Common Genetic Variation in the 3′-BCL11B Gene Desert Is Associated With Carotid-Femoral Pulse Wave Velocity and Excess Cardiovascular Disease Risk

The AortaGen Consortium

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Background—Carotid-femoral pulse wave velocity (CFPWV) is a heritable measure of aortic stiffness that is strongly associated with increased risk for major cardiovascular disease events.

Methods and Results—We conducted a meta-analysis of genome-wide association data in 9 community-based European ancestry cohorts consisting of 20,634 participants. Results were replicated in 2 additional European ancestry cohorts involving 5306 participants. Based on a preliminary analysis of 6 cohorts, we identified a locus on chromosome 14 in the 3′-BCL11B gene desert that is associated with CFPWV (rs7152623, minor allele frequency=0.42, \( \beta = -0.075 \pm 0.012 \) SD/allele, \( P = 2.8 \times 10^{-10} \); replication \( \beta = -0.086 \pm 0.020 \) SD/allele, \( P = 1.4 \times 10^{-6} \)). Combined results for rs7152623 from 11 cohorts gave \( \beta = -0.076 \pm 0.010 \) SD/allele, \( P = 3.1 \times 10^{-15} \). The association persisted when adjusted for mean arterial pressure (\( \beta = -0.060 \pm 0.009 \) SD/allele, \( P = 1.0 \times 10^{-11} \)). Results were consistent in younger (\(<55\) years, 6 cohorts, \( n = 13,914 \), \( \beta = -0.081 \pm 0.014 \) SD/allele, \( P = 2.3 \times 10^{-9} \)) and older (9 cohorts, \( n = 12,026 \), \( \beta = -0.061 \pm 0.014 \) SD/allele, \( P = 9.4 \times 10^{-6} \)) participants. In separate meta-analyses, the locus was associated with increased risk for coronary artery disease (hazard ratio=1.05; confidence interval=1.02–1.08; \( P = 0.0013 \)) and heart failure (hazard ratio=1.10, CI=1.03–1.16, \( P = 0.004 \)).

Conclusions—Common genetic variation in a locus in the BCL11B gene desert that is thought to harbor 1 or more gene enhancers is associated with higher CFPWV and increased risk for cardiovascular disease. Elucidation of the role this novel locus plays in aortic stiffness may facilitate development of therapeutic interventions that limit aortic stiffening and related cardiovascular disease events. (Circ Cardiovasc Genet. 2012;5:81-90.)

Key Words: aorta ■ arterial stiffness ■ pulse wave velocity ■ genetics ■ cardiovascular disease
Several recent studies have demonstrated that carotid-femoral pulse wave velocity (CFPWV), an important indicator of stiffness of the wall of the thoracic and abdominal aorta, is associated with increased risk for major cardiovascular disease (CVD) events. Various risk factors for abnormal CFPWV have been identified, including standard CVD risk factors such as age, glucose intolerance, lipid disorders, and hypertension. In addition, CFPWV is a moderately heritable trait with an estimated residual heritability (h²) of approximately 0.40, although molecular mechanisms contributing to aortic stiffness remain largely undefined. To evaluate associations of common genetic variants with CFPWV, we performed a meta-analysis of genome-wide association study (GWAS) data from 9 community-based cohorts, with replication genotyping in 2 additional cohorts. In addition, in light of the association between CFPWV and CVD risk, we interrogated existing clinical end point GWAS data to determine whether variants associated with CFPWV are associated with CVD risk.

Clinical Perspective on p 90

Methods

Consortium Organization

The AortaGen Consortium includes 9 cohort studies that completed genome-wide genotyping and had measured CFPWV, plus 2 cohort studies that had measured CFPWV and collected DNA for replication genotyping. Each study adopted collaboration guidelines and the consortium established a consensus on phenotype harmonization, covariate selection, and an analytic plan for within-study genome-wide association and prospective meta-analysis of results across studies. Each study received institutional review board approval of its consent procedures, examination and surveillance components, data security measures, and DNA collection and its use for genetic research. All participants in each study gave written informed consent for participation in the study and the conduct of genetic research. Details of study cohort, CFPWV measurement protocols and inclusion and exclusion criteria are provided in the online-only Data Supplement Methods and Table 1.

Phenotyping

Only cohorts that measured CFPWV based on the carotid-to-femoral transit time and distance were included in the meta-analysis. CFPWV increases nonlinearly and exhibits marked variance inflation with advancing age, resulting in a strongly right skewed distribution. In addition, differences in the method used to ascertain transit distance can alter values by up to 30% and the amount of error may be influenced by sex and other anthropomorphic factors such as height and weight. Thus, genetic association analyses were performed using a sex-specific standardized residual that was based on the inverse of CFPWV, which normalizes the distribution, and that was further adjusted for age, age squared, height, and weight. As a result of these transformations, the cohorts had a highly comparable distribution of the phenotype (mean of 0 and standard deviation of 1 with a normal distribution).

Genotyping and Imputation

Genotyping and imputation methods have been described previously and are summarized in online-only Data Supplement Table S1. For genome-wide SNP sets, genotyping was carried out using commercially available arrays. Before imputation, quality control measures were applied as outlined in online-only Data Supplement Table S1. MACH was used by all cohorts for imputation of genotypes to the HapMap set of approximately 2.5 million SNPs. For replication cohorts, genotyping was carried out on the platforms noted in online-only Data Supplement Table S1. All genetic coordinates in tables and Figure refer to HapMap release 22 build 36.

Expression Methods

Details of expression studies, including RNA extraction, cDNA preparation, PCR amplification, and sequencing are provided in the online-only Data Supplement Methods.

Statistical Analyses

The phenotype for meta-analysis was a sex-specific (in Framingham, cohort-specific, and sex-specific) standardized regression residual for 1000CFPWV, adjusted for age, age squared, height, and weight. Genome-wide association analyses were conducted within each cohort using an additive gene-dose model. Linear mixed effects models were fitted to account for relatedness in pedigrees. Within-study associations were combined by prospective meta-analysis using inverse-variance weighting. Meta-analyses were performed using the software program MetaABEL (http://www.genabel.org/packages/MetaABEL). During meta-analysis, SNPs were excluded if weighted mean minor allele frequency was <1%, resulting in 2.41 million SNPs for analysis. The genomic control parameter was calculated to adjust each study and after meta-analysis, was recalculated to adjust for among-study heterogeneity. For the initial meta-analysis, a predetermined threshold of 4.0 × 10⁻⁵ (stage 1) was used to select SNPs for attempted replication. Based on a preliminary analysis of 6 cohorts, we selected SNPs from 2 loci (the SNP with the lowest P and 1 or 2 proxy SNPs to accommodate differing genotyping platforms) for attempted replication. SNPs were genotyped in 2 additional cohorts and analyzed within cohort using a similar analysis plan except that observed rather than imputed genotypes were used in the analyses. Results from the 2 replication cohorts were then combined by meta-analysis. We considered a P < 0.025 (0.05/2) and same direction of effect for the replication meta-analysis as indicative of successful replication.

To assess possible effect modification by age, we performed an age-stratified analysis based on the approximate overall median age of 55 years. For cohorts that spanned this age cutoff (FHS, ERF, Sardinia, ACCT), analyses were repeated in subgroups <55 and ≥55 years of age. Cohorts with predominantly older (AGES, BLSA, HABC, RS-I, RS-II) or younger (HAPI, Asklepios) participants were included in the older or younger group in their entirety to preserve adequate sample size. These groupings resulted in 9 sets of data consisting of predominantly older participants and 6 sets of data consisting of predominantly younger participants. In addition, because of known associations between CFPWV and clinical events, we performed lookups of the top result from our CFPWV GWAS in separate GWAS meta-analyses for clinical end points thought to be related to arterial stiffness, including coronary artery disease, heart failure, stroke, and kidney disease. We also performed a lookup of our top result in an ongoing pulse pressure GWAS meta-analysis in order to determine whether genetic effects on aortic stiffness were detectable as an increase in blood pressure pulsatility in additional cohorts (see the online-only Data Supplement for details of clinical GWAS meta-analyses).

Results

Characteristics of participants at the time of CFPWV measurement in the 11 (9 discovery, 2 replication) AortaGen Consortium cohorts are presented in Table 1. Cohort mean age varied from 34 to 75 years, whereas cohort mean CFPWV varied from 5.5 to 13.6 m/s, corresponding to inverse CFPWV of 193–77 ms/m, respectively. Sample sizes varied from 618 to 6033 participants, with an aggregate of 20 634 and 5306 participants in the discovery and replication phases, respectively.

GWAS meta-analysis results from 9 cohorts are summarized in Figure 1. The quantile-quantile plot shows minimal evidence of test statistic inflation (λgc = 1.03) and a sharp divergence from a slope near unity at a probability
value of approximately $1 \times 10^{-4}$. The negative log $P$ (Manhattan) plot reveals a region of genome-wide significant association on the distal long arm of chromosome 14 (14q32.2, rs1381289, $\beta=-0.073 \pm 0.011$ SD/allele, $P=5.6 \times 10^{-11}$). Imputation quality for the genome-wide significant SNPs in this region was high, with median expected/observed variance ratio of 0.99–1.00. In addition, there is a suggestive region of association on the short arm of chromosome 10 (10p12.32, rs10764094, $\beta=-0.057 \pm 0.011$ SD/allele, $P=2.4 \times 10^{-7}$). A listing of top SNPs from the 9-cohort meta-analysis with a $P<1 \times 10^{-5}$ is presented in Table 2. The table provides results for the top SNP from separate loci defined by linkage disequilibrium (LD) structure ($r^2<0.80$). Results of analyses that further adjusted for mean arterial pressure at the time of CFPWV measurement are presented in online-only Data Supplement Table S3. Comparison of Tables 2 and S3 reveals that several associations were relatively stronger after adjustment for mean arterial pressure ($CFDP1$, $FGFR2$, $NMUR2$, $ADAMTS9$, $OCA2$, $VPS54$), others were unaffected ($C10orf112$, $EFTUD1$, $C14orf64$), and a few were weakened ($ELK3$, $SLCOS5A1$, $MAFB$, $CADPS2$). For the locus on chromosome 14 ($C14orf64$), associations for some SNPs were weaker after adjustment for mean arterial pressure.

**Table 1. Clinical Characteristics of Study Participants**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>Percent Women</th>
<th>Age, y</th>
<th>Percent ≤55 y of Age</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>CFPWV, m/s</th>
<th>Inverse CFPWV, ms/m</th>
<th>Years of CFPWV Assessment</th>
<th>Years of DNA Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGES</td>
<td>967</td>
<td>58</td>
<td>75±5</td>
<td>0</td>
<td>168±9</td>
<td>75±14</td>
<td>13±4.3</td>
<td>83±24</td>
<td>2005</td>
<td>2002–2006</td>
</tr>
<tr>
<td>FHS</td>
<td>6033</td>
<td>54</td>
<td>49±15</td>
<td>68</td>
<td>169±10</td>
<td>77±17</td>
<td>8.5±3.5</td>
<td>131±35</td>
<td>1999–2001*</td>
<td>1996–1999*</td>
</tr>
<tr>
<td>RS-I</td>
<td>3011</td>
<td>57</td>
<td>72±7</td>
<td>0</td>
<td>167±9</td>
<td>74±12</td>
<td>13.6±3.0</td>
<td>77±17</td>
<td>1997–1999</td>
<td>1990–1993</td>
</tr>
<tr>
<td>RS-II</td>
<td>1657</td>
<td>54</td>
<td>64±8</td>
<td>0</td>
<td>169±9</td>
<td>77±13</td>
<td>12.6±3.2</td>
<td>84±18</td>
<td>2000–2001</td>
<td>2000–2001</td>
</tr>
</tbody>
</table>

Replication cohorts

- ACCT
  - 2932
  - 52
  - 34±19
  - 77
  - 171±10
  - 72±14
  - 6.7±2.2
  - 161±40
  - 2001–2009
  - 2001–2009
- Asklepios
  - 2374
  - 52
  - 46±6
  - 93
  - 169±9
  - 74±14
  - 6.6±1.5
  - 157±29
  - 2002–2004
  - 2002–2004

Values are mean±SD except as noted.

CFPWV indicates carotid-femoral pulse wave velocity; AGES, Age, Gene/Environment Susceptibility-Reykjavik Study; BLSA, Baltimore Longitudinal Study of Aging; ERF, Erasmus Rucphen Family Study; FHS, Framingham Heart Study; HABC, Health, Aging, and Body Composition; HAPI, Heredity and Phenotype Intervention; RS, Rotterdam Study; and ACCT, Anglo Cardiff Collaborative Trial.

*Original cohort.
†Offspring cohort.
‡Third-generation cohort.

Figure 1. Quantile-quantile and signal intensity (Manhattan) plots of genome-wide association data for CFPWV. The upper horizontal line corresponds to $P=5.0 \times 10^{-8}$, which was the threshold for genome-wide significance; the lower line corresponds to $P=1.0 \times 10^{-5}$, which was the threshold used to prepare Table 2.
Table 2. Genome-Wide Association Results for Carotid-Femoral Pulse Wave Velocity in 9 Cohorts

<table>
<thead>
<tr>
<th>SNP</th>
<th>No.</th>
<th>Position</th>
<th>Coded</th>
<th>Frequency</th>
<th>Meta-Analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1381289†</td>
<td>14</td>
<td>97 662 117</td>
<td>T</td>
<td>0.436</td>
<td>−0.073 0.011 5.6×10⁻¹¹ C14orf64</td>
</tr>
<tr>
<td>rs897514</td>
<td>14</td>
<td>97 698 696</td>
<td>T</td>
<td>0.436</td>
<td>−0.069 0.011 4.5×10⁻¹⁰ C14orf64</td>
</tr>
<tr>
<td>rs10782490</td>
<td>14</td>
<td>97 619 136</td>
<td>C</td>
<td>0.471</td>
<td>−0.066 0.011 2.7×10⁻⁹ C14orf64</td>
</tr>
<tr>
<td>rs2225442</td>
<td>14</td>
<td>97 692 347</td>
<td>C</td>
<td>0.323</td>
<td>−0.071 0.012 1.2×10⁻⁶ C14orf64</td>
</tr>
<tr>
<td>rs17773233</td>
<td>14</td>
<td>97 652 412</td>
<td>T</td>
<td>0.225</td>
<td>−0.074 0.013 2.1×10⁻⁸ C14orf64</td>
</tr>
<tr>
<td>rs1461587</td>
<td>14</td>
<td>97 673 604</td>
<td>G</td>
<td>0.256</td>
<td>−0.070 0.013 1.5×10⁻⁷ C14orf64</td>
</tr>
<tr>
<td>rs1381273</td>
<td>14</td>
<td>97 718 813</td>
<td>T</td>
<td>0.469</td>
<td>−0.059 0.011 1.9×10⁻⁷ C14orf64</td>
</tr>
<tr>
<td>rs10764094</td>
<td>10</td>
<td>19 950 544</td>
<td>C</td>
<td>0.473</td>
<td>0.057 0.011 2.4×10⁻⁷ C10orf112</td>
</tr>
<tr>
<td>rs8015529</td>
<td>14</td>
<td>97 571 972</td>
<td>G</td>
<td>0.359</td>
<td>−0.066 0.013 2.5×10⁻⁷ C14orf64</td>
</tr>
<tr>
<td>rs4778983</td>
<td>15</td>
<td>80 290 133</td>
<td>C</td>
<td>0.301</td>
<td>0.057 0.012 1.5×10⁻⁶ EFTUD1</td>
</tr>
<tr>
<td>rs7161307</td>
<td>14</td>
<td>97 677 436</td>
<td>T</td>
<td>0.215</td>
<td>−0.065 0.013 1.7×10⁻⁶ C10orf112</td>
</tr>
<tr>
<td>rs6485690</td>
<td>11</td>
<td>46 755 207</td>
<td>A</td>
<td>0.308</td>
<td>0.056 0.012 3.0×10⁻⁶ CKAP5†</td>
</tr>
<tr>
<td>rs10740923</td>
<td>10</td>
<td>19 907 637</td>
<td>G</td>
<td>0.464</td>
<td>−0.052 0.011 3.9×10⁻⁶ C10orf112</td>
</tr>
<tr>
<td>rs7959220</td>
<td>12</td>
<td>95 117 079</td>
<td>G</td>
<td>0.027</td>
<td>0.266 0.059 6.3×10⁻⁶ ELK3</td>
</tr>
<tr>
<td>rs6472483</td>
<td>8</td>
<td>70 791 920</td>
<td>T</td>
<td>0.452</td>
<td>−0.050 0.011 7.1×10⁻⁶ SLC25A1</td>
</tr>
<tr>
<td>rs6101837</td>
<td>20</td>
<td>38 155 981</td>
<td>C</td>
<td>0.416</td>
<td>−0.050 0.011 7.5×10⁻⁶ MAFB</td>
</tr>
<tr>
<td>rs10827649</td>
<td>10</td>
<td>19 949 776</td>
<td>G</td>
<td>0.436</td>
<td>−0.049 0.011 8.6×10⁻⁶ C10orf112</td>
</tr>
<tr>
<td>rs6947805</td>
<td>7</td>
<td>121 844 471</td>
<td>T</td>
<td>0.050</td>
<td>0.117 0.026 9.5×10⁻⁶ CADPS2</td>
</tr>
</tbody>
</table>

*Individual analyses were adjusted for age, age squared, sex, height, and weight.
†R² = 0.93 for rs1381289 and rs7152623.
‡Linkage disequilibrium block includes ARHGAP1, ZNF408, F2, CKAP5, and LRP4.

Based on a preliminary meta-analysis of early GWAS results from 6 cohorts (Table 1; AGES, FHS, ERF, RS-I, RS-II, and Sardinia; 17 854 participants), we selected rs7152623 on chromosome 14 (results from 6 cohorts: β = −0.075 ± 0.012 SD/allele, P = 2.8×10⁻¹⁰) and rs17729837 on chromosome 10 (results from 6 cohorts: β = 0.062 ± 0.012 SD/allele, P = 3.6×10⁻⁷) for attempted replication. Note that rs7152623, the SNP on chromosome 14 selected for replication, falls within the LD block that includes rs1381289 as the SNP with the lowest probability value in the block (Table 2). The 2 SNPs are closely linked (R² = 0.93 for rs1381289 and rs7152623). We successfully replicated the association with rs7152623 on chromosome 14 (replication β = −0.086 ± 0.020 SD/allele, P = 1.4×10⁻⁶). Results for rs7152623 from the full set of 11 cohorts gave a combined β = −0.076 ± 0.010 SD/allele, P = 3.1×10⁻¹⁵ (Figure 2). The effect was attenuated modestly and remained significant when we further adjusted for mean arterial pressure at the time of measurement of CFPWV (β = −0.060 ± 0.009 SD/allele, P = 1.0×10⁻¹⁴). In addition, results were consistent when evaluated separately in subgroups defined by median age, remaining associated in both younger (<55 years of age, 6 cohorts, n = 13 914, β = −0.081 ± 0.014 SD/allele, P = 2.3×10⁻⁸) and older (≥55 years of age, 9 cohorts, n = 12 026, β = −0.061 ± 0.014 SD/allele, P = 9.4×10⁻⁶) participants. The association with rs17729837 on chromosome 10 did not replicate (P = 0.97).

Details of the region of significant association on chromosome 14 are presented in Figure 3. The closest known gene (3’ BCL11B) is nearly 1 MB telomeric to this locus. The associated SNPs are located in a block of LD spanning from approximately 97.60–97.74 MB, which corresponds closely with the location of a cluster of previously documented, overlapping, spliced expressed sequence tags (ESTs), including DB129663 and ARHGAP1, ZNF408, F2, CKAP5, and LRP4.

Figure 2. Forest plot of association results for rs7152623 on chromosome 14. Results for individual cohorts are plotted against the cohort effect size (β coefficient). The size of the box is proportional to the study’s weight in the meta-analysis (inversely proportional to estimated variance of the effect-size estimator). Horizontal lines are the 95% confidence intervals. Diamonds represent the results of meta-analyses; the center denotes overall estimate and the width denotes 95% confidence interval.
BP432414, which are on the minus and plus strand, respectively. One of our highly associated SNPs (rs710285, \( P=5.1 \times 10^{-11} \)) is located in exon 3 of DB129663. In addition, there is a conserved sequence near the center of the chromosome 14 locus (97.67 MB, Figure 3), in overlapping intronic regions of DB129663 and BP432414.

To assess potential functional implications of our findings, we used reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate expression of DB129663 and BP432414 in human aortic samples and various cell lines. In light of the putative role of this region as a remote enhancer of \( BCL11B \) and relative proximity to \( VRK1 \), we also probed for expression of these genes (online-only Data Supplement Table S4). DB129663, BP432414, \( BCL11B \), and \( VRK1 \) were detected in whole aortic rings (online-only Data Supplement Table S4). \( VRK1 \) and DB129663 were expressed in cultured aortic smooth muscle cells, human umbilical vein endothelial cells (HUVECs), and adult cardiac fibroblasts. \( BCL11B \) was expressed in the same samples except cardiac fibroblasts. All transcripts were expressed in CD3+ cells.

To assess the potential clinical relevance of our finding of a locus on Chr14 strongly associated with CFPWV, we performed a lookup of our top SNP for association with CFPWV in the meta-analysis of 9 cohorts (rs1381289, Table 2) in results from separate clinical end point GWAS meta-analyses (see online-only Data Supplement). We found an association with increased risk for coronary artery disease (hazard ratio [HR]=1.05, confidence interval [CI]=1.02–1.08 per allele, \( P=0.0013 \)) and heart failure (HR=1.10, CI=1.03–1.16 per allele, \( P=0.004 \)). To aid interpretation of these CVD end point associations, we used data from FHS to estimate the expected HR for a first major CVD event associated with presence of each allele of rs1381289, assuming that excess CVD risk was mediated by effects of the allele on CFPWV. In FHS, after adjusting for age and sex, CFPWV was associated with an excess CVD risk corresponding to an HR=1.65 per SD. Based on this estimate, and given that each minor allele of rs1381289 was associated with a 0.073 SD increase in CFPWV, the expected HR=1.047 per allele, which is comparable to the GWAS lookup results. In a separate meta-analysis that included 74,011 participants, rs1381289 was associated with higher pulse pressure (\( \beta=0.18\pm0.06 \) mm Hg/allele, \( P=0.002 \)), indicating that the increase in aortic stiffness associated with this SNP is detectable as a modest but significant increase in pressure pulsatility. The SNP was not associated with stroke (\( P>0.7 \)), glomerular filtration rate estimated by using serum creatinine (\( P>0.6 \)) or cystatin (\( P>0.5 \)), or prevalent chronic kidney disease (\( P>0.6 \)).

We also sought to replicate a previously reported association between CFPWV and a SNP (rs3742207) in \( COL4A1 \). After excluding 2 cohorts involved in the original report (Sardinia, HAPI), we found modest evidence of association for this SNP (rs3742207, \( \beta=-0.025\pm0.011 \) SD/allele, \( P=0.017 \)) (online-only Data Supplement Figure S1).

**Discussion**

We performed a meta-analysis of GWAS results for CFPWV from 9 community-based cohorts involving 20,634 participants spanning a broad age range and identified a locus of genome-wide significant association in an apparent gene desert on 14q32.2. This finding was replicated in 2 additional cohorts involving 5306 participants. We identified a conserved sequence within the region of significant association surrounded by a cluster of primate-specific, noncoding RNAs (ncRNAs). We evaluated 2 of these ncRNAs, which have at least 1 associated SNP within an exon and demonstrated that they are expressed in relevant human cardiac and vascular tissues and cell lines, including full thickness aortic rings, aortic smooth muscle cells, cardiac fibroblasts, and HUVECs. In light of the putative role of the region of significant association as a gene enhancer, we also assayed for and demonstrated expression of flanking known genes, \( BCL11B \) and \( VRK1 \), in the same tissues and cell lines. Our findings indicate that the \( VRK1-BCL11B \) gene desert harbors a regulatory locus.
that modulates aortic stiffness. The association was consistent in younger and older participants, suggesting that the effects on CFPWV of genetic variation at this locus manifest early in life, before the marked increase in CFPWV that occurs from midlife onward. In addition, we demonstrated that the locus is associated with increased risk for cardiovascular disease, consistent with the hypothesis that increased aortic stiffness, as assessed by CFPWV, plays a causal role in the pathogenesis of cardiovascular disease. Further elucidation of potential mechanisms of aortic stiffening mediated through this locus may provide novel insights into the pathogenesis of aortic stiffening and could potentially offer insights into currently unavailable targeted interventions that prevent or attenuate aortic stiffening with advancing age and reduce the associated excess risk for major CVD events.

CFPWV has emerged recently as an important risk factor for various afflictions of aging, including CVD, cognitive dysfunction and chronic kidney disease. CFPWV is easily and reproducibly measured in a few minutes using relatively inexpensive technology and is widely considered the present gold standard noninvasive measure of aortic stiffness. Aside from age, with which it is strongly related, CFPWV has consistent but relatively modest relations with standard CVD risk factors and is moderately heritable, rendering the phenotype optimal for genetic studies.

A number of prior studies have evaluated potential genetic correlates of various measures of arterial stiffness including CFPWV. Family-based studies have identified several regions of potential linkage for stiffness measures using a microsatellite-based whole genome approach. Genetic association studies have found relations between measures of arterial stiffness and polymorphisms in various candidate genes, including genes for the angiotensin II type 1 receptor, fibrillin-1, angiotensin-converting enzyme, aldosterone synthase, \( \beta \)-adrenergic receptors, endothelin A and B receptors, matrix metalloproteinasises 3 and 9, endothelial nitric oxide synthase, the large conductance calcium-activated potassium channel, estrogen genes, and various inflammatory genes. However, small sample sizes, ascertainment bias (hypertensive, known coronary artery disease, etc) and relatively weak associations may have limited the generalizability and consistency of findings. Notably, none of the aforementioned candidate genes are in the top hits of the present analysis (Tables 2 and S3). Two prior CFPWV GWAS publications were similarly based on relatively small sample sizes and employed less dense genotyping, which limited power to detect associations. A relative paucity of genome-wide significant findings remains a concern with our present publication involving 9 discovery and 2 replication cohorts and an aggregate sample size of nearly 26,000 individuals. Additional work and larger samples will be required to determine whether any additional loci in the group of excess low probability values below \( 1 \times 10^{-8} \) are true positive associations.

Prior studies provide evidence that the region of association with CFPWV that we have identified on 14q32.2 lies in the vicinity of 1 or more gene enhancers. The region encompasses various regulatory features, including several DNase-I hypersensitive sites and transcription factor binding sites and high levels of nuclear matrix attachment. Chromatin modifications in the region, including high levels of acetylation of histone 3 at lysine 27 (H3K27) and monomethylation at lysine 4 (H3K4) assessed in a lymphoblastoid cell line, are consistent with enhancer function (http://genome.ucsc.edu). Despite considerable genomic separation from the enhancer cluster \( \approx 1 \) MB telomeric, \( BCL11B \) is thought to be a target of one or more of the enhancers in this locus. \( BCL11B \) is located on the minus strand, positioning the enhancer cluster in the remote 3' region of the gene. The closest known gene in the opposite direction, \( VRK1 \), is \( \approx 1.1 \) MB centromeric to the enhancer cluster and is on the plus strand, again positioning the enhancer in the remote 3' region of \( VRK1 \), suggesting that one or both genes could potentially be targets of a remote 3' enhancer in this region.

In support of \( BCL11B \) as a target, numerous translocations have been described that insert fragments of 5q35 at various positions in a breakpoint cluster region that falls between the enhancer region and 3' \( BCL11B \). These translocations interpose the homeobox genes \( TLX3 \) or \( NKK2-5 \) between the enhancer region and 3' \( BCL11B \) and result in ectopic activation of the inserted homeobox gene, dysregulated T-cell proliferation, and acute T-cell lymphoblastic leukemia. \( TLL1 \), a C2H2 zinc finger protein that can directly bind DNA in a sequence specific manner. Acting in part through an interaction with \( SIRT1 \), \( BCL11B \) can effect transcriptional repression of various genes that may be relevant to aortic stiffness. In addition to direct effects of \( BCL11B \) on aortic function, there are potential indirect effects mediated through the known role that \( BCL11B \) plays in T-cell function. T-cell-specific deletion of \( BCL11B \) at the CD4+ single positive stage is associated with increased numbers of proinflammatory T-cells that could potentially infiltrate the aorta and promote inflammation, fibrosis, and stiffening. Additional work will be required to establish the potential role that genetic variation in the chromosome 14 locus may play in \( BCL11B \) expression and aortic function.
We demonstrated that 2 overlapping ESTs that fall completely within the region of highly significant association with CFPWV are expressed in aortic tissue and cell lines. These primate-specific, potentially regulatory ncRNAs are expressed in cDNA extracts from full-thickness human aortic rings and various human cell lines, including aortic smooth muscle cells, HUVECs, and cardiac fibroblasts. One of the highly associated SNPs in the region (rs710285) is located in an exon of DB129663, suggesting a possible functional effect. The enhancer core region mapped by Su et al corresponds to the putative promoter region of DB129663. Thus, enhancer function at our chromosome 14 locus may target DB129663, which appears to be an ncRNA of unknown function. Additional work will be required to test this hypothesis and further define the function of DB129663 and other ncRNAs in the region.

Several additional SNPs with suggestive associations to CFPWV \((10^{-8} < \text{P} < 10^{-5})\) may merit further consideration and additional replication genotyping. The locus on chromosome 10 with the second lowest probability value in our GWAS meta-analysis lies in the vicinity of a putative protein coding gene that may represent a novel member of the low density lipoprotein receptor-related protein (LRP) family.\(^{38}\) We found moderate evidence for association at the LRP4 locus, which is also associated with stroke\(^{39}\) and bone mineral density.\(^{40,41}\) The LRP4 locus includes a nonsynonymous SNP (rs6485702) that has been related to bone mineral density,\(^{40}\) although a separate report involving several of our cohorts positioned the region of highest association with bone mineral density in the promoter region of \\(_{ARHGAP1}\).\(^{41}\) Bone density and arterial stiffness are related phenotypes\(^{42}\) that may share many common pathways. The recently observed inhibitory role that LRP4 plays in Wnt signaling in bone\(^{43}\) coupled with the adverse effects of Wnt signaling in the aorta\(^{44}\) suggests that a mutation that impairs the ability of LRP4 to modulate the Wnt signaling cascade could simultaneously contribute to osteopenia and aortic stiffening. The chromosome 11 locus that encompasses LRP4 and additional potential candidates, including ARHGAP1 and F2, represents a long LD block that was also associated with stroke in a prior meta-analysis that included several of the cohorts in our study.\(^{39}\) The direction of effect in the prior study (higher risk for the minor allele) and ours (stiffer aorta with the minor allele) was consistent with the known association between increased CFPWV and increased risk for stroke. In addition, a prior family-based linkage analysis for myocardial infarction found a single significant linkage peak in the vicinity of our chromosome 14 locus.\(^{45}\) These regions of overlap with prior results involving separate but related phenotypes support the clinical relevance of our associations and suggest that several genetic variants that impact CFPWV may eventually manifest as age-related morbidity and major cardiovascular events.

We also attempted to replicate a previously reported association of CFPWV with a SNP in the COLA4I locus in the only published GWAS that has evaluated CFPWV.\(^{10}\) The present results found modest evidence of association with some heterogeneity of effect, suggesting that additional work will be required to determine whether variation in LD patterns or other factors could potentially account for heterogeneous effects at this locus.

There are limitations of our study that should be considered. The cohorts comprised exclusively white participants of European descent. Thus, our findings may not generalize to other populations. Slightly different methods were used to assess CFPWV in the various cohorts. However, our use of standardized residuals generated within each cohort should have minimized the effects of these technical differences between studies. A major strength of our study is the use of data from 11 large community-based cohorts that routinely ascertained CFPWV, which should enhance generalizability of our findings.

In conclusion, we performed the first large-scale GWAS of CFPWV, which is a moderately heritable measure of aortic stiffness and important risk factor for cardiovascular events. We identified a highly significant locus of association at 14q32.2 in the VRK1-BCL11B gene desert in an LD block that harbors 1 or more gene enhancers. We have also shown that genetic variation at this locus is associated with increased risk for major CVD events, providing strong support for the hypothesis that increased CFPWV contributes to the pathogenesis of CVD. We have shown that 2 potentially regulatory ncRNAs as well as flanking genes, BCL11B and VRK1, are expressed in human aorta. Further work will be required to define precise mechanisms mediating the association between CFPWV and genetic variation in the VRK1-BCL11B gene desert. Elucidation of pathways affected by this locus will provide new insights into the process of aortic stiffening in humans and could yield potential targets for specific interventions that reverse or attenuate aortic stiffening and prevent the associated morbidity and mortality.

**Appendix**

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Disclosures
Dr Mitchell is owner of Cardiovascular Engineering, Inc. a company that designs and manufactures devices that measure vascular stiffness. The company uses these devices in clinical trials that evaluate the effects of diseases and interventions on vascular stiffness. The remaining authors report no conflicts.

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CLINICAL PERSPECTIVE

Carotid-femoral pulse wave velocity (CFPWV) is a heritable measure of aortic stiffness that is strongly associated with increased risk for major cardiovascular disease events. However, the molecular mechanisms contributing to aortic stiffness remain largely undefined. To evaluate associations of common genetic variants with CFPWV, we conducted a meta-analysis of genome-wide association data in 9 community-based European ancestry cohorts consisting of 20,634 participants. Results were replicated in 2 additional European ancestry cohorts involving 5306 participants. We identified a highly significant locus of association at 14q32.2 in the VRK1-BCL11B gene desert in a linkage disequilibrium block that harbors 1 or more gene enhancers. We also showed that variation at this locus is associated with increased risk for major cardiovascular disease events, providing strong support for the hypothesis that increased CFPWV contributes to the pathogenesis of cardiovascular disease. We demonstrated that 2 ncRNAs as well as flanking genes, BCL11B and VRK1, are expressed in human aorta. Further work will be required to define precise mechanisms mediating the association between CFPWV and genetic variation in the VRK1-BCL11B gene desert. Elucidation of pathways affected by this locus will provide new insights into the process of aortic stiffening in humans and could yield potential targets for specific interventions that reverse or attenuate aortic stiffening and prevent the associated morbidity and mortality.
Common Genetic Variation in the 3′-BCL11B Gene Desert Is Associated With Carotid-Femoral Pulse Wave Velocity and Excess Cardiovascular Disease Risk: The AortaGen Consortium


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SUPPLEMENTAL MATERIAL

Common genetic variation in the 3'-BCL11B gene desert is associated with carotid-femoral pulse wave velocity and excess cardiovascular disease risk:
The AortaGen Consortium

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Supplementary Methods

GWAS Sites

The Reykjavik Study cohort originally comprised a random sample of 30,795 men and women born in 1907-1935 and living in Reykjavik in 1967. A total of 19,381 people attended, resulting in 71% recruitment rate. The study sample was divided into six groups by birth year and birth date within month. One group was designated for longitudinal follow up and was examined in all stages. One group was designated a control group and was not included in examinations until 1991. Other groups were invited to participate in specific stages of the study. Between 2002 and 2006, the Age, Gene/Environment Susceptibility-Reykjavik Study (AGES-RS) reexamined 5,764 survivors of the original cohort who had participated before in the Reykjavik Study.1 A reexamination of survivors from AGES-RS is presently being conducted (AGES-II).

Arterial tonometry was added to the original AGES-RS study protocol for all participants beginning January 12, 2005 and continuing through to the end of the examination cycle. Arterial tonometry is being assessed in all participants in AGES-II. Carotid-femoral pulse wave velocity (CFPWV) was evaluated by tonometry and calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that adjusted for parallel transmission in the carotid and aorta by using the suprasternal notch as a fiducial point. Successful genotyping was available for 3,219 AGES-RS participants, of whom 967 had successful measurement of CFPWV. The AGES-RS GWAS was approved by the National Bioethics Committee and the Data Protection Authority.
The Baltimore Longitudinal Study of Aging (BLSA) is an observational study that began in 1958 to investigate normative aging in community dwelling adults who were healthy at study entry. Participants are examined every one to four years depending on their age. Currently there are approximately 1,100 active participants enrolled in the study. Blood samples were collected for DNA extraction, and genome-wide genotyping was completed for 1,231 subjects using Illumina 550K. This analysis focused on a subset of the participants (N=610) of European ancestry for whom CFPW was available. CFPWV was evaluated initially using nondirectional transcutaneous Doppler probes and subsequently with the Complior® SP device (Artech Medical, Pantin, France). CFPWV was calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that adjusted for parallel transmission in the carotid and aorta by using the manubrium as a fiducial point.

The Erasmus Rucphen Family (ERF) study is comprised of a family-based cohort embedded in the Genetic Research in Isolated Populations (GRIP) program in the Southwest of the Netherlands. The aim of this program is to identify genetic risk factors for the development of complex disorders. In ERF, twenty-two families that had a minimum of five children baptized in the community church between 1850 and 1900 were identified with the help of detailed genealogical records. All living descendants of these couples and their spouses were invited to take part in the study. Comprehensive interviews, questionnaires, and examinations were completed at a research center in the area; approximately 3,200 individuals participated. CFPWV was assessed with an automatic device (Complior® Artech Medical, Pantin, France) and calculated from the simultaneously measured foot-to-foot time delay between carotid and femoral pressure...
waves and body surface measurement of the distance between carotid and femoral pulse recording site. Data collection started in June 2002 and was completed in February 2005. In the current analyses, 1,970 participants for whom complete phenotypic, genotypic and genealogical information was available were studied.

The Framingham Heart Study (FHS) began in 1948 with the recruitment of an Original cohort of 5,209 men and women who were 28 to 62 years of age (mean age 44 years; 55 percent women) at entry. In 1971 enrollment of a second generation of study participants took place; this cohort consisted of 5,124 children and spouses of children of the original cohort. The mean age of the Offspring cohort was 37 years; 52 percent were women. A Third Generation cohort of 4,095 children (mean age 40 years; 53 percent women) of offspring cohort participants was enrolled beginning in 2002. Details of study designs for the three cohorts are summarized elsewhere.\textsuperscript{4-6} Tonometry was performed during Examination 26 (1999-2001), Examination 7 (1998-2001) and Examination 1 (2002-2005) for the Original, Offspring and Third Generation cohorts, respectively. CFPWV was evaluated by tonometry and calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that adjusted for parallel transmission in the carotid and aorta by using the suprasternal notch as a fiducial point. Successful genotyping was available for 8,508 FHS participants, of whom 6,033 had successful measurement of CFPWV.

The Health, Aging and Body Composition (Health ABC) Study is a National Institute on Aging-sponsored ongoing cohort study of the factors that contribute to incident disability and the decline in function of healthier older persons, with a particular emphasis on changes in body composition in old age.\textsuperscript{7} Between 4/15/97 and 6/5/98 the
Health ABC study recruited 3,075 participants who were 70-79 year old community-dwelling adults (41% African-American), who were initially free of disabilities that limited mobility and activities of daily living. CFPWV was measured from simultaneous Doppler flow signals obtained from the right carotid and right femoral arteries with nondirectional transcutaneous Doppler flow probes (model 810A, 9.0- to 10-MHz probes, Parks Medical Electronics, Inc). Digitized data were recorded by custom programming for subsequent analysis. A minimum of 10 beats were averaged for each simultaneous recording site using the QRS for synchronization. Three separate runs were recorded for each participant, and all usable runs were averaged. The distance between the carotid and femoral sampling sites was measured above the surface of the body with a metal tape measure. This was done to avoid overestimation of the distance portion of the PWV equation. This distance was divided by the time differentials between the onset of flow at carotid and femoral sites (defined as foot of the flow tracing at each site) to produce CFPWV. Successful genome wide genotyping was available for 2,800 participants, of whom 2,292 had successful measurement of CFPWV. For the purposes of this study, white participants of European descent were included in the analyses, resulting in a final sample of 1,398 participants with CFPWV and genotype data.

The Old Order Amish individuals included in this study were participants of several ongoing studies of cardiovascular health carried out at the University of Maryland. Most participants were from the Heredity and Phenotype Intervention (HAPI) Heart Study and were relatively healthy volunteers from the Old Order Amish community of Lancaster County, Pennsylvania and their family members. Examinations were conducted at the Amish Research Clinic in Strasburg, PA. All protocols were
approved by the Institutional Review Board at the University of Maryland and informed consent was obtained, including permission to use their DNA for genetic studies. Study participants were enrolled within the 2003-2008 time period. CFPWV was measured with a Complior® device (Artech Medical, Pantin, France). All subjects were measured in the morning after an overnight fast and abstained from smoking or exercise prior to the test. In brief, after a 10 minute rest period and while in the supine position the carotid and femoral pulse wave were recorded simultaneously. The time delay between the rapid upstroke of the carotid and femoral waveforms represented the transit time for the wave. The average of ten cycles was used to determine transit time. The distance traveled by the pulse wave from the carotid to the femoral site was measured by tape. The final PWV for each subject was then calculated by dividing the distance traveled over the average transit time and expressed in meters per second. Measures with variance of over 9% were excluded. All scans were scored by a single blinded experienced cardiologist.

The Rotterdam Study (RS) is a prospective population-based cohort study comprising 7,983 subjects (78% response rate) aged 55 years or older. Baseline data (RS-I) were collected between 1990 and 1993. In 1999, inhabitants who turned 55 years of age or moved into the study district since the start of the study were invited to participate in an extension of the RS (RS-II) of whom 3,011 participated (67% response rate). The rationale and design of the RS have been described in detail elsewhere. During the third examination phase from the RS-I (1997-1999) and the first examination phase from the RS-II (1999-2001), measurements of arterial stiffness were performed. CFPWV was assessed with an automatic device (Complior® Artech Medical, Pantin,
France) and calculated from the simultaneously measured foot-to-foot time delay between carotid and femoral pressure waves and body surface measurement of the distance between carotid and femoral pulse recording site. All RS participants with available DNA were genotyped using Illumina Infinium II HumanHap BeadChips at the Department of Internal Medicine, Erasmus Medical Center following manufacturer’s protocols. Participants with call rate < 97.5%, excess autosome heterozygosity, sex mismatch, or outlying identity-by-state clustering estimates were excluded. After quality control 5,974 RS-I participants and 2,157 RS-II participants were included. Of these, 3,011 RS-I participants and 1,657 RS-II participants had successful measurement of CFPWV.

The SardiNIA Study is a longitudinal study of aging-related quantitative traits comprising individuals from a circumscribed region on the island of Sardinia, Italy. The sample constitutes a genetically isolated founder population by virtue of their geographic isolation and ethnic homogeneity. In the SardiNIA study, 6,148 men and women over the age of 14 were recruited from a cluster of four towns in the Lanusuei Valley in the Ogliastro province of the island, which has a total population of 11,000. In this cohort, 3,329 and 1,412 individuals were genotyped with the Affymetrix 10K and Affymetrix 500K Mapping array set, respectively, with 436 individuals generating an overlapping dataset. Given the relatedness among individuals, the full genotypes on the 2,893 individuals typed with only the 10K panel were imputed based on stretches of shared haplotype, permitting analyses on 4,305 individuals. CFPWV was evaluated with nondirectional transcutaneous Doppler probes and calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that
adjusted for parallel transmission in the carotid and aorta by using the manubrium as a fiducial point. CFPWV and genotype data was available in 4,216 subjects.

**Replication Sites**

The Anglo Cardiff Collaborative Trial (ACCT) consists of ~12,000 community-derived individuals selected at random from the local general practice lists and open-access cardiovascular risk assessment clinics across East Anglia and Wales in the United Kingdom. Approval for studies was obtained from the local research ethics committees, and written informed consent obtained from each participant. The overall response rate was 85%. All participants completed a detailed questionnaire and measurements were conducted in a quiet temperature controlled room. Height and weight were assessed with standard methods. After 20 minutes of supine rest, peripheral blood pressure was recorded in the brachial artery of the non-dominant arm using a validated oscillometric technique (HEM-705 CP, Omron Corporation). CFPWV was measured using tonometry and calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that adjusted for parallel transmission in the carotid and aorta by using the suprasternal notch as a fiducial point as described previously. A subset of the ACCT sample (n=3,101) aged 16-87 years were selected for replication of the hits from the primary GWAS. The age distribution of the cohort was bimodal, so the dataset was analyzed globally and separately based on individuals aged <55 years and ≥55 years. Quantitative associations were investigated using the program PLINK (version 1.05) ([http://pngu.mgh.harvard.edu/~purcell/plink](http://pngu.mgh.harvard.edu/~purcell/plink)) and SPSS (version 15.0).
The Asklepios Study (AS) is a prospective population-based cohort study. Participants are a random sample (n=2,524) drawn from the Belgian population who were 35-55 years old and free from overt cardiovascular disease. Subjects were extensively phenotyped including echocardiography, carotid and femoral vascular ultrasound and arterial tonometry. All measurements were single observer, site, protocol and device. Baseline data were collected between 2002 and 2004. The rationale and design of the AS have been described in detail elsewhere.\textsuperscript{13} CFPWV was evaluated by Doppler and calculated from the foot-to-foot delay between carotid and femoral waveforms and body surface measurements that adjusted for parallel transmission in the carotid and aorta by using the suprasternal notch as a fiducial point. Successful genotyping and measurement of CFPWV was available for 2,380 participants. Quantitative associations were investigated using SPSS (version 17.0).

Clinical Endpoint Consortia

The Coronary ARtery DIsease Genome-wide Replication And Meta-analysis (CARDIoGRAM) consortium combines data from 14 GWAS in individuals with European ancestry including >22,000 cases with coronary artery disease (CAD) or myocardial infarction (MI) and >60,000 controls, and unifies samples from Atherosclerotic Disease VAscular functioN and genetiC Epidemiology study, CADomics, Cohorts for Heart and Aging Research in Genomic Epidemiology, deCODE, the German Myocardial Infarction Family Studies I, II, and III, Ludwigshafen Risk and Cardiovascular Heath Study/AtheroRemo, MedStar, Myocardial Infarction Genetics Consortium, Ottawa Heart Genomics Study, PennCath, and the Wellcome Trust Case Control Consortium. These
studies have a case-control design or are prospective cohort studies both having detailed phenotyping for CAD or MI as previously described. Control subjects have been derived from population-based studies in most investigations. For all of the participating studies, genome-wide scans were performed in the years 2006-2009 using either Affymetrix or Illumina platforms followed by imputation of genotypes in most studies. Statistical methods have been standardized across the studies, and an analysis platform has been created to allow summarized analyses on CAD, MI, and related phenotypes.

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium heart failure GWAS included data from 4 prospective, population-based cohorts of adults in the USA and the Netherlands: the Atherosclerosis Risk in Communities (ARIC) Study, the Cardiovascular Health Study (CHS), the Framingham Heart Study (FHS), and the Rotterdam Study (RS). In brief, ARIC recruited 15,792 participants 45 to 64 years of age from 1987 to 1989 from 4 US communities. The CHS recruited participants 65 years of age and older from 4 US communities in 2 waves: 5201 participants in 1989–1990 and an additional 687 African Americans in 1992–1993. The FHS recruited 2 generations of participants 28 to 72 years of age in 2 time periods: 5209 Original Cohort participants were recruited in 1948 from Framingham, Massachusetts, and 5124 Offspring Cohort participants were recruited in 1971–1975. The RS recruited 7983 participants 55 years of age and older from 1990 to 1993 from Rotterdam, the Netherlands. The ARIC and CHS studies included African American participants, 27% and 15%, respectively, whereas FHS and RS included almost exclusively participants of European ancestry. Each study independently conducted
genome-wide scans of study participants, and within-study analyses were conducted according to a prespecified plan.

The Pulse Pressure/Mean Arterial Pressure working group on behalf of the International Consortium for Blood Pressure GWAS combines data from 35 GWAS in individuals with European ancestry, including AGES, ARIC, ASPS, B58C-T1DGC, B58C-WTCCC, BLSA, BHS, CARL, CHS, CoLaus, CROATIA, DGI controls, EGCUT, EPIC Norfolk, ERF, Fenland, FHS, FUSION controls, FVG, INCHIANTI, KORA S3, MICROS, MIGen controls, NESDA, NFBC1966, NTR, NSPHS, ORKNEY, ProCARDIS controls, RSI, RSII, SHIP, SUVIMAX, TwinsUK and Val Borbera. For all participating studies, genome-wide scans were performed using either Affymetrix or Illumina platforms followed by imputation of genotypes. Statistical methods have been standardized across studies. Pulse pressure was defined as systolic blood pressure minus diastolic blood pressure. The combined analyses included 74,011 subjects.

The neuroCHARGE consortium combined data from white participants in four large, prospective population based cohort studies: ARIC, CHS, FHS, and RS to study the genetics of stroke. All participating studies approved guidelines for collaboration, and a neurology working-group arrived at a consensus on phenotype harmonization, covariate selection and analytic plans for within-study analyses and meta-analysis of results. Each study has an Institutional Review Board that approved the consent procedures, examination and surveillance components, data security processes, genotyping protocols and current study design. Stroke was defined as a focal neurologic deficit of presumed vascular cause with a sudden onset and lasting for at least 24 hours or until death if the participant died less than 24 hours after the onset of
symptoms. The combined analysis included 1544 incident strokes (1164 ischemic strokes) among 19,602 persons followed for an average of 11 years.

The CKDGen consortium performed a genome-wide association meta-analysis in 67,093 study participants of European ancestry from 20 predominantly population-based cohorts. They analyzed glomerular filtration rate (GFR) estimated from serum creatinine by the Modification of Diet in Renal Disease (MDRD) Study equation (eGFRcrea) as well as chronic kidney disease (CKD). To discriminate true susceptibility loci for renal function from those related to creatinine production and secretion, they used GFR estimated from a second serum marker of kidney function, cystatin c (eGFRcys).

Expression Methods

Commercially available cultured human aortic smooth muscle cells, adult human cardiac fibroblasts and human umbilical vein endothelial cells (HUVEC) were purchased and cultured according to the protocol recommended by the manufacturer (Cell Application Inc); CD3+ enriched cells from a healthy donor were provided by Dr. P. Olkhanud (NIA, Baltimore, USA). Aortic tissue samples were obtained from cadaveric donors or beating heart donors through transplant coordinators from the Addenbrooke’s Hospital, Cambridge. Fresh thoracic and abdominal aorta removed by the surgical team at the time of organ donation was immediately placed in tissue medium and transported to the Addenbrooke’s Hospital, where it was processed immediately. Each specimen was trimmed free of blood vessels, fat and any surrounding deposits. A sample of tissue from the ascending aortic rings was chopped into small pieces and preserved
overnight at 4°C in a tube containing RNA\textit{later} solution. Solution was removed the following day and sample stored at -80°C for RNA extraction. All samples and patient data were handled in accordance with the policies and procedures of the Human Tissue Act, and the study was approved by the Local and Regional Ethics Committees. Informed consent was also obtained from the relatives.

**RNA Extraction, cDNA Preparation, PCR Amplification and Sequencing**

Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc) with an additional on-column DNase digestion step, according to the protocol recommended by the manufacturer. Commercially available total RNA samples extracted from human heart, human skeletal muscles, human kidney and human brain were obtained from Cell Application Inc. For cDNA synthesis, 2 $\mu$g of total RNA were used with the cDNA Archive Kit (Applied Biosystems Inc) using oligo (dT) primers in 25 $\mu$g of final volume. A control sample lacking reverse transcriptase was processed along with each cDNA synthesis in order to detect genomic DNA contamination. For subsequent PCR reactions, 1 $\mu$l of cDNA mixture was used together with Platinum Taq-Polymerase (Invitrogen Inc) or KOD-polymerase (Novagen Inc) in a final volume of 25 $\mu$l. Primers were designed with Vector NTI 11.0 software (**Supplementary Table S2**). Amplification products of appropriate size were excised from agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen Inc); recovered DNA fragments were cloned with TOPO TA Cloning® Kit (Invitrogen Inc). Three independent clones for each sample were selected for follow-up sequencing to avoid possible reading errors. We used BigDye® Terminator
v1.1 kit (Applied Biosystems Inc) for sequencing reaction and samples were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems Inc).

Total RNA was extracted from human aortic tissue using the TRIzol® Plus RNA Purification System (Invitrogen). Extraction of RNA was conducted according to the manufacturer’s protocol and was further purified using PureLink™ silica-gel spin columns followed by DNase I digestion to minimize genomic DNA contamination (Invitrogen, PureLink™ RNA Mini Kit). First strand cDNA synthesis was performed on 1 µg total RNA using AMV reverse transcriptase according to manufacturer instructions (Reverse Transcription System, Promega). Reverse transcription was initiated using random hexamer primers and the reaction carried out at 42°C for 60 min, followed by heat inactivation at 95°C for 5 min. PCR primers were designed to target BP432414, DB129663, BCL11B and VRK1 (Supplementary Table S2). A 5 µl aliquot of cDNA was used as template DNA in a 25 µl PCR reaction. Each reaction contained 5 pmol of each primer, 0.1 mM dNTPs, 1 U AGSGold™ DNA polymerase, 2.5 mM MgCl₂, 75 mmol/L Tris-HCL (pH 9.0), 20 mM (NH₄)₂SO₄, and 0.01% TWEEN-20. The PCR protocol consisted of 10 min at 95°C, followed by a touchdown procedure of 15 cycles of 95°C for 15 s, 68°C for 15 s, and 72°C for 15 s, decreasing annealing by 1°C per cycle. Following the initial 15 cycles the method consisted of 30 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s, with a final extension of 72°C for 10 min. To verify that the amplified product was the targeted gene, the product from one sample was sequenced at Geneservice (www.geneservice.co.uk) using Sanger sequencing followed by analysis on Applied Biosystems 3730 DNA Analyzer.
## Supplementary Table S1. Genotyping methods.

<table>
<thead>
<tr>
<th>Study</th>
<th>Genotyping Platform</th>
<th>Genotyping Calling Algorithm</th>
<th>Exclusion of SNPs used for Imputation</th>
<th>Imputation Method</th>
<th>Imputation Backbone (NCBI Build)</th>
<th>Filtering of Imputed Genotypes</th>
<th>Data Handling and Statistical Tests</th>
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<tbody>
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<td>AGES</td>
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<td>R, GenABEL and ProbABEL</td>
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<td>MACH 1.0.15</td>
<td>Hapmap CEU Release 22 Build 36</td>
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<td>LMEKIN package in R – linear mixed effects model incorporating familial covariance based on degree of relatedness</td>
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<td>Phased CEU haplotypes, HapMap Release 22 Build 36</td>
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<td>Measured genotype accounting for polygenic component</td>
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## Supplementary Table S1 (continued).

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<tr>
<th>Study</th>
<th>Genotyping Platform</th>
<th>Genotype Calling Algorithm</th>
<th>Exclusion of SNPs used for Imputation</th>
<th>Imputation Method</th>
<th>Imputation Backbone (NCBI build)</th>
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<td>SARDINIA</td>
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<td>Excess Mendelian inconsistencies</td>
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### Replication Cohorts

- **ACCT**  
  ABI Prism 7900

- **Aスクレピオン**  
  Sequenom
Supplementary Table S2. Primers used for reverse transcriptase polymerase chain reaction (RT-PCR) amplification.

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<th>Primer Name</th>
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<td>GCTATGACAAGTTCCACTGTGG</td>
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<tr>
<td>DB129663_R1</td>
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<tr>
<td>DB129663_F2</td>
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<tr>
<td>DB129663_R2</td>
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<td>BP432414.1_F</td>
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<tr>
<td>BCL11B_F2</td>
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*Isoform 2 / Isoform 1
**Supplementary Table S3.** Genome wide association results for CFPWV with additional adjustment for mean arterial pressure.

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<th>SNP</th>
<th>Chromosome</th>
<th>Allele</th>
<th>Meta-analysis*</th>
<th>Closest Gene</th>
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<td>rs17773233</td>
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<td>Allele</td>
<td>Meta-analysis*</td>
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*Individual analyses were adjusted for age, age^2, sex, height, weight and mean arterial pressure. †LD block includes ARHGAP1, ZNF408, F2, CKAP5 and LRP4.
Supplementary Table S4. Summary of expression data for genes and expressed sequence tags (EST’s) associated with the chromosome 14 locus in human tissues and cell lines.

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<tr>
<th>RNA source</th>
<th>DB129663</th>
<th>BP432414</th>
<th>BCL11B</th>
<th>VRK1</th>
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<td>-</td>
<td>+*</td>
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<td>+</td>
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<td>Human aortic rings</td>
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<td>CD3+ cells</td>
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</table>

DB129663 and BP432414 are human EST’s. *Detected only with primers BCL11B_F1/_R1. NT, not tested.
Supplementary Figure S1. Forest plot for COL4A1.

Results for individual cohorts are plotted against the cohort effect size (beta coefficient). Sardinia and HAPI were involved in the original report and are therefore presented separately from the 9 replication cohorts. The final point represents the meta-analysis of all 11 cohorts. The size of each box is inversely proportional to the estimated variance of the effect-size estimator. Horizontal lines are the 95% confidence intervals. Diamonds represent the results of meta-analyses.
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Replication Sites

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Clinical Endpoint Consortia

Please refer to cited sources for a full listing of investigators, sources of funding and acknowledgements for the CARDIoGRAM consortium,\(^{14}\) the CHARGE heart failure GWAS consortium,\(^{15}\) the Pulse Pressure/Mean Arterial Pressure working group of the International Consortium for Blood Pressure GWAS,\(^{16;17}\) the neuroCHARGE consortium\(^{18}\) and the CKDGen consortium.\(^{19}\)
Supplemental References


