Genetic Architecture of Ambulatory Blood Pressure in the General Population

Insights From Cardiovascular Gene-Centric Array


See Editorial Commentary, pp 1035–1037

Abstract— Genetic determinants of blood pressure are poorly defined. We undertook a large-scale, gene-centric analysis to identify loci and pathways associated with ambulatory systolic and diastolic blood pressure. We measured 24-hour ambulatory blood pressure in 2020 individuals from 520 white European nuclear families (the Genetic Regulation of Arterial Pressure of Humans in the Community Study) and genotyped their DNA using the Illumina HumanCVD BeadChip array, which contains \( \approx 50 \ 000 \) single nucleotide polymorphisms in >2000 cardiovascular candidate loci. We found a strong association between rs13306560 polymorphism in the promoter region of MTHFR and CLCN6 and mean 24-hour diastolic blood pressure; each minor allele copy of rs13306560 was associated with 2.6 mm Hg lower mean 24-hour diastolic blood pressure \( (P=1.2 \times 10^{-8}) \). rs13306560 was also associated with clinic diastolic blood pressure in a combined analysis of 8129 subjects from the Genetic Regulation of Arterial Pressure of Humans in the Community Study, the CoLaus Study, and the Silesian Cardiovascular Study \( (P=5.4 \times 10^{-6}) \). Additional analysis of associations between variants in gene ontology-defined pathways and mean 24-hour blood pressure in the Genetic Regulation of Arterial Pressure of Humans in the Community Study showed that cell survival control signaling cascades could play a role in blood pressure regulation. There was also a significant overrepresentation of rare variants (minor allele frequency: <0.05) among polymorphisms showing at least nominal association with mean 24-hour blood pressure indicating that a considerable proportion of its heritability may be explained by uncommon alleles. Through a large-scale gene-centric analysis of ambulatory blood pressure, we identified an association of a novel variant at the MTHFR/CLCN6 locus with diastolic blood pressure and provided new insights into the genetic architecture of blood pressure. (Hypertension. 2010;56:1069-1076.) *Online Data Supplement*

Key Words: gene ■ genetics ■ blood pressure ■ single nucleotide polymorphism ■ association ■ heritability

Rais ed blood pressure (BP) is the single most important risk factor for cardiovascular diseases worldwide. BP is a complex trait with significant heritability. However, a majority of the causative genes and related molecular mechanisms remains largely unknown. Recent candidate gene studies and the first genome-wide association scans (GWAS) have revealed that at least a fraction of BP-associated genes map to functionally and/or clinically important signaling cascades of cardiovascular regulation. This indicates that BP gene discovery may be greatly facilitated by large-scale systematic analysis of variants in pathways of cardiovascular regulation. The recently developed Illumina HumanCVD BeadChip, herewith called the 50K IBC array, permits simultaneous genotyping of \( \approx 50 \ 000 \) common and low-frequency single nucleotide polymorphisms (SNPs) in >2000 candidate genes and loci with the highest functional relevance.

Received April 28, 2010; first decision May 24, 2010; revision accepted October 6, 2010.

From the Departments of Cardiovascular Sciences (M.T., R.D., P.S.B., C.P.N., P.C., M.D., V.C., S.R., P.v.d.H., N.J.S.), Genetics (R.H.), and Health Sciences (P.R.B., J.R.T., M.D.T.), University of Leicester, Leicester, United Kingdom; Leicester National Institute for Health Research Biomedical Research Unit in Cardiovascular Disease (M.T., P.R.B., N.J.S.), Glenfield Hospital, Leicester, United Kingdom; University Medical Center Groningen (P.v.d.H.), University of Groningen, Groningen, the Netherlands; GlaxoSmithKline (D.W., K.S., V.M.), Philadelphia, Pa; Department of Medicine (P.V., G.W.), Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; Department of Internal Medicine, Diabetology, and Nephrology (E.Z.-S.), Medical University of Silesia, Zabrze, Poland; School of Science and Engineering (F.J.C.), University of Ballarat, Ballarat, Australia.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Correspondence to Nilesh J. Samani, Department of Cardiovascular Sciences, University of Leicester, Clinical Sciences Wing, Glenfield Hospital, Leicester LE3 9QP, United Kingdom. E-mail njs@le.ac.uk

© 2010 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.110.155721
to cardiovascular system. The overall genetic coverage for many genes related to cardiovascular disease is denser on this array than on traditional genotyping platforms used in GWAS. Such a selection of genetic variants enables investigations of major cardiovascular pathways at an unprecedented scale without compromising the depth of coverage and allows important questions about the genetic architecture of BP to be examined.

Here we report the results of the first large-scale experiment using the new 50K IBC array in relation to mean 24-hour systolic and diastolic BP (SBP and DBP) in a cohort of subjects whose clinic BP values and the prevalence of hypertension are representative of the British general population.

### Methods

#### Studies, Subjects, and Phenotypes

The major stages of our research strategy are presented in Figure 1. The primary analysis using the 50K IBC array was performed in subjects recruited as part of the Genetic Regulation of Arterial Pressure of Humans in the Community (GRAPHIC) Study. Validation of the main findings from the GRAPHIC Study was carried out in subjects from 2 additional European white cohorts, the CoLaus Study and the Silesian Cardiovascular Study (SCS).

**GRAPHIC Study**

Details of recruitment and phenotyping of the GRAPHIC subjects are described elsewhere. Briefly, nuclear families (all of white European ancestry) with both parents (aged 40 to 60 years) and 2 adult offspring (aged ≥18 years) were identified through general practices in Leicestershire. Participants had a detailed history taken and were examined by research nurses following standard protocol. Three clinic BP readings were made using an OMRON HEM-705CP digital BP monitor. Clinic BP was defined as the mean of the second and third readings. Ambulatory BP was measured using a SpaceLabs 90207 monitor (SpaceLabs) for 26 hours. The first 2 hours of each record was discarded to avoid an alerting response. Readings were taken at 30-minute intervals between 8:00 AM and 10:00 PM and hourly between 10:00 PM and 8:00 AM. The ambulatory BP data were summarized weighing each time period proportional to its length. A total of 2037 subjects from 520 families underwent successful ambulatory BP monitoring and were genotyped in this project. Because of low overall genotyping call rate (<90%) or incomplete phenotypic information, 17 individuals were excluded from the current association analysis.

**CoLaus Study and SCS**

The recruitment strategy and phenotyping of these cohorts have been described elsewhere, and further details are given in online Data Supplement (please see http://hyper.ahajournals.org). Full genetic and phenotypic information was available for 5356 and 753 genetically unrelated subjects from CoLaus and SCS, respectively. The studies were conducted according to the principles expressed in the Declaration of Helsinki, approved by their respective local bioethical committees, and all of the subjects gave informed, written consent for participation.

#### Genotyping and Quality Control Filters

**Genotyping Platform**

Detailed description of the strategy for the selection of genes on the Illumina HumanCVD BeadChip are provided elsewhere. A list of the loci on the array is available at http://bmic.upenn.edu/cvdsnp/. Further description of tagging of loci is provided in the online Data Supplement.

**Primary Genetic Association Analysis**

A total of 200 ng of leukocyte DNA from each subject in GRAPHIC Study was hybridized to 50K IBC arrays (version 2) on an Illumina Bead Station 500 (Illumina, Inc), using protocols specified by the manufacturer. Alleles were called using the Illumina BeadStudio (v3) Genotyping Module (based on GenCall Software algorithm for clustering, calling, and scoring genotypes). The quality of cluster plots was visually inspected by 2 independent investigators.

**DNA Analysis in the CoLaus Cohort and the SCS**

Genotypes of SNPs showing significant and suggestive associations with mean 24-hour BP in GRAPHIC Study (n=17) were extracted in silico from GWAS conducted in the CoLaus cohort using the Affymetrix Genome-Wide Human SNP Array 5.0. Of these, 5 SNPs were directly genotyped by the Affymetrix array, and 10 were imputed. Information on 2 SNPs (rs4648310 and rs17037388) was not available. The SNP that showed consistent association with BP in the GRAPHIC Study was hybridized to 50K IBC arrays (version 2) on an Illumina HumanCVD BeadChip and was genotyped in the SCS using commercially available TaqMan assays on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

#### Statistical Analysis

Heritability of BP was estimated using an algorithm implemented in Sequential Oligogenic Linkage Analysis Routines (SOLAR v2.0) software. The x² test was used in the parental generation of the

---

**Figure 1.** Major stages of the research strategy.
GRAPHIC Study to examine whether distributions of genotypes for each SNP on the 50K IBC array were concordant with Hardy-Weinberg equilibrium in the parental generation of the GRAPHIC Study. SNPs that passed this and additional filters (see the online Data Supplement) were examined for their associations with BP under an additive model of inheritance using generalized estimating equations with exchangeable correlation structure to account for familial correlations and with adjustment for age, age^2, and sex. Association analysis of top SNPs in the GRAPHIC Study with clinic BP in the CoLaus and SCS cohorts was conducted on age-, age^2-, and sex-adjusted linear regression models fitted in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) under additive model of inheritance. BP values of subjects on antihypertensive treatment in all of the cohorts were adjusted for the BP-lowering effect of therapy using a semiparametric algorithm in GRAPHIC and SCS or adding a constant of 15 mm Hg (to SBP) and 10 mm Hg (to DBP) in CoLaus. Genetic effects are shown as \( \beta \)-coefficients (\( \beta \)) per each extra minor allele copy of an SNP with the respective SE. Two sensitivity analyses (no treatment correction or exclusion of subjects on antihypertensive treatment) were undertaken to examine whether antihypertensive therapy had any impact on the observed associations.

To correct for multiple testing in the GRAPHIC Study for each association with the 2 principal phenotypes (mean 24-hour SBP and DBP), we calculated false-positive discovery rate (q-values) based on the method proposed by Storey and Tibshirani and available in the QVALUE software (http://genomine.org/qvalue/). The q-value thresholds of 0.05 and 0.25 were selected to identify the findings that represent significant and suggestive associations, respectively.

Fixed-effect meta-analysis of association between rs13306560 and clinic BP in the GRAPHIC, CoLaus, and SCS was conducted under additive model of inheritance using inverse variance-weighted averages of \( \beta \)-coefficients and SE in METAL (http://www.sph.umich.edu/csg/abecasis/metal/index.html). The between-study heterogeneity was evaluated using the \( \chi^2 \) test.

Two-tail Fisher exact test was used to analyze the distributions of rare, exonic, and nonsynonymous variants across different strata of associations with mean 24-hour mean SBP and DBP in the GRAPHIC Study.

The power estimates of the GRAPHIC Study to detect both nominal (\( P < 0.05 \)) and Bonferroni-adjusted (33577 tests) effects of various sizes for mean 24-hour SBP and DBP across a range of different minor allele frequencies were derived using the average SE observed at each allele frequency and constructed in STATA.

An extended description of the statistical analysis is available in the online Data Supplement.

Bioinformatic Analysis

All of the SNPs on the 50K IBC array were annotated to specific loci using resources available in the public domain, including information on design of the array at http://bmc.cavu.mpimp-golm.mpg.de/50k SNP, National Center for Biotechnology Information (Built 36.3), and the SNP Function Portal web-based application for analysis of human genetic variants. To examine in more detail associations between mean 24-hour BP and genes linked previously to SBP and DBP, we identified such genes using the Gene Ontology Annotation Database (http://www.ebi.ac.uk/GOA/downloads.html). The details of this Gene Ontology Annotation Database–based strategy are shown in Table S1 (available in the online Data Supplement). Identification of processes associated with mean 24-hour SBP and DBP was conducted using the Gene Set-Based Analysis of Polymorphisms web interface. The Gene Ontology (GO) database was used as a reference repository of functionally annotated biological processes.

An extended description of the bioinformatic analysis is provided in the online Data Supplement.

Results

Subjects

The characteristics of the GRAPHIC subjects included in the analysis are shown in Table 1. The relevant characteristics of the CoLaus and SCS cohorts are listed in Table S2.

### Table 1. Characteristics of the GRAPHIC Study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fathers (n=511)</th>
<th>Mothers (n=512)</th>
<th>Sons (n=508)</th>
<th>Daughters (n=498)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>53.8 (4.3)</td>
<td>51.8 (4.4)</td>
<td>25.0 (5.1)</td>
<td>25.9 (5.4)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.8 (3.9)</td>
<td>27.1 (4.5)</td>
<td>24.9 (4.1)</td>
<td>24.6 (5.0)</td>
</tr>
<tr>
<td>Clinic SBP, mm Hg</td>
<td>137.3 (18.4)</td>
<td>128.5 (18.3)</td>
<td>127.7 (13.0)</td>
<td>113.8 (11.9)</td>
</tr>
<tr>
<td>Mean 24-h SBP, mm Hg</td>
<td>124.3 (11.5)</td>
<td>117.0 (11.4)</td>
<td>120.8 (8.1)</td>
<td>112.8 (7.2)</td>
</tr>
<tr>
<td>Clinic DBP, mm Hg</td>
<td>86.0 (10.7)</td>
<td>80.8 (10.4)</td>
<td>75.9 (9.6)</td>
<td>73.6 (8.6)</td>
</tr>
<tr>
<td>Mean 24-h DBP, mm Hg</td>
<td>77.7 (7.2)</td>
<td>71.7 (7.6)</td>
<td>69.2 (6.5)</td>
<td>67.9 (5.2)</td>
</tr>
<tr>
<td>Antihypertensive treatment, %</td>
<td>77 (15.1)</td>
<td>53 (10.4)</td>
<td>3 (0.6)</td>
<td>1 (0.2)</td>
</tr>
</tbody>
</table>

Data are means and SDs or counts and percentages.

**Heritability of Mean 24-Hour and Clinic BP in the GRAPHIC Study**

The narrow-sense heritability (\( h^2 \)) of mean 24-hour SBP was marginally higher than that of clinic SBP (\( h^2 = 0.33, SE = 0.05 \), \( P = 8.4 \times 10^{-14} \) versus \( h^2 = 0.31, SE = 0.04, P = 1.2 \times 10^{-13} \), respectively). The mean 24-hour DBP showed more significant heritability than clinic DBP (\( h^2 = 0.41, SE = 0.05, P = 3.7 \times 10^{-20} \) versus \( h^2 = 0.32, SE = 0.04, P = 3.3 \times 10^{-15} \), respectively).

**Associations Between SNPs on the 50K IBC Array and Mean 24-Hour SBP and DBP in the GRAPHIC Cohort**

Of 49,094 genotyped SNPs, 15,517 SNPs were excluded from analysis for reasons given in the online Data Supplement. Of 33,577 SNPs that passed all of the quality filters and were included in further analysis, 32,939 were annotated to 3036 loci. The remainder (n=638) were mapped to hypothetical genes.

The distributions of nominal \( P \) values for the associations of mean 24-hour SBP and DBP are shown in Figure S1. The power to detect associations with each principal phenotype is shown in Figure S2. The genomic control (\( \lambda \)) coefficients computed for mean 24-hour SBP and DBP showed no inflation of the association statistic driven by stratification (\( \lambda = 0.972 \) and \( \lambda = 0.973 \), respectively). A total of 1782 and 1842 SNPs that passed quality filters showed at least nominal (\( P < 0.05 \)) association with mean 24-hour SBP and DBP, respectively. These nominal associations mapped to 738 (mean 24-hour SBP) and 762 (mean 24-hour DBP) loci.

After calculation of false-positive discovery rate, only 1 SNP (rs2797221) in the Usher syndrome type IIa protein gene (USH2A) showed a significant association with mean 24-hour SBP; each extra minor allele copy of rs2797221 was associated with 3.9-mm Hg lower adjusted mean 24-hour SBP (\( P = 8.6 \times 10^{-7}, q = 0.0285 \); Table 2). Four other SNPs (rs9892909 in SPHK1, rs2283210 in KCNQ1, rs2623410 in PDE1A, and rs443095 in THBS4) showed suggestive associations with mean 24-hour SBP (Table S3). The associations associated with mean 24-hour DBP were suggestive of associations with mean 24-hour BP in the GRAPHIC Study.
of these 5 SNPs with mean 24-hour DBP are presented in Tables 2 and S4. Only 1 SNP (rs13306560 in the region between the 5,10-methylenetetrahydrofolate reductase [NADPH] gene [MTHFR] and chloride channel 6 gene [CLCN6]) showed a significant association with mean 24-hour DBP after calculation of false-positive discovery rate; each minor allele copy of rs13306560 was associated with 2.6 mm Hg lower mean 24-hour DBP (Table S9). We also examined the association of rs13306560 with mean daytime DBP at both time periods; the association with nocturnal DBP was stronger than that of daytime DBP (daytime: \( \beta = -2.30, \text{SE}=0.56, P=3.4\times10^{-5} \); nighttime: \( \beta = -3.37, \text{SE}=0.51, P=2.9\times10^{-11} \)).

**Evaluation of Significant and Suggestive Association Signals Identified in Analysis of Mean 24-Hour BP Using Clinic BP in the GRAPHIC Study, CoLaus Cohort, and SCS**

The majority of SNPs showing significant or suggestive associations with mean 24-hour SBP (n=5) and DBP (n=13) were also associated (in most instances at a lower level of significance) with clinic BP in the GRAPHIC Study (Tables 3 and S4). However, only one of the associations identified in analysis of mean 24-hour BP in the GRAPHIC Study was confirmed in relation to clinic BP in the CoLaus cohort (rs13306560 and clinic DBP; \( P=0.0322 \)).

**Table 2. SNPs Showing Significant Associations With Mean 24-Hour BP in the GRAPHIC Study**

<table>
<thead>
<tr>
<th>Ch</th>
<th>Locus</th>
<th>SNP</th>
<th>Minor (Coded)/ Major Allele</th>
<th>MAF</th>
<th>HWE</th>
<th>( \beta ) (SE)</th>
<th>( P )</th>
<th>( q )</th>
<th>Mean 24-Hour SBP</th>
<th>( \beta ) (SE)</th>
<th>( P )</th>
<th>( q )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>USH2A</td>
<td>rs2797221</td>
<td>T/C</td>
<td>0.0112</td>
<td>1</td>
<td>-3.92 (0.79)</td>
<td>8.6\times10^{-7}</td>
<td>0.0285</td>
<td></td>
<td>-2.44 (0.98)</td>
<td>0.0131</td>
<td>0.7796</td>
</tr>
<tr>
<td>1</td>
<td>MTHFR/CLCN6</td>
<td>rs13306560</td>
<td>A/G</td>
<td>0.0527</td>
<td>0.1963</td>
<td>-3.27 (0.72)</td>
<td>6.1\times10^{-6}</td>
<td>0.0815</td>
<td></td>
<td>-2.63 (0.46)</td>
<td>1.2\times10^{-8}</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

Ch indicates chromosome; HWE, statistical significance of Hardy-Weinberg equilibrium test (in parents); \( \beta \), estimated quantitative effect of each SNP minor allele copy on mean 24-hour BP (adjusted for age, age\(^2\), sex, and antihypertensive medication); \( q \), lower BP in carriers of the minor allele; \( q \) value, false positive discovery rate.

**Table 3. rs13306560 and Clinic BP in the GRAPHIC Study and Validation Cohorts**

<table>
<thead>
<tr>
<th>Study</th>
<th>Informative Subjects</th>
<th>Minor (Coded) Allele</th>
<th>( \beta ) (SE)</th>
<th>( P )</th>
<th>( \beta ) (SE)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRAPHIC</td>
<td>2020*</td>
<td>A</td>
<td>-3.07 (1.06)</td>
<td>0.0039</td>
<td>-2.60 (0.68)</td>
<td>0.0001</td>
</tr>
<tr>
<td>CoLaus†</td>
<td>5356*</td>
<td></td>
<td>-1.20 (0.85)</td>
<td>0.1589</td>
<td>-1.16 (0.54)</td>
<td>0.0322</td>
</tr>
<tr>
<td>SCS</td>
<td>753*</td>
<td></td>
<td>-6.17 (3.01)</td>
<td>0.0407</td>
<td>-4.53 (1.74)</td>
<td>0.0094</td>
</tr>
<tr>
<td>Combined‡</td>
<td>8129*</td>
<td></td>
<td>-2.12 (0.65)</td>
<td>0.0011</td>
<td>-1.87 (0.41)</td>
<td>5.4\times10^{-6}</td>
</tr>
</tbody>
</table>

\( P \) values were obtained from tests adjusted for age, age\(^2\), sex, antihypertensive treatment, and familial correlations (where appropriate).

*Data show the no. of subjects with available genotype and phenotype information.

†rs13306560 was imputed in the CoLaus cohort (\( r^2 \text{hat}=0.88 \)).

‡Data are based on inverse variance-weighted fixed-effects meta-analysis; \( P \) values of heterogeneity test were 0.1512 and 0.0744 for SBP and DBP, respectively.

**MTTHFR/CLCN6/NPPB Locus: Linkage Disequilibrium and Functional Analysis In Silico**

rs13306560 maps to the promoter region of MTHFR and CLCN6 (http://genome.ucsc.edu/cgi-bin/hgTracks). Four other MTHFR SNPs (rs17037388, rs17037390, rs17367504, and rs13306561) were also associated with mean 24-hour DBP at \( q<0.25 \) (Table S5). One of these SNPs, rs17367504, was identified as the lead association signal at this locus in a recent GWAS meta-analysis of clinical BP. These 4 additional SNPs are in strong linkage disequilibrium (LD; as measured.
by $r^2$ ($r^2>0.8$) with each other (Figure 2). However, they are only in a very weak LD with rs13306560 ($r^2=0.27$ to 0.28), rs13306560 is also in weak LD ($r^2=0.3$ in the GRAPHIC Study), with rs11801879—natriuretic peptide precursor B (NPPB) polymorphism showing suggestive association with mean 24-hour DBP in our discovery cohort (Figure 2 and Table S5). Genetic variation within this locus was associated previously with BP in subjects of European ancestry. However, our conditional analysis (see the online Data Supplement) indicated that the association signals at rs17367504 and rs11801879 were both driven by their LD with rs13306560. HapMap-based LD examination in Utah residents with ancestry from northern and western Europe (CEU population) revealed only 1 polymorphism (rs13306567) as a statistically similar proxy of rs13306560 ($r^2=0.83$) within its adjacent 500 kb (Figure S3). The proxy maps to intron 3 of MTHFR (5178 bp from rs13306560) and is not present on the 50K IBC array. All of the other SNPs within the 500 kb distance of rs13306560 exhibit very weak LD with it (all $r^2<0.4$) (Figure S3). rs13306560 is the only confirmed polymorphism within the intergenic junction of MTHFR and CLCN6 (Figure S4). This short segment shows a high GC content (64.4%) and is a part of the larger 1104-bp CpG island spanning the 5’ flanking regions of both genes (Figure S4). The region lies within 6 quantitative trait loci for BP identified in rats and 1 quantitative trait loci for atherosclerosis in the mouse (Figure S4). It exhibits significant conservation across placental mammals and scores high in evolutionary and sequence pattern extraction through reduced representations program (that discriminates enhancer elements from neutral DNA sites with >90% accuracy). rs13306560 lies in the center of the most conserved part of this region; its ancestral allele is conserved not only in primates and other mammals but also in higher vertebrates (opossum).

**Associations Between Mean 24-Hour SBP and DBP and BP-Relevant Candidate Genes**

To specifically investigate genes on the 50K IBC array with direct relevance to BP, we first identified GO categories that include “blood pressure” terms using the Gene Ontology Annotation database (Table S1). This revealed 113 GO processes with 129 genes, of which 110 were present on the array. A total of 105 of these genes were well tagged (tier 1 or 2; Tables S10 and S11). In total, 1982 SNPs were typed in these genes. Of 105 sufficiently tagged genes, only 3 (2.9%; NPPB, KCNJ1, and PTGS2) had SNPs within the stratum of suggestive associations ($q<0.25$) with mean 24-hour SBP or DBP. Of the remaining 102 genes, only 1 (NPPA) had SNPs associated with mean 24-hour BP below the threshold of significance, above which associations could be attributed to pure chance only ($q>0.5$; Tables S10 and S11).

We also examined the coverage on the 50K IBC array for 17 SNPs implicated recently in BP regulation through large-scale meta-analyses of GWAS. Apart from the MTHFR variant discussed earlier, only 4 other SNPs were directly represented or had good proxies on the array. Of these, 2 SNPs (rs3184504 in the SH2B3 locus and rs17696736 in the C12orf30 locus; both on chromosome 12) showed evidence of association. Both the direction of allelic association and effect size estimates of these 2 SNPs were consistent with the data from the meta-analyses of GWAS (Table S12).

**Pathwayomic Analysis of Mean 24-Hour SBP and DBP**

Gene set-based analysis of polymorphisms analysis identified 5 biological processes as significantly associated with mean 24-hour BP after correction for multiple testing (Table S13). Of these, 2 processes (protein kinase cascade, GO:0007243, and regulation of cell proliferation, GO:0042127) were implicated in the regulation of both mean 24-hour SBP and DBP (Table S13).

**Rare, Exonic, and Nonsynonymous Variants and Mean 24-Hour SBP and DBP**

There were no significant differences in distribution of exonic or nonsynonymous variants among SNPs showing at least nominal ($P<0.05$) associations with mean 24-hour BP and those represented overall on the genotyping platform (Table 4). On the other hand, rare SNPs (those with minor allele frequency $|MAF|<0.05$) were overrepresented within the stratum of variants nominally associated with both mean

---

**Figure 2. MTHFR/CLCN6/NPPB locus:** association with mean 24-hour DBP in the GRAPHIC Study. Associations of individual SNPs with mean 24-hour DBP in the GRAPHIC Study are plotted as $-\log_{10}P$ against chromosomal bp position. rs13306560 is shown as a red diamond, and its LD relationship with the other markers (including rs17367504 and rs11801879) is indicated by color shading (whereby: red is $r^2>0.8$ and white is $r^2<0.2$). The locations of known genes in the region are shown in green, and the recombination hotspots are represented as blue peaks. MTHFR, 5,10-methylenetetrahydrofolate reductase (NADPH) gene; CLCN6, chloride channel 6 gene; NPPB, natriuretic peptide precursor B gene; NPPA, natriuretic peptide precursor A gene; AGTRAP, angiotensin II receptor-associated protein gene.
24-hour SBP and DBP ($P=8.7 \times 10^{-5}$ and $P=0.0036$, respectively; Table 4). This difference became even more striking when applying a lower threshold for definition of uncommon polymorphisms (MAF < 0.02); SNPs with MAF below this cutoff were almost two times more common among variants associated with mean 24-hour BP than expected by chance ($P=3.0 \times 10^{-6}$ and $P=2.4 \times 10^{-7}$ for mean 24-hour SBP and DBP, respectively; Table 4). However, neither exonic nor exclusively nonsynonymous SNPs were overrepresented within the stratum of significant rare variants (Table 4), irrespective of the criterion of rare variant definition. Sensitivity analysis conducted using a lower level of statistical significance ($P<0.01$) confirmed these findings (Table S14).

### Discussion

Our study has uncovered novel alleles, genes, and pathways of BP regulation while showing that a majority of the previously examined candidates are not (or only weakly) associated with mean 24-hour BP. Mean 24-hour BP probably does not provide distinct information compared with clinic SBP and DBP. However, it shows higher reproducibility, closer correlations with indices of target organ damage and better predictive value of cardiovascular morbidity.\(^{22,23}\)

The higher heritability of mean 24-hour BP than that of clinic measurements demonstrated particularly well for DBP in the GRAPHIC families suggests that 24-hour ambulatory BP monitoring may be particularly informative in gene discovery. However, because of unavailability of the replication resource with 24-hour ambulatory BP monitoring, we validated our primary association findings by using clinic BP measurements in other independent cohorts.

Our findings need to be interpreted in the context of the power of our study. At a nominal level of significance, given the greater precision of our BP phenotypes and the population size, our study had a good power to detect effect sizes of as low as 1 mm Hg for variants with MAF $\geq 10\%$ (Figure S2). With Bonferroni correction, the power was significantly diminished because of the large number of SNPs analyzed (Figure S2). However, Bonferroni adjustment is probably overconservative in the context of SNPs on this array, given their significant LD-driven correlations and nonindependence. Nonetheless, our top signal (rs13306560) from analysis of mean 24-hour DBP survived correction for multiple testing based on q-value calculations. The identified association would retain its statistical significance even after application of more conservative thresholds based on Bonferroni correction ($1.5 \times 10^{-6}$) or those used in previous GWAS ($5.0 \times 10^{-8}$). rs13306560 also gave consistent associations in relation to clinic BP in $>8000$ subjects from 3 European populations, making it unlikely that it represents a false-positive finding.

Indeed, our conditional analysis indicates that the association with rs13306560 may largely explain the association between BP and other SNPs at the MTHFR/CLCN6/NPPB locus reported in recent GWAS.\(^{8,9}\) Quantitatively, the magnitude of rs13306560 effect on BP is one of the largest genetic effects that has been reported to date for a relatively common variant. Furthermore, rs13306560 shows a significant functional potential itself in silico. Indeed, the alleles of rs13306560 are conserved in mammals and the SNP maps to CpG island in the MTHFR/CLCN6 promoter with evidence of operation of selective pressure (to maintain the sequence in this DNA fragment) throughout mammalian evolution. One possible mechanism by which rs13306560 could, therefore, mediate the association with BP is through differential methylation of the MTHFR/CLCN6 promoter in the 2 alleles. MTHFR has a strong biological potential to influence BP regulation. It encodes an enzyme that catalyzes conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine.\(^{24}\) Circulating concentrations of homocysteine have been associated with risk of cardiovascular diseases, including hypertension, possibly through promotion of free radical-mediated endothelial damage and dysfunction.\(^{25}\) One of the common nonsynonymous MTHFR polymorphisms (rs1801133, $-C677T$ or $A222V$) known to produce a thermostable enzyme isofrom with reduced activity in TT homozygotes\(^ {29}\) was overrepresented in patients with hypertension.\(^ {26}\) However, rs1801133 is not in LD with rs13306560 ($r^2=0.02$) and showed only nominal association with mean

### Table 4. Distribution of SNPs Associated Nominally ($P<0.05$) With Mean 24-Hour BP According to Their Characteristic and Frequency

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Illumina Gene-Centric Array</th>
<th>SNPs Associated With Mean 24-Hour SBP at $P&lt;0.05$</th>
<th>$P$</th>
<th>SNPs Associated With Mean 24-Hour DBP at $P&lt;0.05$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All SNPs</td>
<td>33,577</td>
<td>1,782</td>
<td>...</td>
<td>1,842</td>
<td>...</td>
</tr>
<tr>
<td>Exonic SNPs</td>
<td>2,298 (6.8%)</td>
<td>126 (7.1%)</td>
<td>0.7004</td>
<td>138 (7.5%)</td>
<td>0.2770</td>
</tr>
<tr>
<td>Nonsynonymous SNPs</td>
<td>1,634 (4.9%)</td>
<td>84 (4.7%)</td>
<td>0.8211</td>
<td>94 (5.1%)</td>
<td>0.6175</td>
</tr>
<tr>
<td>All SNPs with MAF &lt; 0.05</td>
<td>4,355 (13.0%)</td>
<td>290 (16.3%)</td>
<td>$8.7 \times 10^{-5}$</td>
<td>283 (15.4%)</td>
<td>0.0036</td>
</tr>
<tr>
<td>Exonic SNPs with MAF &lt; 0.05*</td>
<td>516 (11.8%)</td>
<td>32 (11.0%)</td>
<td>0.7778</td>
<td>34 (12.0%)</td>
<td>0.9244</td>
</tr>
<tr>
<td>Nonsynonymous SNPs with MAF &lt; 0.05*</td>
<td>406 (9.3%)</td>
<td>24 (8.3%)</td>
<td>0.6021</td>
<td>24 (8.5%)</td>
<td>0.7507</td>
</tr>
<tr>
<td>All SNPs with MAF &lt; 0.02</td>
<td>1,188 (3.5%)</td>
<td>104 (5.8%)</td>
<td>$3.0 \times 10^{-6}$</td>
<td>112 (6.1%)</td>
<td>$2.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>Exonic SNPs with MAF &lt; 0.02†</td>
<td>162 (13.6%)</td>
<td>14 (13.5%)</td>
<td>1.0</td>
<td>20 (17.9%)</td>
<td>0.2530</td>
</tr>
<tr>
<td>Nonsynonymous SNPs with MAF &lt; 0.02†</td>
<td>128 (10.8%)</td>
<td>10 (9.6%)</td>
<td>0.8685</td>
<td>14 (12.5%)</td>
<td>0.5292</td>
</tr>
</tbody>
</table>

\*Data show the percentage calculated in relation to total No. of SNPs with MAF < 0.05.
†Data show the percentage calculated in relation to total No. of SNPs with MAF < 0.02.
24-hour DBP in the GRAPHIC Study. rs1801133 was also only weakly associated with clinic DBP in the recent GWAS meta-analysis of BP.¹⁹ Thus, it is unlikely that rs13306560 mediates its effect on BP through the thermolabile form of MTHFR.

CLCN6 belongs to a family of molecules that function as either transmembrane ion channels or electroneutral Cl⁻/H⁺ exchangers.²⁷ CLCN6 protein is mainly expressed in the central and peripheral nervous system, where it resides within vesicular endosomes.²⁷ Experimental disruption of CLCN6 leads to a lysosomal storage disease; CLCN6 knockout mice show reduced pain sensitivity and mild behavioral abnormalities.²⁷ CLCN6 mRNA expression was confirmed in the kidney but whether, like other members of the chloride transporters family (CLCNKB and CLCN5), it may contribute to renal ion handling is not known. Importantly, mutations in several chloride channel genes underlie monogenic forms of low BP (CLCNKB)²⁸ and defects in tubular ion reabsorption leading to chronic kidney disease (CLCN5).²⁹ Although the location of rs13306560 in the promoter of both MTHFR and CLNC6 makes them strong candidates, recent studies have shown that polymorphisms can act by influencing more distally located genes.²⁹ Therefore, at this stage we cannot exclude MTHFR, CLCN6, or in fact other genes in this locus (ie, NPPA and NPPB) as the mediator(s) of the identified association (Figure 2).

A notable finding in our study is that very few variants of the most frequently examined candidates for BP (eg, genes of the renin-angiotensin and sympathetic nervous systems), which were present on 50K IBC array, showed associations with mean 24-hour BP. These data are consistent with recent GWAS, which also did not report any definite associations with such genes.⁹ The very good genetic coverage (exceeding that of the GWAS platforms) for a majority of these strong candidate genes makes it unlikely that any of their common variants (although not genotyped directly) were not captured in this analysis. This makes our investigation one of the most comprehensive and detailed studies of these systems for their genetic impact on BP. Collectively, these results clearly suggest that common genetic variants underlying familial predisposition to hypertension may reside outside classic systems of BP regulation.¹⁰ In respect to this, our pathwayomic analysis has uncovered that signaling cascades that control cell survival through promotion of cellular growth (ie, mitogens activated through protein kinase cascades) and death (via apoptosis) may play roles in BP regulation. Future systematic analyses of these pathways may identify novel genetic determinants of human hypertension and illuminate novel molecular mechanisms of BP regulation.

Our data also show a consistent overrepresentation of rare alleles among the SNPs associated with mean 24-hour BP. Indeed, irrespective of the threshold of rare variant definition, stratum of statistical significance, and the phenotype, rare SNPs were associated with BP more frequently than expected by chance. Most strikingly, rare SNPs (MAF<0.05) also constitute the majority of significant and suggestive associations with mean 24-hour SBP (Tables 2 and S3). Interestingly, these overrepresented rare variants do not lead to amino acid substitutions within the encoded proteins and map to extraxonic regions. These results lend support to the hypothesis that rare alleles play a role in genetic predisposition to blood pressure elevation³¹ and indicate that more subtle mechanisms than those leading to changes in amino acid structure of encoded proteins may underlie human hypertension.

**Perspectives**

This large-scale gene-centric analysis identified a novel association between a potentially functional variant at the MTHFR/CLCN6/NPPB locus and BP. We also show that both rare variants and genes that reside outside the classic physiological pathways of BP regulation may be important elements of the genetic architecture of BP. Future studies should focus on replication of these findings in larger cohorts and elucidation of the functional mechanisms that underlie the uncovered associations.

**Acknowledgments**

We thank the research nurses and other staff who undertook the recruitment and phenotyping in the studies.

**Sources of Funding**

Recruitment and genotyping of the Genetic Regulation of Arterial Pressure of Humans in the Community cohort was funded by the British Heart Foundation. The CoLaus Study was supported by research grants from GlaxoSmithKline and from the Faculty of Biology and Medicine (Lausanne, Switzerland) and is currently supported by the Swiss National Science Foundation (33CSCO-122661). The Silesian Cardiovascular Study was supported by a National Institutes of Health Fogarty International Research Collaboration Award (R03 TW007165 to M.T.). N.J.S. holds a British Heart Foundation Chair of Cardiology, and V.C. is supported by the British Heart Foundation. M.D.T. holds a Medical Research Council Clinician Scientist Fellowship (G0501942). This study is part of the research portfolio supported by the Leicester National Institute for Health Research Biomedical Research Unit in Cardiovascular Disease.

**Disclosures**

D.W., K.S., and V.M. are employees of GlaxoSmithKline.

**References**


15. AC, rells B, Leitl B, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81:559–575.


