 45 or nearby.

Discussion

We conducted a GWAS in the SardiNIA cohort, and found that SNP rs3742207 in the *COL4A1* gene was significantly associated with PWV. Furthermore, this locus was successfully replicated both in an independent sample within the SardiNIA cohort, and in the Old Order Amish population, an external genetically distant founder population of European ancestry, suggesting that this *COL4A1* variant may have an effect in other populations.

Collagen Type 4

There are 6 different types of type 4 Collagen alpha chains (α1–6). Each one is encoded by a different gene and comprises repeating triple-helical domains with a characteristic G-X-Y motif interposed between the amino terminus and a globular C-terminus. These alpha chains assemble into triple helices to form type 4 Collagen. The Collagen type 4 α1 molecule consists of twenty triple helical repeats G-X-Y(X), where the first position is always a Glycine residue. Mutations in this Glycine residue cause Mendelian disorders characterized by small vessel[24], or small and large vessels25 angiopathy.

Type 4 Collagen had not previously been considered to be involved in regulating arterial stiffness. Unlike Collagen types 1 and 3, which are constituents of the extracellular matrix that are found in the medial layer of the arterial walls where they impart the tensile strength to the arteries, type 4 Collagen is a structural component of basement membranes. At the present time there is no functional evidence implicating *COL4A1* as a determinant of PWV. Nonetheless a speculative discussion of putative mechanisms though which *COL4A1* may influence arterial stiffness is provided in the supplementary materials.

Studies of the Genetics of Arterial Stiffness

Heritability studies have consistently concluded that a genetic component likely underlies the variance in arterial stiffness[26–28]. Similarly, results of linkage studies for non-invasive indices of arterial stiffness[11, 27, 28] suggested an underlying genetic component, even though the findings among these studies did not necessarily overlap.

Previous association studies that examined the genetic underpinnings of arterial stiffness focused mostly on polymorphisms in candidate genes that are believed to be involved in regulating arterial structure and/or function, such as nitric oxide synthase,[29] angiotensin II type 1 receptor,[30] collagen 1,[26] G-protein β-3 subunit,[31] β-adrenergic receptors,[32] fibrillin 1,[33] and C-reactive protein.[34] These candidate gene studies, which were conducted in single populations, did not attempt replication in other populations, and often yielded discrepant results[27, 29].

The first GWAS evaluating SNPs associated with arterial stiffness was performed in approximately 644 participants in the Framingham Heart Study, using a 100K Affymetrix GeneChip array[11]. None of the associations with the various markers of arterial stiffness

reached genome-wide significance in that study. The 100K chip that was used did not include rs3742207 or any neighboring SNP in the same LD block (Supplementary Figure 3). It did include 7 other SNPs in the *COL4A1* gene region, 3 of which were associated ($0.01<p<0.05$) with PWV in age- and sex-adjusted analyses (available at http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=search&db=gap&term=carotid-femoral%20pulse%20wave%20velocity&doptcmdl=SAnalyses). Of note, some of these markers were also associated with PWV in SardiNIA (Figure 3A) but with higher p-values than SNP rs3742207.

The GWAS in the SardiNIA study was performed in a larger cohort and with a much denser SNP map, and showed, for the first time, a link between a polymorphism in the Collagen Type 4 α1 gene and arterial stiffness. Each copy of the minor allele (C) of rs3742207 is associated with a 21 cm/s higher PWV, such that homozygotes for this minor allele have an approximately 42 cm/s (6.3%) higher PWV than homozygotes for the major allele, an effect size comparable to those reported in GWAS of other traits, including ones recently studied in the Sardinian cohort.[18, 35, 36] Importantly, in a cohort of 1,678 Danes aged 40–70 years, a PWV increase of 34 cm/s was associated with a 2.9% increase in age- and sex-adjusted cardiovascular mortality[5]. Interestingly, rs3742207 was recently found to be associated with the prevalence of myocardial infarction in Japanese individuals[37].

Limitations

This study was conducted primarily in a founder population in SardiNIA, so caution should be exercised in extending these results to other populations. However, the values and distributions of PWV in our study did not significantly differ from those obtained in other out-bred populations. Furthermore, and in contrast to the micro-isolates where the gene pool is restricted to a few variants, the number of founders of the current day Sardinian population is large enough to encompass most of the existing alleles in the European population, although with some differences in frequency. Importantly, recent studies from the SardiNIA project[18, 35, 36] have demonstrated that findings in SardiNIA are reproducible in other populations; and specifically in this study, we were able to replicate the association of our top finding in an independent and genetically distant founder population of European ancestry. Nonetheless, additional studies in populations of different ethnic origin are needed to further replicate and extend our finding.

Conclusions

Using GWAS, we found that a SNP in the *COL4A1* gene is strongly associated with PWV, an established independent predictor of adverse cardiovascular outcomes. Collagen type 4 is the major structural component of basement membranes, suggesting that previously unrecognized cell-matrix interactions may exert an important role in regulating arterial stiffness. Further work is needed to elucidate these mechanisms, and this could potentially lead to the development of novel interventions aimed at delaying or preventing the risks associated with accelerated arterial stiffening. This would help fulfill, in part, the high expectations for breakthroughs in basic science and in clinical medicine that are engendered by modern era genetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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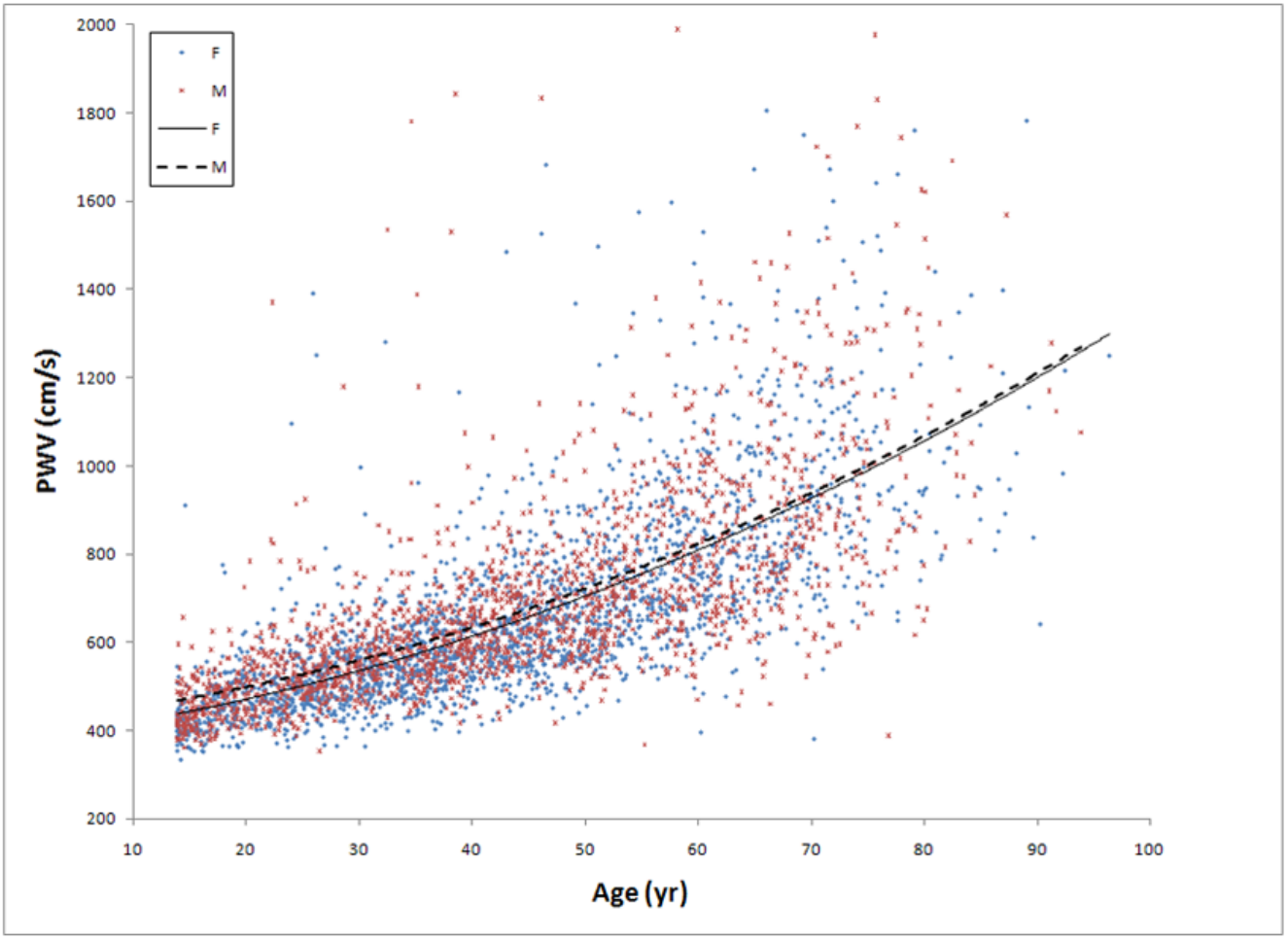

Figure 1.
The relationship of pulse wave velocity (PWV) and age in men and women in the SardiNIA study cohort who were genotyped with the 500K/10K Gene Array Set. The best fit regression curves (quadratic) are shown.

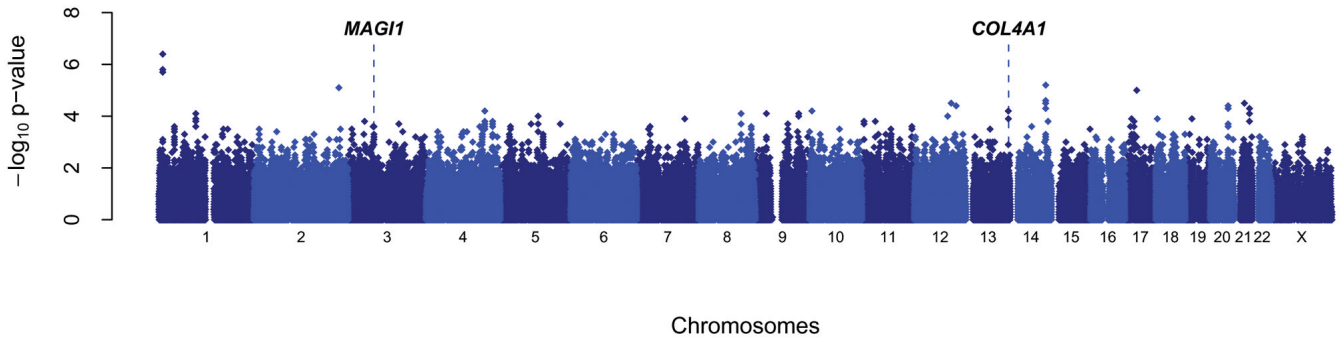

Figure 2.

Summary of genome-wide association studies (GWAS) for pulse wave velocity (PWV) in the SardiNIA study cohort. The −log10 of the p-values for the associations of PWV with 362,169 SNPs that passed quality control filters are plotted according to the positions of these SNPs along the p to q arms of each chromosome (1–22, X). The positions of COL4A1 and MAGI1, the two genes that were replicated in the SardiNIA stage 2 analysis, are highlighted.

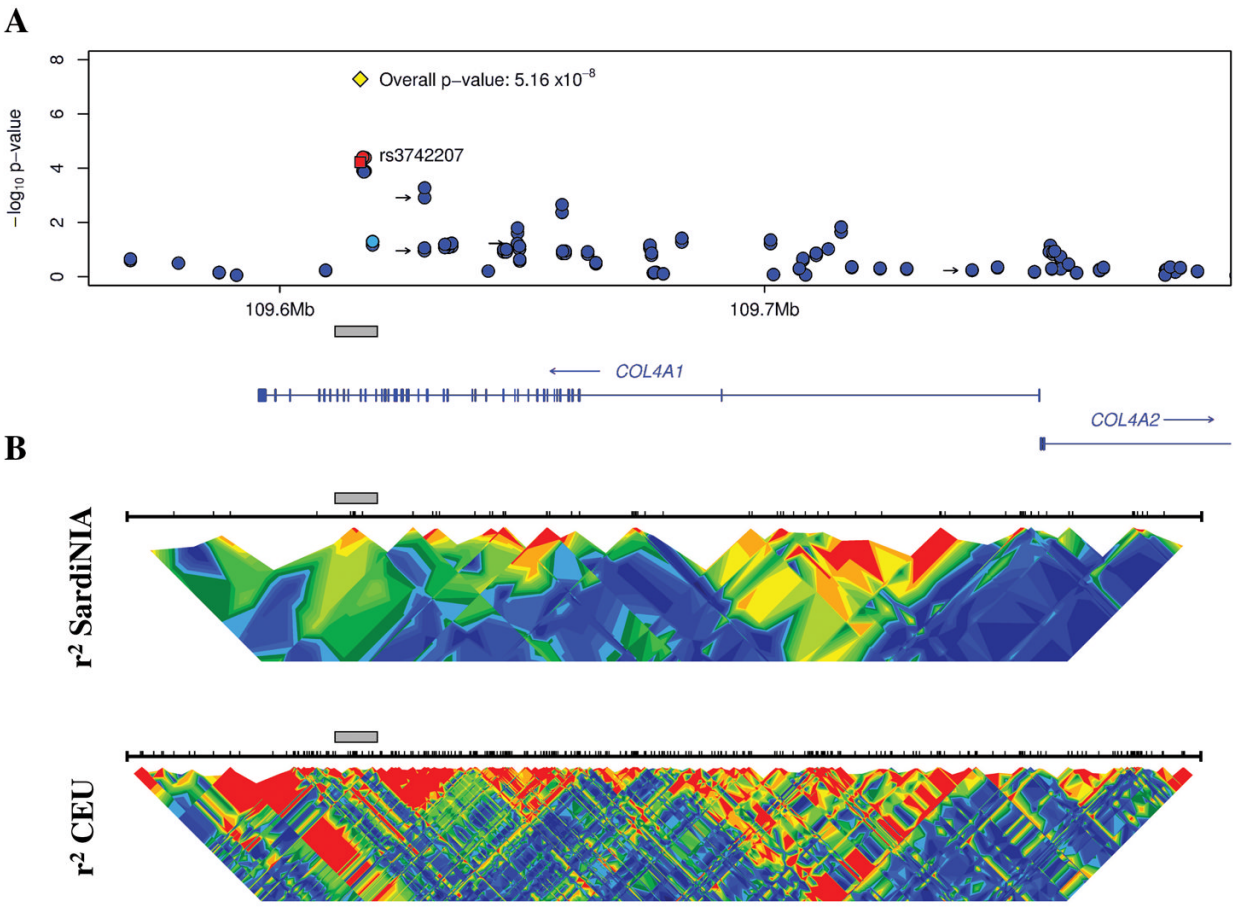

Figure 3.

A) Summary of the association with PWV in the COL4A1 region in the SardiNIA study. Dots represent all the markers in this region that were analyzed, colored according to their linkage disequilibrium (r2) with rs3742207, which is represented by a red square. LD coloring ranges from red (high LD), to green (moderate LD), to blue (low LD). The yellow diamond indicates the overall p-value for rs3742207 obtained from combining the results of SardiNIA, SardiNIA Stage 2 and HAPI Heart. Black arrows denote markers that were also analyzed by the Framingham Heart Study12, and they are from left to right: rs2131939, rs10492497, rs496916 and rs2391823. B) A summary of the patterns of disequilibrium in the SardiNIA study cohort and in the CEU (Utah residents with ancestry from Northern and Western Europe) HapMap population, 22 where r2 values are calculated as described23 and colored as in panel A. The gray bar marks the region of association and facilitates comparisons among the panels.

Table 1

Demographic and clinical characteristics of the SardiNIA cohort (overall, and stratified according to the phase of genotyping), and of the Old Order Amish study cohort

	SardiNIA			Old Order Amish
	Overall	Initial GWAS	Stage 2	
	N=6,049	N=4,221	N=1,828	N=826
Age (years)	43.7±17.6	43.1±17.4	44.1±16.9	46.4±15.1
Sex (male)	2569 (42.5%)	1841 (43.6%)	728 (39.8%)	443 (53.6%)
Smoking (ever)	1237 (20.3%)	850 (20.1%)	387 (21.2%)	211 (25.5)
Height (cm)	159.9±9.1	159.9±9.0	160.1±9.1	167.1±8.8
Weight (kg)	64.9±13.3	64.8±13.3	65.0±13.2	73.8±12.7
BMI (kg/m^2)	25.3±4.7	25.3±4.7	25.3±4.6	26.4±4.3
Systolic Blood Pressure (mmHg)	125±18	125±18	125±18	120±15
Diastolic Blood Pressure (mmHg)	77±11	77±11	77±11	75±9
Heart Rate (beats/min)	67±11	67±11	68±11	64±9
PWV (cm/sec)	670±217	669±209	672±207	547±143
Total cholesterol (mg/dL)	209±43	208±42	210±42	210±47
Triglycerides (mg/dL)	88±68	87±68	89±69	72±44
LDL (mg/dL)	127±36	127±35	127±36	139±42
HDL (mg/dL)	64±15	64±15	64±15	56±15
Fasting glucose (mg/dL)	90±23	90±23	90±25	87±10
Creatinine (mg/dL)	0.8±0.2	0.8±0.2	0.8±0.2	0.8±0.2
Antihypertensive medications	752 (12.4%)	544 (12.2%)	208 (11.4%)	18 (2.2%)

Data are expressed as Mean±SD or N (%)

Table 2

Summary of association results for rs3742207 (A) and rs1495448 (B)

This table summarizes the association results with PWV for the top SNPs rs3742207 (*COL4A1*) and rs1495448 (*MAGI1*). Alleles refer to the forward strand, and are ordered such that the first allele (+) is associated with increased PWV values. Effect sizes and standard errors (in cm/s) are derived from models that analyzed non-normalized PWV, whereas p-values are calculated from models that analyzed the normalized trait as described in the text.

A) *COL4A1*					
Study	**N**	**Allele (+/−)**	**Freq**	**Effect (SE)**	**P-value**
SardiNIA	4,221	C/A	0.44	21.0 (4.7)	5.94×10^{-5}
SardiNIA stage 2	1,828	C/A	0.42	15.2 (5.1)	0.0035
				Combined SardiNIA	7.08×10^{-7}
Old Order Amish	813	C/A	0.55	16.4 (8.2)	0.0218
				Combined SardiNIA and Old Order Amish	5.16×10^{-8}
B) *MAGI1*					
Study	**N**	**Allele (+/−)**	**Freq**	**Effect (SE)**	**P-value**
SardiNIA	4,221	T/G	0.42	8.5 (4.5)	2.76×10^{-4}
SardiNIA stage 2	1,828	T/G	0.46	11.0 (5.0)	0.013
				Combined SardiNIA	1.07×10^{-5}
Old Order Amish	813	T/G	0.50	2.3 (8.7)	0.49
				Combined SardiNIA and Old Order Amish	1.23×10^{-5}