

Loci influencing blood pressure identified using a cardiovascular gene-centric array

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Blood pressure (BP) is a heritable determinant of risk for cardiovascular disease (CVD). To investigate genetic associations with systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP) and pulse pressure (PP), we genotyped ~50 000 single-nucleotide polymorphisms (SNPs) that capture variation in ~2100 candidate genes for cardiovascular phenotypes in 61 619 individuals of European ancestry from cohort studies in the USA and Europe. We identified novel associations between rs347591 and SBP (chromosome 3p25.3, in an intron of *HRH1*) and between rs2169137 and DBP (chromosome 1q32.1 in an intron of *MDM4*) and between rs2014408 and SBP (chromosome 11p15 in an intron of *SOX6*), previously reported to be associated with MAP. We also confirmed 10 previously known loci associated with SBP, DBP, MAP or PP (*ADRB1*, *ATP2B1*, *SH2B3/ATXN2*, *CSK*, *CYP17A1*, *FURIN*, *HFE*, *LSP1*, *MTHFR*, *SOX6*) at array-wide significance ($P < 2.4 \times 10^{-6}$). We then replicated these associations in an independent set of 65 886 individuals of European ancestry. The findings from expression QTL (eQTL) analysis showed associations of SNPs in the *MDM4* region with *MDM4* expression. We did not find any evidence of association of the two novel SNPs in *MDM4* and *HRH1* with sequelae of high BP including coronary artery disease (CAD), left ventricular hypertrophy (LVH) or stroke. In summary, we identified two novel loci associated with BP and confirmed multiple previously reported associations. Our findings extend our understanding of genes involved in BP regulation, some of which may eventually provide new targets for therapeutic intervention.

INTRODUCTION

Blood pressure (BP) is a cardinal risk factor for cardiovascular disease (CVD). Systolic BP (SBP) and diastolic (DBP) levels are associated with increased risk of atherosclerotic vascular disease and other cardiovascular causes of death (1). Much of the excess CVD risk imparted by BP elevation can be ameliorated through interventions to decrease BP (2). The identification of novel genes and pathways involved in BP regulation may highlight new ways of reducing BP and CVD risk associated with hypertension. The mean arterial pressure (MAP) and pulse pressure (PP, the difference between SBP and DBP) are single BP components associated with CVD risk (3–5). The latter is an indicator of conduit artery stiffness and is known to increase with age, as aortic elasticity decreases. To date, ~50 common genetic variants associated with BP and hypertension have been reported, largely through genome-wide association studies (GWAS), meta-analyses and admixture mapping approaches (6–16). The identification of common variants associated with BP may be enriched through further gene-centric approaches (17–19). Accordingly, we tested the hypothesis that candidate gene analysis would identify known and novel associations with SBP, DBP, MAP and PP and would confirm previously reported associations. To further investigate and discover associations with BP, we genotyped approximately 50 000 single-nucleotide polymorphisms (SNPs) on a gene-centric array (ITMAT-Broad_CARE [IBC] array, Illumina San Diego, CA, USA) that captures variation in ~2100 candidate genes for cardiovascular traits including BP (20) in 61 619 individuals of European ancestry. We identified two novel BP-associated loci, at the candidate genes *MDM4* and *HRH1* and have validated these associations through *in silico* replication analysis in a large set of independent samples.

RESULTS

Discovery association analyses

In the primary discovery meta-analysis, four BP traits were analyzed in 61 619 individuals from 27 cohorts, as described in Table 1. We analyzed SBP, DBP, MAP and PP as continuous traits. Cohort characteristics, including age, sex, BP values and the proportion of individuals treated with BP lowering medications, are provided in Table 1. The details of the cohorts are provided in the Supplementary Material, Table S1 and Supplementary Materials.

Association analyses were successfully carried out for up to 48 372 SNPs, and summaries of the quality-control (QC) steps and numbers of SNPs removed at each step are provided in Supplementary Material, Table S2A. Cohort-specific genomic control inflation factors, λ_{GC} , did not suggest the presence of inflation (Supplementary Material, Table S3). Meta-analysis quantile–quantile plots are shown in Supplementary Material, Fig. S1 and *P*-values for association for all SNPs are provided in Supplementary Material, Table S4. We identified 22 significant SNP-trait associations with SBP, DBP, MAP and PP at 12 different loci ($P < 2.4 \times 10^{-6}$), including two novel loci near *HRH1* and *MDM4* for BP traits and one novel SNP-trait association in the *SOX6* locus, a region previously described in association with MAP.

Replication analyses

Replication testing was performed in 65 866 additional individuals, including 43 266 individuals in seven cohorts with genome-wide SNP genotypes imputed to HapMap (Supplementary Material, Table S2B) and 22 600 individuals genotyped on the same IBC chip used for the discovery analyses. Through the joint analysis of SNPs considered relevant during the discovery phase combined with replication data, we identified robust association of 22 SNP-trait associations at 12 independent loci meeting our array-wide significance threshold of $P < 2.4 \times 10^{-6}$: six loci were associated with DBP (*MTHFR*, *MDM4*, *HFE*, *SH2B3/ATXN2*, *CSK*, *FURIN*), nine loci were associated with SBP (*MTHFR*, *HRH1*, *CYP17A1*, *LSP1*, *SOX6*, *ATP2B1*, *SH2B3/ATXN2*, *CSK*, *FURIN*), six loci were associated with MAP (*MTHFR*, *ADRB1*, *ATP2B1*, *ATXN2*, *CSK*, *FURIN*) and one locus associated with PP (*CYP17A1*). The association findings are summarized in Table 2.

We confirmed previously reported BP associations at 10 loci, and identified two novel loci: rs347591 associated with SBP (chromosome 3p25, in an intron of *HRH1*, $P = 1.57 \times 10^{-8}$) (Fig. 1A); rs2169137 associated with DBP (chromosome 1q32, in an intron of *MDM4*, $P = 5.9 \times 10^{-8}$) (Fig. 1B). We additionally found evidence of association of rs281413 (chromosome 19p13 in an intron of *ICAM3*) with DBP, in our discovery analysis, although it was not confirmed in the replication analysis ($P = 3.08 \times 10^{-6}$ in discovery, $P = 1.4 \times 10^{-5}$ in the joint analysis). Finally, one of the SNP-trait associations we identified was novel in our analysis, with the association of rs2014408 with SBP (chromosome 11p15, in an intron of *SOX6*, $P = 5.71 \times 10^{-10}$), whereas previously only association with MAP had been reported. A second Bonferroni correction of our results for testing four traits did not result in a change in the overall results, so we present the original results here, as the four traits are highly correlated. Full association results for SNPs in our discovery analysis with association $P < 1 \times 10^{-5}$ are reported in Supplementary Material, Table S5. Despite ascertainment biases in some of the discovery cohorts, due to inclusion or exclusion based upon BP or hypertension status (as noted in Supplementary Material, Table S1), we show replication of the key findings. We comprehensively compared the results of our analysis with all published associations at the time of this report (6–13,19) (Supplementary Material, Table S6). We reviewed 77 previously reported loci for our BP traits of interest and found that 43 were represented on our genotyping array, with one region containing a proxy SNP ($r^2 = 0.66$) rather than the index SNP previously reported (Supplementary Material, Table S4). At a nominal association threshold ($P = 0.05$), 32 SNPs were associated with one or more BP traits in our study, and with a multiple testing correction ($P < 0.00116$), we observed 21 SNPs with BP associations.

Sex interaction

In a secondary sex-specific analysis of our discovery sample (Supplementary Material, Table S7), we had no new significant associations. To follow-up possible sex differences in the two novel associations identified in our discovery efforts, we tested for interactions of rs347591 (*HRH1*) and rs2169137 (*MDM4*) with sex and identified a modest sex-specific effect for all four continuous BP traits at rs2169137

Table 1. Clinical characteristics of discovery and replication cohorts

	Age	Female/ male	SBP	DBP	MAP	PP	BMI	Taking anti- hypertensive medication
Discovery cohorts								
AMC-PAS	42.9 ± 5.3	180/563	128.6 ± 17.9	79.9 ± 10.7	96.1 ± 12.1	48.8 ± 13.1	26.9 ± 4.1	33.2%
Amish	47.6 ± 15.0	713/691	121.9 ± 16.5	75.3 ± 9.4	90.8 ± 10.8	46.6 ± 12.2	27.3 ± 5.0	16.4%
ARIC	54.2 ± 5.7	5124/4453	118.3 ± 17	71.5 ± 10	87.1 ± 11.3	46.7 ± 12.7	26.9 ± 4.9	25.1%
BHS	22.5 ± 4.4	291/228	111.5 ± 10.2	71.8 ± 8.5	85 ± 8.3	39.7 ± 8	24.7 ± 6.1	2.7%
CARDIA	40.6 ± 4.1	703/623	102.2 ± 30.5	71.9 ± 11.2	84.5 ± 11.4	37.7 ± 8.8	26.9 ± 6.4	3.7%
CCCS	64.2 ± 9.7	555/1402	136.94 ± 19.04	78.33 ± 10.61	156.48 ± 23.57	58.61 ± 15.69	29.2 (4.8)/ 29.8 (6.5)	88.7%
CFS	40.9 ± 19.9	302/252	121.1 ± 16.6	71.8 ± 11.7	88.2 ± 12.2	49.3 ± 12.4	30.1 ± 8.8	8.8%
CHS	72.6 ± 6.3	2208/1722	135.3 ± 21.5	69.9 ± 11.6	91.7 ± 12.9	65.4 ± 18.6	26.3 ± 4.8	39.7%
CLEAR	67.8 ± 9.6	0/1365	151.4 ± 22.4	82.2 ± 12.3	105.3 ± 13.8	69.2 ± 18.4	28.1 ± 5.0	67.0%
EPIC_NL	54.06 ± 10.11	4057/1137	133.13 ± 21.22	80.46 ± 10.93	97.98 ± 13.20	52.65 ± 15.68	26.77 ± 4.45	N/A
FHS	40.9 ± 9.1	3775/3134	118.6 ± 14.3	76.4 ± 9.8	90.5 ± 10.6	42.2 ± 9.4	26.1 ± 5	5.5%
GIRaFH	44.5 ± 11.7	882/812	134.9 ± 19.2	82.0 ± 10.5	99.7 ± 12.3	52.9 ± 14.4	25.1 ± 3.5	9.3%
GQ2	65.5 ± 10.5	385/93	130.51 ± 22.40	72.71 ± 12.89	149.78 ± 27.66	57.81 ± 18.47	29.7 (7.7)/ 29.5 (6.3)	76.2%
INVEST	69.4 ± 9.5	467/580	160.8 ± 17.4	90.6 ± 10.1	114.0 ± 10.6	70.1 ± 15.9	29.0 (4.7)/ 28.4 (6.2)	82.6%
LURIC	58.1 ± 8.6	558/1480	151.2 ± 24.4	89.8 ± 12.1	110.3 ± 15.0	61.4 ± 17.6	27.7 ± 4.2	85.6%
MEDAL	62.9 ± 9.0	1178/2820	136.8 ± 16.0	81.7 ± 9.6	94.9 ± 8.6	52.9 ± 11.5	30.5 ± 6.3	1.4%
MESA	62.7 ± 10.3	1199/1097	123.5 ± 20.8	70.1 ± 10.2	87.9 ± 12.3	53.4 ± 16.7	27.8 ± 5.1	33.3%
MONICA/ KORA F3	57.6 ± 8.1	755/649	131.8 ± 19.4	83.3 ± 10.3	99.5 ± 12.6	48.5 ± 13.2	27.8 ± 4.5	30.1%
MONICA/ KORA S12	51.8 ± 9.9	431/549	133.6 ± 19.1	81.5 ± 11.1	98.8 ± 12.5	52.1 ± 14.6	27.2 ± 4.0	16.6%
NSHS95	49.4 ± 18.4	857/899	126.6 ± 17.7	76.7 ± 11.6	93.3 ± 11.8	49.9 ± 15.8	27.1 ± 5.5	N/A
PEAR	50.1 ± 9.4	194/244	151.8 ± 12.4	98.0 ± 5.7	115.9 ± 6.9	53.7 ± 10.8	[30.3 (4.4)/ 30.4 (6.1)]	0%
PennCAC	56.0 ± 8.0	631/1145	132 ± 23.2	72.4 ± 11.2	52.6 ± 12.6	59.5 ± 19.7	29.8 ± 5.9	N/A
PennCath	52.0 ± 9.0	739/1386	127 ± 15.1	76.7 ± 9.5	62.9 ± 10.5	51.9 ± 12.3	30.1 ± 5.9	32.8%
SMART	59.36 ± 12.25	206/299	158.64 ± 18.57	94.76 ± 11.80	116.06 ± 12.87	63.88 ± 13.78	27.35 ± 4.62	39.4%
WHI	68.0 ± 6.6	7606/0	133.0 ± 18.8	75.0 ± 9.7	94.3 ± 11.0	58.0 ± 16.2	28.3 (6.2)	33.3%
Replication cohorts								
AIBIII	52.8 (9.2)	249/209	119.9 (13.7)	75.4 (7.6)	90.2 (8.8)	44.5 (10.3)	25.7 (3.6)	100.0%
ASCOT	63 (8.1)	224/1015	161.4 (17.8)	92.9 (9.9)	115.7 (10.6)	68.5 (16.1)	29.1 (4.6)	52.9%
BRIGHT (controls)	58.7 (8.9)	1088/647	123 (10.5)	76.4 (7.2)	91.9 (7.5)	46.7 (8.3)	25.3 (3.3)	100.0%
BRIGHT (cases)	58 (10.3)	1144/775	154.3 (21.1)	93.9 (11.3)	114 (13.3)	60.4 (15.7)	58 (10.3)	51.8%
BWHHS	68.85 (5.51)	3373/0	146.53 (26.59)	79.16 (12.85)	102 (15)	67.6 (19)	27.25 (5.95)	76.8%
GRAPHIC	39.30 (14.50)	1004/1020	127.09 (17.84)	79.12 (10.96)	95.1 (12.5)	48 (11.9)	26.11 (4.61)	93.7%
LIFELINES	47.3 ± 11.2	4640/3483	127.9 ± 15.7	75.1 ± 9.1	52.7 ± 11.8	92.7 ± 10.3	26.3 ± 4.3	15.5%
MDC	57.8 (5.9)	1074/772	115.6 (5.8)	73.6 (5.3)	87.6 (4.7)	42 (6)	24.3 (3.3)	100.0%
NBS	41.36 (12.37)	1183/1169	N/A	N/A	N/A	N/A	N/A	N/A
NESDA	41.5 ± 12.7	1166/551	135.3 ± 20.2	81.7 ± 11.8	99.6 ± 13.9	53.6 ± 12.7	25.5 ± 4.9	12.8%
NORDIL	56 (4)	979/940	177.3 (14.6)	105.9 (5.5)	129.7 (7.1)	71.5 (13.9)	28.3 (4.6)	100.0%
PREVEND	49.6 ± 12.5	1752/1869	129.1 ± 19.9	74.1 ± 9.9	54.9 ± 13.9	92.4 ± 12.5	26.1 ± 4.3	14.2%
Procardis	59.34 (9.93)	1634/1564	130.75 (17.11)	79.63 (10.03)	96.7 (11.2)	51.1 (13.4)	26.81 (4.37)	83.8%
Rotterdam Study	69.4 (9.1)	3327/2327	144.1 (24.2)	76.9 (12.6)	99.3 (15.0)	67.1 (18.5)	26.3 (3.7)	32.5%
TRAILS clinical cohort	15.8 ± 0.6	97/217	119.1 ± 12.6	61.0 ± 6.6	80.4 ± 7.3	58.1 ± 11.4	21.5 ± 3.6	N/A
TRAILS population cohort	16.2 ± 0.7	693/642	118.1 ± 12.4	61.1 ± 6.9	80.1 ± 7.4	57.7 ± 10.6	21.2 ± 3.2	N/A
WGHS	54.2 ± 7.1	22 625/0	125.5 ± 16.4	78.0 ± 10.7	93.8 ± 11.9	47.6 ± 10.4	25.9 ± 5.0	12.9%
WHII	60.83 (6.0)	1845/3210	128.1 (16.7)	74.6 (10.5)	92.4 (11.8)	53.5 (11.2)	26.7 (4.3)	81.4%

Mean ± standard deviation is given for each phenotype, except % were indicated. BP values shown are actual values without modification for medication treatment.

(*P*-value for interaction was 0.0058 for SBP; 0.055 for DBP; 0.014 for MAP and 0.033 for PP) (Supplementary Material, Table S8), with the association observed in women but not

in men (in females only SBP beta was 0.041, se 0.322, and in males only SBP beta was 0.82, se 0.307, *P*-value for interaction of rs2169137 with sex was 0.0058).

Table 2. Loci associated with hypertension traits using discovery and replication data (Betas and SEs corresponding to the trait are highlighted in bold)

Gene	SNP	CHR	BP	A1	A2	CAF	Beta	SE	Discovery results			PP	SBP	Replication results			PP	SBP	Discovery + replication results			SBP
									DBP	MAP	DBP			MAP	DBP	MAP			PP	SBP		
DBP																						
MTHFR	rs13306561	1	11788391	G	A	0.154	-0.5224	0.0852	8.641E-10	9.93E-12			1.33E-09	2.6E-13	6.91E-14	8.51E-04	3.82E-11	1.75E-21	4.49E-24		3E-19	
MDM4	rs2169137	1	202764536	G	C	0.271	-0.3552	0.0702	4.201E-07					0.007492				5.86E-08				
HFE	rs1799945	6	26199158	G	C	0.146	0.406	0.0864	2.615E-06					6.34E-12				2.78E-16				
ATXN2	rs10774625	12	110394602	A	G	0.505	0.4929	0.0764	1.103E-10	4.61E-10			1.56E-07	5.98E-09	8.88E-11		9.37E-10	4.94E-18	2.4E-19		8.12E-16	
CSK	rs7085	15	72882536	T	C	0.281	0.4403	0.0677	7.936E-11	5.01E-11			6.68E-11	1.58E-05	3.54E-08		1.33E-05	2.5E-14	2.14E-17		3.48E-14	
FURIN	rs2071410	15	89221944	G	C	0.371	0.3661	0.066	2.882E-08	4.30E-08			0.00013	1.19E-08	2.27E-09		2.36E-09	1.78E-15	5.31E-16		2.92E-12	
MAP																						
MTHFR	rs13306561	1	11788391	G	A	0.154	-0.6665	0.0979	8.641E-10	9.93E-12			1.33E-09	2.6E-13	6.91E-14	8.51E-04	3.82E-11	1.75E-21	4.49E-24		3E-19	
ADRB1	rs7076938	10	115779365	C	T	0.282	-0.3869	0.0776		6.05E-07				9.72E-09				3.14E-14				
ATP2B1	rs2681472	12	88533090	G	A	0.172	-0.612	0.1129		5.87E-08			3.99E-09		6.95E-10		3.3E-11		2.24E-16		7.68E-19	
ATXN2	rs10774625	12	110394602	A	G	0.505	0.5343	0.0857	1.103E-10	4.61E-10			1.56E-07	5.98E-09	8.88E-11		9.37E-10	4.94E-18	2.4E-19		8.12E-16	
CSK	rs7085	15	72882536	T	C	0.281	0.5109	0.0778	7.936E-11	5.01E-11			6.68E-11	1.58E-05	3.54E-08		1.33E-05	2.5E-14	2.14E-17		3.48E-14	
FURIN	rs6227	15	89226236	T	C	0.316	0.764	0.1506	4.081E-08	3.65E-09			3.88E-07	1.86E-06	1.02E-10		3.77E-09	9.83E-13	3.35E-18		8.01E-15	
PP																						
CYP17A1	rs3824755	10	104585839	C	G	0.096	-0.6438	0.1289			5.93E-07	2.46E-07				6.06E-04	0.000384			1.52E-09	3.77E-10	
SBP																						
MTHFR	rs13306561	1	11788391	G	A	0.154	-0.8657	0.1428	8.641E-10	9.93E-12			1.33E-09	2.6E-13	6.91E-14	8.51E-04	3.82E-11	1.75E-21	4.49E-24		3E-19	
HRH1	rs347591	3	11265122	G	T	0.345	-0.5284	0.1071					8.14E-07				0.001192				1.57E-08	
CYP17A1	rs3824755	10	104585839	C	G	0.096	-0.6438	0.1289			5.93E-07	2.46E-07				6.06E-04	0.000384			1.52E-09	3.77E-10	
LSP1	rs661348	11	1861868	C	T	0.437	0.472	0.1038				5.43E-06					7.55E-11				3.39E-15	
SOX6	rs2014408	11	16321858	T	C	0.21	0.5571	0.1246				7.74E-06					1.03E-05				5.71E-10	
ATP2B1	rs2681472	12	88533090	G	A	0.172	-0.9733	0.1654		5.87E-08		3.99E-09		6.95E-10		3.3E-11		2.24E-16		7.68E-19		
ATXN2	rs10774625	12	110394602	A	G	0.505	0.6614	0.1261	1.103E-10	4.61E-10		1.56E-07	5.98E-09	8.88E-11		9.37E-10	4.94E-18	2.4E-19		8.12E-16		
CSK	rs7085	15	72882536	T	C	0.281	0.7412	0.1135	7.936E-11	5.01E-11		6.68E-11	1.58E-05	3.54E-08		1.33E-05	2.5E-14	2.14E-17		3.48E-14		
FURIN	rs6227	15	89226236	T	C	0.316	0.764	0.1506	4.081E-08	3.65E-09		3.88E-07	1.86E-06	1.02E-10		3.77E-09	9.83E-13	3.35E-18		8.01E-15		

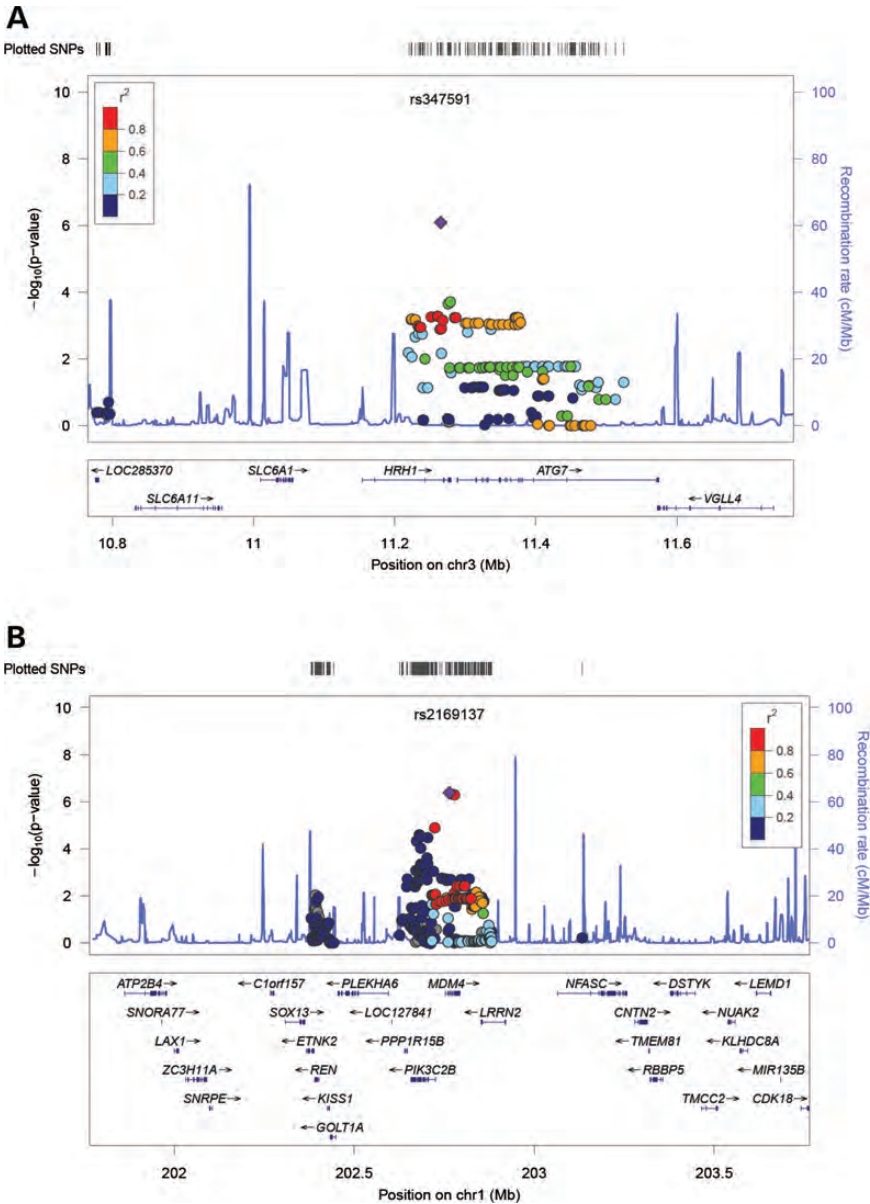


Figure 1. Regional association plots of the (A) *HRH1* and (B) *MDM4* loci are shown with negative $\log_{10}(P\text{-value})$ on the y-axis and chromosomal position on the x-axis.

Conditional analyses

For the loci described in Table 2, except those containing fewer than three genome-wide significant SNPs, conditional analyses were conducted using the allele dosage of the SNPs within a 500 kb (± 250 kb) window around the most significant SNP per locus as a covariate in a subset of discovery cohorts (ARIC, CARDIA, CHS and MESA). Statistical models were identical to those used in the discovery analyses except for the additional SNP covariates. No SNPs remained significant in these conditional models after correcting the total number of tests (SNPs) in the 500 kb window, suggesting that only the strongest signal of association at each locus explained our findings. The complete results for these analyses are shown in Supplementary Material, Table S9.

Annotations of the identified loci

Annotations for the 12 loci associated with BP traits in our study showed that the majority of the variants we identified were located within an intron of the corresponding gene (rs13306561 in *MTHFR*; rs2169137 in *MDM4*; rs3824755 in *CYP17A1*; rs661348 in *LSP1*; rs2014408 in *SOX6*; rs2681472 in *ATP2B1*; rs4766578 and rs10774625 in *ATXN2*; rs8032315 and rs2071410 in *FURIN*; rs347591 in *HRH1*). In aggregate, the associated SNPs accounted for 0.4–0.6% of the phenotypic variance in SBP, DBP, MAP and PP (Supplementary Material, Table S10). These estimates are in line with other association studies in which $<1\%$ of the overall phenotypic variance was explained by the common variants identified (6).

Expression QTL (eQTL) analysis showed that several of our associated SNPs (or proxy SNPs that were highly correlated with our lead SNP of interest [$r^2 > 0.8$]) were associated with the expression levels of nearby genes, as assessed by the microarray analysis of RNA expression in various tissues, including blood, liver, lymph tissues, lymphoblastoid cell lines (LCLs) and peripheral blood T cells and monocytes, brain, adipose tissue and liver. These results are summarized in Supplementary Material, Table S11. Testing for eQTL associations at our novel loci showed no eQTL associations in the *HRH1* region, but we did observe significant eQTL associations in the *MDM4* region. Expression of *MDM4* transcripts in lymph tissues, LCLs and monocytes was associated with rs4951401 ($P = 1.59 \times 10^{-7}$ in lymph tissue) and rs4245739 ($P = 9.9 \times 10^{-11}$ in LCLs and $P = 6.68 \times 10^{-12}$ in monocytes). Both rs4951401 and rs4245739 are highly correlated with the lead SNP we identified in our association analyses, rs2169137 in *MDM4* (r^2 0.963 and 0.927, respectively). Additional eQTL associations were identified for rs129128 with the *HFE* transcript (chromosome 6p22, $P = 3.03 \times 10^{-8}$), rs3184504 and rs653178 with *SH2B3* and *ATXN2* transcripts (12q24, $P = 3.5 \times 10^{-7}$); rs2470893 with the *CSK* transcript (15q24, $P = 4.6 \times 10^{-9}$).

Additionally, we queried the ENCODE database (<http://www.regulomedb.org/>). We examined the annotations in the region of *MDM4* SNPs (these SNPs include rs4245739, rs4951401, rs2169137). These SNPs show no new ENCODE regulatory annotations. We also queried *HRH1* (rs347591) which did not have an eQTL association. This SNP does show overlap via position weight matrix for the HTF (*HER2*) transcription factor and also via DNA footprinting. This is potentially encouraging but no ChIP-seq data are available for HTF, so this is not necessarily strongly confirmed. The SNPs which do overlap with features are those with eQTL associations in *HFE* (rs129128), *SH2B3* (rs3184504) and *CSK* (rs2470893).

Pleiotropy evaluations

We tested association of rs347591 (chromosome 3p25, *HRH1*) and rs2169137 (chromosome 1q32, *MDM4*) with traits known to be associated with hypertension, including coronary artery disease (CAD), left ventricular hypertrophy (LVH) and stroke. In these analyses, no additional trait associations with these SNPs were identified (Supplementary Material, Table S12).

DISCUSSION

Using a genotyping array that covers common genetic variation in ~2100 candidate genes for several cardiovascular traits including BP, we identified robust associations at 11 known and two novel loci associated with continuous BP traits. In our primary discovery experiment, we identified 21 loci associated with our traits of interest in an analysis of 61 619 individuals ($P < 2.4 \times 10^{-6}$). Through the joint analysis of SNPs considered relevant during the discovery phase combined with replication data, in a total of 127 485 individuals, we identified robust association of 22 SNP-trait associations at 12 independent loci meeting our significance threshold

of $P < 2.4 \times 10^{-6}$. Associations at two of these loci were not previously known to be associated with BP. The association of SBP with variants in the *HRH1* locus, and the association of DBP with variants in the *MDM4* locus were novel and replicated *in silico* in an additional 65 866 individuals. Additionally, the *SOX6* locus contains a novel SNP-trait association for SBP with rs2014408; previously an association with MAP was shown for this locus (19). Finally, one additional region, chromosome 19p13.2 containing *ICAM3*, was significant in our discovery experiment but was not replicated in the joint analysis of discovery and replication samples. Only one of the replication cohorts had data available for this SNP (UK, $P = 0.466$). Although still meeting the array-wide significance criterion in the combined analysis, we do not consider this association to be robust in the replication experiment.

The histamine receptor H1 (*HRH1*) gene product is expressed in numerous tissues, such as smooth muscle and neurons. *HRH1* is expressed in the nucleus tractus solitarius, where it has a role in regulating arterial pressure in rats (21). Congenic mapping in a rodent model linked *HRH1* to autoimmune T-cell responses and vascular responses regulated by histamine after Bordetella pertussis toxin sensitization (22), and this locus appears to have a role in regulating blood brain barrier permeability (23). In a mouse model of atherosclerosis, apolipoprotein E-null mice treated with a histamine H1 receptor selective antagonist developed 40% fewer aortic plaques compared with mice treated with a H2 receptor antagonist and higher levels of inflammatory markers within the plaques and higher numbers of inflammatory cells, despite equivalent plasma lipoprotein levels, suggesting that the H1 receptor enhances low density lipoprotein cholesterol permeability into the intimal space of the artery (24). *HRH1* was selected for the IBC array as a lower priority gene based on the presence in a Protein ANalysis Through Evolutionary Relationships (PANTHER) inflammatory/immune response pathway. The *HRH1* gene is located ~249 kb from the *ATP2B2* gene with no linkage disequilibrium (LD) detected in HapMap CEU between common variants in these genes. Although *ATP2B2* has not been directly implicated in BP or hypertension, it is one of the Ca(2+)-ATPases, a family of plasma membrane pumps encoded by at least three additional genes: *ATP2B1* on chromosome 12q21; *ATP2B3* on Xq28 and *ATP2B4* on 1q25. *ATP2B1* contains a robust SNP association for SBP, DBP and hypertension (6). Mice null for *Atp2b1* are embryolethal (25), but recently a mouse with conditional knockout for the *Atp2b1* in vascular smooth muscle cells was generated and found to have significantly elevated BP (26). In humans, no associations have been found between variants in the *ATP2B2* gene and BP or other vascular traits. Additionally, within the recombination interval containing *HRH1* and the peak association signal, SNPs within the *ATG7* gene were associated but well below the statistical significance threshold for association, decreasing the likelihood that variants in *ATG7* are driving the signal in this region.

Mouse double minute 4 homolog (*MDM4*) encodes a nuclear protein that is a critical regulator of p53 tumor suppressor protein by binding to this protein and inhibiting its activity, promoting cell viability and growth. *MDM4* does not have a described vascular function, but it has a critical role in regulating p53, a transcription factor which plays an

important role in regulating target genes that induce cell cycle arrest, apoptosis and cell senescence (27). *MDM4* was also selected for the IBC array as a lower priority gene, based on the presence in a PANTHER apoptosis pathway. Mice null for p53 shows a variety of aging-related phenotypes (28). *MDM2*, another regulator of p53, has been shown to have a functional role in aldosterone-induced vascular remodeling (29), and *MDM2* and *MDM4* have been shown to have non-overlapping but a similar regulation of p53 pathways (30–32). A genome-wide linkage analysis for loci associated with PP has shown suggestive linkage on chromosome 1 (BP QTL 77, LOD = 2.7) for blacks in the HyperGEN Network (33), with *MDM4* overlapping with this linkage peak. An overlap with the *MDM4* region has also been shown for 10 quantitative trait loci (QTL) related to BP in rats (Human *MDM4*: <http://rgd.mcw.edu/rgdweb/report/gene/main.html?id=1319584>; Rats *MDM4*: [http://rgd.mcw.edu/rgdweb/search/qtls.html?term=Mdm4\[gene\]&speciesType=3](http://rgd.mcw.edu/rgdweb/search/qtls.html?term=Mdm4[gene]&speciesType=3)). The sex interaction we observed is of unclear significance since it was not confirmed in independent samples. *MDM4* has also been associated with measures of cognitive performance in a GWAS (34). Within the recombination interval containing *MDM4*, correlated SNPs in *PIK3C2B* were associated, so we cannot exclude that the association signal may in part be due to variants in this gene. *PIK3C2B* has no known function related to BP or vascular disease.

The association of the *SOX6* locus with SBP expands our prior knowledge of associations in this region with associated BP traits, with this locus previously reported as associated with MAP (11). MAP is derived from a calculation incorporating SBP and therefore these traits are highly correlated. Additionally, we replicated previous reports of 32 SNP-trait associations with BP, with acknowledgement that some of the cohorts in the published literature were genotyped on multiple platforms and have therefore been included in not only our IBC analysis but also previous reports.

The strengths of this study include the large size ($n = 61\,619$) of the discovery meta-analysis with replication in an additional 65 866 individuals and access to gene expression data from humans subjects. This meta-analysis has led to the identification of two novel signals not previously detected by prior association studies. Another unreported locus was found in the discovery phase, e.g. *ICAM3*, which could not be replicated due to the inadequate sample size in the replication cohorts with high-quality genotypes for this SNP. The non-replication may also be explained by GWAS with less dense coverage used for the replication phase. In addition, future functional studies are needed to fully comprehend the underlying mechanisms responsible for the detected associations. However, we did find that the *MDM4* locus was related to expression of *MDM4* transcripts in several tissues, and the lead SNP identified in our study is in tight LD with the expression-associated SNPs, suggesting a transcriptional effect of the associated SNP we identified. Finally, it needs to be emphasized that the results were generated in European ancestry populations and that additional studies are needed in other ethnic groups.

In summary, our study has identified two novel loci containing the *HRH1* and *MDM4* genes associated with BP traits of clinical significance. The identification of these loci expands our understanding of the genetic determinants of BP.

MATERIALS AND METHODS

Study subjects

The phenotype and genotype data of 61 619 individuals of European ancestry, belonging to 27 participating studies (Supplementary Material, Table S1), were analyzed in the discovery phase, and additional 65 866 individuals of European ancestry from 18 additional studies were used in the replication phase. Individuals of European ancestry, as confirmed by principal component analysis of genetic ancestry, were analyzed in this study. All individuals in these studies provided informed consent, and each study was approved by its own local ethics committee. More detailed information about each participating cohort is provided in the Supplementary Material.

Phenotype

BP ascertainment in each study was performed according to the protocols described in the Supplementary Material. PP was defined as SBP minus DBP, and MAP was defined as 2/3 DBP plus 1/3 SBP. In the discovery analyses, each cohort provided regression models for its data, adjusted for age, age-squared, body mass index (BMI) and study-specific corrections for population substructure (based on principal component analysis). For individuals taking BP lowering medications, the BP values were adjusted by adding 15 mmHg to the SBP and 10 mmHg to the DBP in the discovery and replication cohorts. These adjustments were also implemented prior to the calculation of estimated off-treatment MAP and PP.

Genotyping and quality control

A total of 51 859 SNPs were genotyped and after filtering for an mismatches >30% with HapMap, removing SNPs without an rsID in dbSNP129 and SNPs with more than two possible bases for a single SNP, 48 372 SNPs included in the discovery meta-analyses, all present in at least one of the three versions of the Illumina HumanCVD BeadChip ('Cardiochip', ITMAT-Broad_CARE [IBC] array, Illumina San Diego, CA, USA) (20), which was used by all cohorts participating in the discovery analysis.

For the IBC array, gene and specific SNP information was assimilated from 2400 published studies systematically analyzed up until May 2007. An emphasis was placed on the sample size, data quality and strength of the described associations. Genes with known or putative association with phenotypes for sleep, lung and blood diseases were also nominated. Several pathway-based tools were used to identify additional biologically plausible candidate genes: Kyoto Encyclopedia of Genes and Genomes; PANTHER and BioCarta. These tools were employed to collate additional genes from key pathways including lipid metabolism, thrombogenesis, circulation and gas exchange, insulin resistance, metabolism, and inflammation, oxidative stress and apoptosis.

Early access was provided to a number of unpublished mouse atherosclerosis expression QTL (eQTL) datasets. Genes predicted to be causal for the atherosclerotic lesion size in genetic crosses of mice with differing susceptibility to atherosclerosis were identified. Early access was provided to a number of key findings from a number of CVD-related GWASs.

Genotypes were called using Beadstudio (Illumina) and the data were processed using stringent QC filters, as summarized in Supplementary Material, Tables S2 and S3. Variants with minor allele frequencies <1% were excluded from the analysis. Further details on genotyping methods are provided in the Supplementary Material, Tables S2 and S3.

QC measures were taken during the various steps of this work. Individuals with <90% call rate (completeness) across all SNPs were removed. SNPs with <95% call rate (completeness) or SNPs causing heterozygous haploid genotype calls were removed across all remaining individuals. SNPs with $P < 1 \times 10^{-7}$ for the Hardy–Weinberg Equilibrium test were also removed. In the NHLBI Candidate Gene Association Resource (CARE) samples (35), SNPs associated with chemistry plate effects were also removed.

Statistical association and meta-analysis

Initial association analyses were calculated within each cohort for males and females separately, adjusting for age, age-squared, BMI and study center when appropriate. In each cohort, except FHS, CFS and Amish, association analysis was performed using PLINK (36) using linear regression under an additive genetic model. The family structure was modeled using a linear mixed effects model implemented in R (37) in FHS and CFS, and the Mixed Model Analysis for Pedigrees software program in the Amish (38).

Meta-analysis was conducted using the summary statistics contributed by each discovery study, using an inverse variance weighted, fixed-effects method. At the meta-analysis stage of analysis, SNPs with frequencies incompatible with HapMap frequencies were removed (defined as >30% difference in the allele frequencies). Two analysis groups independently performed the meta-analysis using different software packages: METAL (39) and MANTEL (40); both applied a fixed-effects model weighted by inverse variance. The results from both the groups were compared and a concordance check was performed as a validation of the results (data not shown). Genomic control (41) was applied to each study result and then to the meta-analysis summary data to control effects possibly due to population stratification or cryptic relatedness. Quantile–quantile plots are shown for each trait in Supplementary Material, Fig. S1. Previous studies using the IBC array have used different significance thresholds from $P < 1 \times 10^{-5}$ to 3×10^{-6} [(42) and (43), respectively]. The CARE IBC array studies (35), which are included in this meta-analysis, determined that after accounting for LD, the effective number of independent tests was ~20 500 for Europeans producing an experimental or ‘array-wide’ statistical threshold of $P = 2.4 \times 10^{-6}$, respectively, to maintain a false-positive rate of 5% (44) and thus, we have adopted these thresholds for this study. Since we have analyzed four traits, although highly correlated, we examined the effect of further Bonferroni correction for four tests. For each associated locus, the LD patterns were examined and independence between the loci identified in this study was verified using SNAP (45) ($r^2 < 0.3$).

For loci with multiple SNPs showing association with the traits, we also conducted conditional analyses to evaluate independent signals. At each locus with variants associated with

BP traits, we added the most significant SNP within the locus as a covariate in the association tests in each cohort in the NHLBI CARE consortium. Then, we performed meta-analysis of cohort-specific conditional analysis results. This conditional analysis was performed for the SNPs within a 500 kb region around the most significant SNP. The P -values for SNP association testing were then recorded, respectively, for associated locus for each trait.

Replication analysis

Independent SNPs with $P < 1.0 \times 10^{-5}$ in the discovery analysis were carried forward for replication analysis using independent samples for each trait. The significance threshold for association in the replication phase was a Bonferroni-corrected P -value based on an $\alpha = 0.05$ and the final number of independent ($r^2 < 0.3$) SNPs tested, and we also combined the discovery and replication data in a meta-analysis, in which evidence of positive replication was defined by $P < 2.4 \times 10^{-6}$ in the meta-analysis of combined discovery and replication samples. Associated loci were tested for replication by carrying forward to replication testing the lead SNP (minimum P -value) at each locus.

Interaction testing

For the lead SNP identified in novel genes, we tested for interactions with sex by first calculating the residual after adjusting for age, age-squared, BMI, sex and 10 principle components, and then performed sex-specific linear regression on a SNP for each cohort separately. This analysis was done using the PLINK $G \times E$ function. The sex-specific estimates were further combined by meta-analysis for men and women separately, using METAL (39). The interaction between gene and sex was tested by comparing the regression coefficients in men and women. That is, we calculated a T statistic: $T = (\hat{\beta}_M - \hat{\beta}_F) / \sqrt{\hat{s}_M^2 + \hat{s}_F^2}$, where $\hat{\beta}_M$, $\hat{s}_M^2$, $\hat{\beta}_F$ and $\hat{s}_F^2$ are the estimated regression coefficients and their standard errors from male and female meta-analysis, respectively. T follows the standard normal distribution.

Variance explained

The proportion of the trait variance explained by the discovered associations was calculated by first obtaining the residuals after adjusting for age, age-squared, BMI and 10 PCs and then performing linear regression on all 21 identified associated variants together in a subset of the cohorts comprising the NHLBI CARE consortium cohorts (ARIC, CHS, MESA, CARDIA, CFS). The variance explained was calculated by a standard analysis of variance.

Annotation

eQTL analysis

We identified alias rsIDs for significant index SNPs using SNAP (45). Further proxy SNPs in high LD ($r^2 = 1.0$) were identified with SNAP using multiple HapMap CEU builds. Current and alias rsIDs were searched for primary SNPs and LD proxies against a collected database of expression SNP (eSNP) results including the following tissues: fresh lymphocytes (46), fresh

leukocytes (47), leukocyte samples in individuals with celiac disease (48)), LCLs derived from asthmatic children (49), HapMap LCL from three populations (50), a separate study on HapMap CEU LCL (51), peripheral blood monocytes (52,53), omental and subcutaneous adipose (54,55), stomach (55) and whole blood samples (54,56), endometrial carcinomas (57), brain cortex (52,58), three large studies of brain regions including prefrontal cortex, visual cortex and cerebellum, respectively (Emilsson, personal communication), liver (55,59,60), osteoblasts (61), skin (62) and additional fibroblast, T-cell and LCL sample datasets (63). The collected eSNP results met the criteria for statistical thresholds for association with gene transcript levels as described in the original papers. In each case, where an index or proxy SNP was associated with a transcript, we further examined the strongest eSNP for that transcript within that dataset (best eSNP), and the LD between the best eSNP and BP-selected eSNPs to assess the concordance of the BP association and expression signals. Annotation of SNPs in the region was done with the SNAP web-based tool (45).

Evaluation of pleiotropy of BP variants with cardiovascular disease

We evaluated the effect of the novel loci identified in our study with traits known to be related to elevated hypertension including CAD, LVH and stroke. The definitions of the CAD, LVH and stroke phenotypes and association tests were carried out as described in the Supplementary Material. Evidence of association for the additional traits was alpha of 0.05 adjusted by the two SNPs tested for association ($P = 0.025$).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiment, alphabetically F.A., P.deB., S.G., B.K., D.L., X.Z. Performed the experiments:

individual cohorts conducted genotyping and cohort-level analyses, except for the NHLBI CARE program samples were genotyped under a contract with the Broad/MIT. Analyzed the data: F.A., P.deB., S.G., W.G., B.K., D.L., V.T., X.Z. Contributed reagents/materials/analysis tools: all authors. Wrote the paper: F.A., P.deB., S.G., W.G., B.K., D.L., V.T., X.Z., with contributions from all authors.

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