Resequencing Epithelial Sodium Channel Genes Identifies Rare Variants Associated With Blood Pressure Salt-Sensitivity: The GenSalt Study

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BACKGROUND
A resequencing study of renal epithelial sodium channel (ENaC) genes was conducted to identify rare variants associated with blood pressure (BP) salt-sensitivity.

METHODS
The Genetic Epidemiology Network of Salt-Sensitivity (GenSalt) study was conducted among 1,906 participants who underwent a 7-day low-sodium followed by a 7-day high-sodium feeding-study. The 300 most salt-sensitive and 300 most salt-resistant GenSalt participants were selected for the resequencing study. Three ENaC genes (*SCNN1A*, *SCNN1B*, and *SCNN1G*) were resequenced using capillary-based sequencing methods. Traditional burden tests were utilized to examine association between rare variants and BP salt-sensitivity. Associations of low-frequency and common variants were tested using single-marker analyses.

RESULTS
Carriers of *SCNN1A* rare variants had a 0.52 [95% confidence interval (CI): 0.32–0.85] decreased odds of BP salt-sensitivity compared with noncarriers. Neither *SCNN1B* nor *SCNN1G* associated with salt-sensitivity of BP in rare variant analyses ($P = 0.65$ and 0.48, respectively). In single-marker analyses, 3 independent common variants in *SCNN1A*, rs11614164, rs4764586, and rs3741914, associated with salt-sensitivity after Bonferroni correction ($P = 4.4 \times 10^{-4}$, 1.1×10^{-8}, and 1.3×10^{-3}). Each copy of the minor allele of rs4764586 was associated with a 1.36-fold (95% CI: 1.23–1.52) increased odds of salt-sensitivity, whereas each copy of the minor allele of rs11614164 and rs3741914 was associated with 0.68-fold (95% CI: 0.55–0.84) and 0.69-fold (95% CI: 0.54–0.86) decreased odds of salt-sensitivity, respectively.

CONCLUSIONS
This study demonstrated for the first time a relationship between rare variants in the ENaC pathway and BP salt-sensitivity. Future replication and functional studies are needed to confirm the findings in this study.

CLINICAL TRIAL REGISTRY
Trial Number NCT00721721

Keywords: blood pressure; capillary-based sequencing; dietary sodium; epithelial 1 alpha subunit (*SCNN1A*); genetics; hypertension; mean arterial pressure; rare variants; salt sensitivity; single nucleotide polymorphism; sodium channel epithelial 1 beta subunit (*SCNN1B*); sodium channel epithelial 1 gamma subunit (*SCNN1G*).

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The absolute burden of hypertension increased from 921 million in 2000 to 1.39 billion in 2010 globally.[1,2] As an important risk factor for cardiovascular disease morbidity and mortality,[3,4] high systolic blood pressure (SBP) accounted for 208.1 million disability-adjusted life-years and 10.4 million deaths worldwide in 2013.[5] Observational studies and randomized controlled trials have identified dietary sodium intake as an important modifiable determinant of BP.[6–10] However, there is substantial interindividual variability in BP response to salt loading and depletion, a phenomenon described as BP salt-sensitivity.[11,12] Despite its heritable nature, the genomic mechanisms underlying BP salt-sensitivity remain largely unknown.[13–16] A better understanding of the genetic underpinnings of this complex phenotype may provide important biological insights into BP regulation and hypertension pathophysiology.

The renal epithelial sodium channel (ENaC) plays an important role in BP control, acting as a key regulator of sodium reabsorption in epithelial cells located in the distal part of the renal tubule.[17] An abnormal increase or decrease

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in ENaC activity can lead to hypertension or hypotension phenotypes.[18] The 3 ENaC subunits (α, β, and γ) are encoded by the *SCNN1A*, *SCNN1B*, and *SCNN1G* genes, respectively.[19] Recent genome-wide association and candidate gene studies have identified many common and low-frequency variants, including those located in ENaC, contributing to BP salt-sensitivity.[20–26] However, the effect of these identified variants is modest and can explain only a limited fraction of the heritability of BP salt-sensitivity. The association between rare variants which may have relatively strong and causal effects on BP salt-sensitivity has not been clearly elucidated.

The Genetic Epidemiology Network of Salt-Sensitivity (GenSalt) resequencing study, which was conducted among the 300 most salt-sensitive and 300 most salt-resistant participants of the GenSalt study, is the first resequencing study of the BP salt-sensitivity phenotype. We aimed to identify novel common, low-frequency, and rare ENaC variants associated with BP salt-sensitivity by employing aggregate rare variant and single-marker analyses among the 600 GenSalt sequencing study participants.

METHODS

Study population

The GenSalt study was a family-based dietary feeding-study conducted in rural areas of northern China from 2003 to 2005. Details of the study design and eligibility criteria have been presented previously.[27] Briefly, a community-based BP screening was conducted among participants aged 18–60 years in the study villages to identify potential probands and their siblings, spouses, and offspring aged 16 or older. Those with mean SBP between 130 mm Hg and 160 mm Hg, mean diastolic BP (DBP) between 85 mm Hg and 100 mm Hg, and no use of antihypertension medication were identified as probands. Persons who had stage 2 hypertension (SBP ≥ 160 mm Hg and/or DBP ≥ 100 mm Hg), secondary hypertension, a history of clinical cardiovascular disease, diabetes, or chronic kidney disease, used antihypertensive medications less than 1 month before the screening visit, or were pregnant, heavy alcohol drinkers, or currently on a low-sodium diet were excluded from the study. Among 1,906 participants who were eligible to receive the dietary intervention, 1,860 (97.6%) completed a 7-day low-sodium intervention (3 g of sodium chloride or 51.3 mmol sodium/day) followed by a 7-day high-sodium intervention (18 g of sodium chloride or 307.8 mmol sodium/day). Those 300 participants with the highest and 300 participants with lowest mean arterial pressure (MAP) response to the high-sodium intervention were selected to participate in the current resequencing study.

Institutional review boards or ethnic committees at all participating institutions approved the GenSalt study. Written informed consent was obtained from each GenSalt study participant before baseline data collection and intervention.

Dietary intervention

After a 3-day baseline observation on their usual diet, study participants received a 7-day low-sodium diet (3 g of sodium chloride or 51.3 mmol of sodium per day) followed by a 7-day high-sodium diet (18 g of sodium chloride or 307.8 mmol of sodium per day). During the period of sodium intervention, dietary potassium intake remained unchanged. Total energy intake varied according to each participant's baseline energy intake. All foods were cooked without salt, and prepackaged salt was added to the individual study participant's meal when it was served by the study staff. To ensure compliance with the intervention program, study participants were required to have breakfast, lunch, and dinner at the study kitchen under the supervision of the study staff during the entire study. The study participants were instructed to avoid consuming any foods or beverages that were not provided by study. Three timed urinary specimens (one 24-hour specimen and two 8-hour overnight specimens) were collected during the 3-day baseline and at the last 3 days of each intervention period. Overnight urinary sodium excretion was converted to 24-hour values based on formulas developed from data obtained from a subgroup of study participant whose 24-hour and overnight urine samples were collected on the same days. The results from the 24-hour urinary excretions of sodium and potassium showed excellent compliance with the study diet. The mean (SD) of 24-hour urinary excretions of sodium and potassium were 242.2 (66.7) mmol and 36.9 (9.6) mmol at baseline, 47.5 (16.0) mmol, and 31.4 (7.7) mmol during the low-sodium intervention, and 244.3 (37.7) mmol and 35.7 (7.5) mmol during the high-sodium intervention, respectively.

Data collection

A standard questionnaire was administered to participants by trained staff at the baseline examination to obtain information about demographic characteristics, personal and family medical history, and lifestyle risk factors. SBP and DBP were measured 3 times every morning during the 3-day baseline and on days 5, 6, and 7 of each intervention period. All BP was measured by trained and certified observers using a random zero sphygmomanometer according to a standard protocol.[28] BP was measured with the participants in the sitting position after resting for 5 minutes. Participants were advised to avoid consumption of alcohol, coffee, or tea; cigarette smoking; and exercise for at least 30 minutes before BP measurements. BP observers were blinded to the participants' dietary intervention. Body weight and height were measured twice with the participants in light indoor clothing without shoes. Body mass index was calculated as weight in kilograms divided by height in square meters.

MAP was calculated using the following equation: MAP = DBP + (SBP − DBP)/3. MAP response to high-sodium intervention was calculated as the mean of 9 MAP measures on days 5, 6, and 7 of the high-sodium intervention minus the mean of 9 measures on days 5, 6, and 7 of the low-sodium intervention. The 300 participants with the highest MAP response to the high-sodium intervention and the 300 participants with the lowest MAP response to the high-sodium intervention were identified as the most salt-sensitive and most salt-resistant

participants, respectively, and were subsequently selected for the GenSalt resequencing study.

Candidate gene selection, sequencing data, and quality control

Three ENaC genes including the sodium channel epithelial 1 alpha subunit (*SCNN1A*), sodium channel epithelial 1 gamma subunit (*SCNN1G*), and sodium channel epithelial 1 beta subunit (*SCNN1B*) were selected based on their potential biological effect on BP regulation. Functional regions of these 3 ENaC genes were resequenced using the VariantSEQr system (Applied Biosystems, Foster City, CA). Resequencing identified 117 single nucleotide variants in the selected ENaC genes, including 63 rare variants (minor allele frequency, MAF < 0.01) and 54 common and low-frequency variants (MAF ≥ 0.01). Variants were updated to the Genome Reference Consortium Human genome build 38 (GRCh38) and annotated using ANNOVAR software (http://annovar.openbioinformatics.org/en/latest/).[29] Quality control including checks of Mendelian consistency, genotyping call rate, Hardy–Weinberg equilibrium, and MAF was performed using the Haploview software (version 4.2; MIT/Harvard Broad Institute, Cambridge, MA).[30] Single nucleotide variants with low genotyping call rates (<85%) and deviations from Hardy–Weinberg equilibrium after adjustment for multiple comparisons were excluded. Furthermore, variants that were examined in our previous candidate gene study [or in linkage disequilibrium with those examined previously] were also excluded.[24] Of 117 single nucleotide variants sequenced in this study, 2 rare variants not passing quality control and 30 common and low-frequency variants assessed previously were excluded (Supplementary Table). A total of 61 rare variants and 24 common and low-frequency variants passed quality control and were included in this analysis. Annotation information for identified variants, including genomic locations, MAFs, call rates, and *P* values for the Hardy–Weinberg equilibrium test, is presented in the Supplementary Table.

Statistical analysis

Baseline characteristics of salt-sensitive and salt-resistant GenSalt resequencing study participants were compared using the chi-square test for categorical variables and *t*-tests for continuous variables.

Aggregated effect of rare variants on BP salt-sensitivity. Traditional burden tests were utilized to analyze the aggregate effects of rare variants on BP salt-sensitivity.[31] Variants with MAF <0.01 within the entire ENaC pathway and within each ENaC gene were collapsed separately. A binary indicator variable was created to categorize participants based on the presence or absence of rare variants within the entire ENac pathway and within each ENaC gene. Generalized estimating equations were utilized to accommodate family structure and test the association between the collapsed indicator variables and BP salt-sensitivity after multivariable adjustment for age, gender, field center, and body mass index using the PROC GENMOD procedure in SAS (version 9.4; SAS Institute, Cary, NC). Bonferroni correction was employed to account for testing the 3 genes and entire gene pathway (α threshold = 0.05/4 = 0.0125).

Effects of single common and low-frequency variants on BP salt-sensitivity. To analyze the additive associations between single common and low-frequency variants (MAF ≥ 0.01) and BP salt-sensitivity, generalized estimating equation was again used to account for the correlation of individuals within families and adjust for the fixed effects of age, gender, field center, and body mass index. This analysis was carried out using the PROC GENMOD procedure in SAS. Statistical significance was determined using a Bonferroni correction for testing 24 single common and low-frequency variants (α-threshold = 0.05/24 = 2.08×10^{-3}).

RESULTS

Characteristics of the GenSalt resequencing study participants are shown in Table 1. Compared with their salt-resistant counterparts, salt-sensitive participants were, on average, older and more likely to be female. Baseline MAP levels were also higher among salt-sensitive participants, while MAP levels during low-sodium intervention were similar between salt-resistant and salt-sensitive participants. As expected, salt-sensitive participants exhibited larger increases in MAP levels in response to the high-sodium intervention compared with salt-resistant participants.

Table 1. Characteristics of GenSalt resequencing study participants

	Salt sensitive (*n* = 299)	Salt resistant (*n* = 303)	*P* value
Age, years	41.42 ± 8.33	37.75 ± 8.72	<0.001
Female, (%)	52.51	35.64	<0.001
BMI, kg/m^2	23.65 ± 3.15	23.19 ± 3.33	0.08
Baseline MAP, mm Hg	92.10 ± 11.26	87.29 ± 10.05	<0.001
MAP during low-sodium intervention, mm Hg	84.83 ± 10.14	86.40 ± 9.36	0.05
MAP during high-sodium intervention, mm Hg	94.89 ± 10.61	82.62 ± 9.13	<0.001
MAP response to high sodium, mm Hg	10.06 ± 3.37	−3.77 ± 2.42	<0.001

Data are presented as mean ± SD or percentage. Abbreviations: BMI, body mass index; GenSalt, Genetic Epidemiology Network of Salt-Sensitivity; MAP, mean arterial pressure.

Results of the aggregate analysis of the entire ENaC pathway and each individual ENaC gene are shown in Table 2. A total of 61 rare variants were identified in this pathway, with 20, 17, and 24 rare variants identified in *SCNN1A*, *SCNN1G*, and *SCNN1B* genes, respectively. In the entire ENaC pathway analysis, no significant association with salt-sensitivity was identified after Bonferroni correction. In gene-based analyses, the *SCNN1A* gene showed significant association with salt-sensitivity after Bonferroni correction for multiple comparisons ($P = 0.009$). The odds ratio of participants carrying rare variants in *SCNN1A* gene was 0.52 (95% confidence interval [CI]: 0.32–0.85) compared with those not carrying these rare variants. Neither *SCNN1G* nor *SCNN1B* associated with salt-sensitivity in rare variant analyses.

In single common and low-frequency variant analyses, 3 of 24 variants associated with salt-sensitivity of BP (Figure 1). Intronic *SCNN1A* markers rs11614164, rs4764586, and rs3741914 achieved significance after Bonferroni correction for multiple testing with $P = 4.4 \times 10^{-4}$, 1.1×10^{-8}, and 1.3×10^{-3}, respectively (Table 3). Each copy of the minor alleles of rs4764586 associated with 1.36-fold (95% CI: 1.23–1.52) increased odds of salt-sensitivity, whereas each copy of the minor alleles of rs11614164 and rs3741914 was associated with 0.68-fold (95% CI: 0.55–0.84) and 0.69-fold (95% CI: 0.54–0.86) decreased odds of salt-sensitivity, respectively. Three other variants, including rs13306614, rs191480979 in *SCNN1A*, and rs13306665 in *SCNN1G*, also showed nominally significant associations ($P < 0.05$) with salt-sensitivity (Figure 1).

DISCUSSION

In the first study to examine the effects of rare ENaC variants on salt-sensitivity of BP, aggregate rare variant analyses identified the *SCNN1A* gene as a potentially important contributor to this complex phenotype. Those who carried rare *SCNN1A* variants had 0.52-fold decreased odds of salt-sensitivity compared with noncarriers. Similarly, single-marker–based analyses identified 3 novel intronic variants of the *SCNN1A* gene associated with salt-sensitivity after stringent correction for multiple testing. The minor alleles of rs4764586 predicted higher risk and that of rs11614164 and rs3741914 predicted lower risk of BP salt-sensitivity compared with their corresponding major alleles. These findings highlight the role for ENaC, especially the *SCNN1A* gene, in BP salt-sensitivity.

Table 2. Pathway and gene-based findings of rare variants among GenSalt sequencing study participants

	Number of rare variants	OR (95% CI)[a]	*P* value
ENaC pathway	61	0.89 (0.61–1.29)	0.53
SCNN1A	20	0.52 (0.32–0.85)	0.009
SCNN1G	17	0.88 (0.50–1.54)	0.65
SCNN1B	24	1.23 (0.69–2.19)	0.48

Abbreviations: CI, confidence interval; ENaC, epithelial sodium channel; OR, odds ratio.

[a]Adjusted for age, gender, field center, and body mass index.

The *SCNN1A* gene plays an essential role in electrolyte and BP homeostasis.[32] Numerous studies have demonstrated a causal role of rare *SCNN1A* variants in pseudohypoaldosteronism type I (PHA-1), a Mendelian salt-wasting hypotensive disorder resulting from unresponsiveness to mineralocorticoids.[33–35] While *SCNN1A* mutations have been demonstrated to contribute to monogenic BP disorders *via* their influence on sodium regulation, this is the first study to directly implicate rare variants in the *SCNN1A* gene in MAP responses to dietary sodium intake. Previous studies have identified common *SCNN1A* variants with small effects on BP responses to dietary sodium intake.[24] In contrast, the rare genetic variants identified by the current study conferred large effects on the salt-sensitivity phenotypes, with carriers showing a nearly 2-fold reduced odds of BP salt-sensitivity. These finding highlight the potential importance of identifying rare variants for common complex traits like BP salt-sensitivity, suggesting that they may in aggregate help to explain some of the "lost heritability" of these phenotypes. Since MAP is a function of both SBP and DBP, we conducted *post-hoc* analyses to explore whether *SCNN1A* also associated with these BP response phenotypes. In our analyses, the nominally significant association was identified between *SCNN1A* gene and SBP but not DBP response to sodium, suggesting that the observed *SCNN1A*-MAP response association was probably driven by the *SCNN1A*-SBP response relation.

In contrast to findings for *SCNN1A*, no significant associations with salt-sensitivity were identified in gene-based analyses of rare variants in the *SCNN1B* and *SCNN1G* genes or in the entire ENaC pathway. Previous monogenic studies have identified mutations in the cytoplasmic C terminus of either the β or γ subunit of ENaC responsible for Liddle's syndrome, characterized by increased channel activity.[18,36] Meanwhile, it was also demonstrated that loss of function mutations in the β or γ subunit of ENaC can lead to the PHA-1 phenotype, characterized by decreased channel activity.[34,37] We speculated that bidirectional channel activity resulting from both beneficial and deleterious mutations could explain the null findings from the traditional burden tests employed here. However, *post-hoc* sequence kernel association test (SKAT) analyses, which should have increased power in the presence of variants with opposite effects,[38] also produced nonsignificant results. Replication studies are needed to verify the results of the current study.

Single common and low-frequency variant analyses revealed 3 variants in the *SCNN1A* gene associated with the BP salt-sensitivity phenotype. These 3 independent variants (all pairwise $r^2 < 0.17$) are all located in intronic regions of *SCNN1A* and are not in linkage disequilibrium with any variants of obvious functional relevance. Using the Combined Annotation Dependent Depletion (CADD) score, which integrates multiple annotation databases for *in-silico* functional prediction of single nucleotide variants, significant *SCNN1A* marker rs4764586 was in the top 20th percentile of

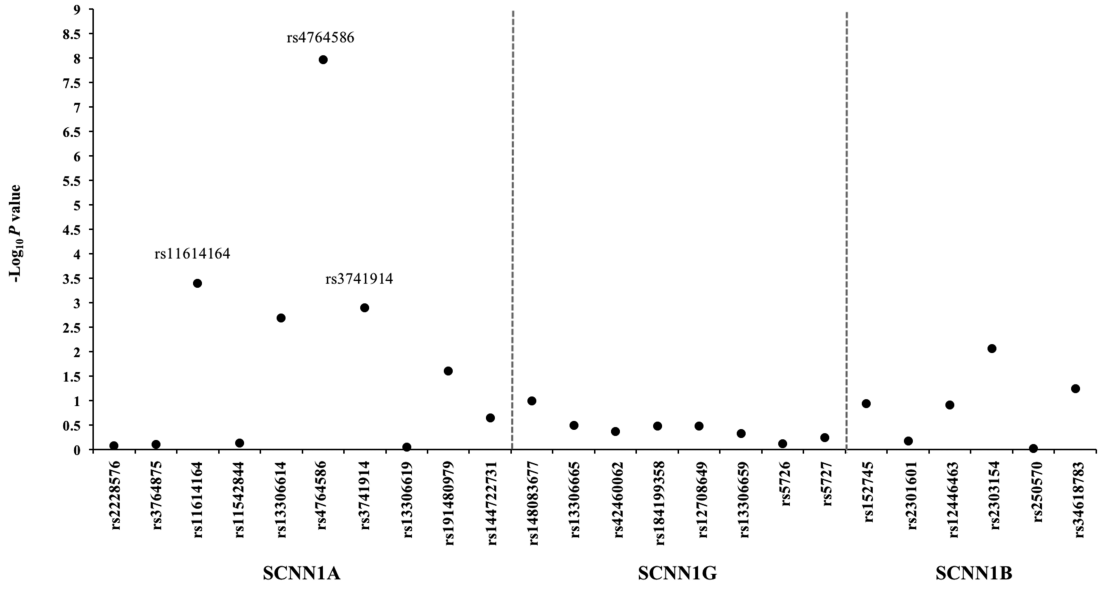

Figure 1. $-\text{Log}_{10}$ *P* values for the 24 low-frequency and common variants in *SCNN1A, SCNN1G,* and *SCNNs1B* with blood pressure salt-sensitivity. Three labeled variants were significantly associated with salt-sensitivity after Bonferroni correction for multiple testing (α threshold = $0.05/24 = 2.08 \times 10^{-3}$).

Table 3. Top common and low-frequency variant findings from the GenSalt sequencing study

HGNC symbol	Chromosome	SNP	Physical position	Gene region	Major allele	Minor allele	MAF	OR (95% CI)[a]	*P* value
SCNN1A	12	rs11614164	6354696	intronic	A	G	0.23	0.68 (0.55–0.84)	4.4×10^{-4}
	12	rs4764586	6361905	intronic	C	A	0.35	1.36 (1.23–1.52)	1.1×10^{-8}
	12	rs3741914	6374297	intronic	C	T	0.20	0.69 (0.54–0.86)	1.3×10^{-3}

Abbreviations: CI, confidence interval; HGNC, HUGO gene nomenclature committee; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphism.

[a]Adjusted for age, gender, field center, and body mass index.

CADD scores for their potential gene regulatory functions.[39] Unfortunately, *SCNN1A* markers rs11614164 and rs3741914 were not included in the CADD score database; so, their potential functional relevance could not be assessed. This was the first study to identify an association of markers rs11614164, rs4764586, and rs3741914 with salt sensitivity of BP. No variants in *SCNN1B* and *SCNN1G* associated with MAP response to high-sodium intervention in our resequencing study. Replication studies are needed to confirm the findings reported here, along with functional studies to verify any regulatory role of the reported variants.

The current study has several important strengths. It is the first to explore the role of rare ENaC variants with the BP salt-sensitivity phenotype. Considering the participants of this study were recruited from an isolated Han Chinese population in rural north China, the results of this study should be robust to population stratification. With MAP calculated based on 9 BP measurements collected at the end of each phase of the high- and low-sodium interventions, measurement error should be reduced. Furthermore, gold-standard capillary sequencing was used for variant discovery. However, several limitations of this study should also be addressed. The GenSalt study excluded participants with SBP ≥160 mm Hg and/or DBP ≥100 mm Hg and minimized the number with very low BP values through its enrichment for participants with pre-hypertension and stage 1 hypertension. Since BP is highly correlated with salt-sensitivity of BP, this may have limited the number of participants with extreme salt-sensitive and salt-resistant phenotypes despite our extreme trait design. As such, our ability to detect variants with very large effects on the BP response phenotype may have been reduced due to the sampling strategy. Although we aimed to sequence all functional regions of the ENaC genes, when mapped to the newest genome build some likely nonfunctional regions (e.g., introns and intergenic areas) were included and some functional regions were missed (e.g. parts of exons and promoters) (see Supplementary Table). The inclusion of nonfunctional variants and inadvertent exclusion of functional variants may have reduced the power of aggregate rare variant analyses to detect gene-based associations. In addition, the relatively small sample size may have reduced power further. Finally, external replication analyses were not feasible due to the unique phenotype examined in this study.

In conclusion, our report provides the first evidence of a relationship between rare variants in the ENaC pathway and salt-sensitivity of BP. A protective, aggregate effect of rare variants in the *SCNN1A* gene on MAP response to the sodium intervention was identified. In addition, several common variants in the *SCNN1A* gene were observed to associate with BP salt-sensitivity. While these findings are promising, future work is needed. External replication is warranted to confirm the robustness of the variants reported here. In addition, functional studies are needed to verify *in-silico* predicted functional relevance of the common variants identified and also to pinpoint the rare variants responsible for the strong gene-based signal.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *American Journal of Hypertension* online.

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DISCLOSURE

The authors declared no conflict of interest.

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