Metabolomics and Incident Hypertension Among Blacks
The Atherosclerosis Risk in Communities Study

Yan Zheng, Bing Yu, Danny Alexander, Thomas H. Mosley, Gerardo Heiss, Jennifer A. Nettleton, Eric Boerwinkle

Abstract—Development of hypertension is influenced by genes, environmental effects, and their interactions, and the human metabolome is a measurable manifestation of gene–environment interaction. We explored the metabolomic antecedents of developing incident hypertension in a sample of blacks, a population with a high prevalence of hypertension and its comorbidities. We examined 896 black normotensives (565 women; aged, 45–64 years) from the Atherosclerosis Risk in Communities study, whose metabolome was measured in serum collected at the baseline examination and analyzed by high-throughput methods. The analyses presented here focus on 204 stably measured metabolites during a period of 4 to 6 weeks. Weibull parametric models considering interval censored data were used to assess the hazard ratio for incident hypertension. We used a modified Bonferroni correction accounting for the correlations among metabolites to define a threshold for statistical significance (P<3.9×10^{-4}). During 10 years of follow-up, 38% of baseline normotensives developed hypertension (n=344). With adjustment for traditional risk factors and estimated glomerular filtration rate, each +1SD difference in baseline 4-hydroxyhippurate, a product of gut microbial fermentation, was associated with 17% higher risk of hypertension (P=2.5×10^{-4}), which remained significant after adjusting for both baseline systolic and diastolic blood pressure (P=3.8×10^{-4}). After principal component analyses, a sex steroids pattern was significantly associated with risk of incident hypertension (highest versus lowest quintile hazard ratio, 1.72; 95% confidence interval, 1.05–2.82; P for trend, 0.03), and stratified analyses suggested that this association was consistent in both sexes. Metabolomic analyses identify novel pathways in the pathogenesis of hypertension. (Hypertension. 2013;62:398-403.) • Online Data Supplement

Key Words: blacks ■ hypertension ■ metabolomics ■ risk factors

Hypertension is a leading risk factor for cardiovascular disease mortality, causing >7 million deaths every year worldwide.1 Blacks have greater prevalence and severity of hypertension than European-Americans,2 and are, thus, prime candidates for primary prevention efforts. Hypertension and its underlying pathophysiology may be present for years before clinical diagnosis, when irreversible pathology has already occurred. Current clinical evaluation of hypertension risk, such as blood pressure measurement, provides limited insight into relevant abnormal mechanisms for a particular patient. Because blood pressure is regulated and hypertension is controlled by a multiple physiological and anatomic systems,3 it has been proposed that a systems-biology approach to hypertension risk management and control would be beneficial.4 The metabolome represents the outcome of multiple physiological and metabolic processes and the ultimate downstream expression of the interaction between gene action and environmental exposure.5,6 Therefore, the metabolome may provide a high-resolution, multifactorial phenotypic signature of the pathogenesis, manifestation, or pathophysiology7 of hypertension.

To date, no study has explored the metabolomic antecedents of incident hypertension in blacks, who have the highest prevalence and rate of incident hypertension among all races.2 Therefore, we measured the metabolome in a well-characterized sample of blacks from the Atherosclerosis Risk in Communities (ARIC) study and identified individual metabolites and metabolite patterns that are significantly associated with incident hypertension during ≈10 years of follow-up.

Methods

Study Sample

The ARIC Study consists of a prospective cohort designed to identify the causes and outcomes of cardiovascular diseases. The ARIC study population was selected as a probability sample of 15792 men and
women aged 45 to 64 years from 4 communities (Forsyth County, NC; Jackson, MS; suburbs of Minneapolis, MN; and Washington County, MD). Detailed descriptions of its study design, objectives, and procedures have been published elsewhere.6 Eligible participants were interviewed at home and then invited to a baseline clinical examination between 1987 and 1989. Participants returned for multiple follow-up clinical examinations. The ARIC study was approved by the institutional review boards at each site (University of Minnesota, Johns Hopkins University, University of North Carolina, University of Mississippi Medical Center, University of Texas Health Sciences Center at Houston, and Wake Forest University). Metabolomic profiles were measured in a subsample of ARIC black participants (n=1977) randomly selected from the Jackson, MS, field center from all of those who provided informed consent for genetic research, provided quality dietary data, and fasted ≥8 hours before the baseline examination.

Assessment of Baseline Covariates and Metabolites

The baseline examinations included standardized medical history, physical examination with anthropometric measures, and laboratory testing. Sitting blood pressure was measured by trained technicians on the left arm of the participants with an appropriately sized cuff 3× with a random-zero mercury sphygmomanometer after a 5-minute rest, and the average of the last 2 readings was used. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or currently taking antihypertensive medication. Incident hypertension was defined as new occurrences of hypertension during the follow-up examinations among the baseline normotensives. Leisure-time physical activity was measured using a modification of the Baecke Physical Activity questionnaire.8 Alcohol intake was ascertained from a standardized questionnaire and the alcohol amount in grams per week was used in these analyses. Cigarette smoking status was self-reported and categorized as current and noncurrent smoker. Diabetes mellitus was defined as a fasting glucose level ≥7.0 mmol/L, a nonfasting level ≥11.1 mmol/L, a self-reported physician diagnosis, or pharmacological hypoglycemic treatment. Estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation.10

Among all normotensive participants (n=927) at the baseline examination, 31 were excluded from the analysis because of prevalent coronary heart disease (by history or ECG criteria, n=23), prevalent heart failure (by Gothenburg criteria or heart failure medication use,11 n=12), or prevalent stage 4 or 5 chronic kidney disease (estimated glomerular filtration rate, <30 ml/min per 1.73 m²; n=1) leaving 896 normotensive participants (men, n=331) for the analyses presented here.

Detection and quantification of metabolites in fasting serum samples continuously stored at –80°C were completed by Metabolon Inc (Durham, NC) using an untargeted, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry-based metabolomic quantification protocol. Metabolites were identified by comparison to library entries of purified standards or recurrent unknown entities.11 This untargeted approach identified and quantified 602 metabolites, including named compounds whose chemical identity is known (n=361; 90 in amino acid metabolism pathways, 16 in carbohydrate, 12 in cofactors and vitamins, 8 in energy, 147 in lipid, 14 in nucleotide, 29 in peptide, and 45 in xenobiotics) and unnamed compounds that do not currently have a chemical standard (n=241).11 The unknown chemical identities are tagged beginning with X and followed by numbers, such as X-12345. Measurement methods of these metabolites and rigorous laboratory quality control process were described in detail elsewhere.11 A repeatability study was carried out to evaluate the biological stability of metabolites in fasting serum collected 4 to 6 week apart. On the basis of these analyses, 204 of total 602 metabolites were selected on the basis of having a reliability coefficient ≥0.6 and having <80% of the values below the detection limit or missing (BDL/missing).11 and these values were assigned the lowest detected value for that metabolite in all samples. These 204 metabolites consisted of 187 metabolites treated as continuous variables in the analyses (<50% BDL/missing observations; 1=BDL/missing values, 2=values below the median, and 3=values equal or above the median; 10 named and 7 unnamed compounds; Table S1 in the online-only Data Supplement).

For descriptive analyses across groups, χ² tests were used for categorical variables, and 2-sample t tests or Wilcoxon rank-sum tests were used for continuous traits. The Weibull parametric model for interval censored data, which is an accelerated failure time proportional-hazards model, was used to estimate the hazard ratio (HR) of developing incident hypertension. Two multivariable models were used to assess the relation between metabolites (either an individual metabolite or metabolomic pattern) and incident hypertension. Covariates were selected on the basis of published reports.15–18 The basic model (Model 1) adjusted for traditional risk factors (ie, age, sex, leisure-time physical activity, alcohol intake, current cigarette smoking status, body mass index, and diabetes mellitus status). Model 2 included the covariates in Model 1 with the addition of a measure of kidney function (ie, estimated glomerular filtration rate). For the identified individual metabolomic biomarker candidates and the potential hypertension-related principal components, we also investigated whether the others and the corresponding robust test further adjustment for baseline systolic and diastolic blood pressure.

In analyses of association between an individual metabolite and hypertension, all HRs were calculated and reported per +1 SD for the continuous variables or per +1 category change in the categorical variables. A modified stepwise Bonferroni procedure, the Dubey/Armitage-Parmar algorithm,11,19 was used to correct for multiple comparisons and a significance level of 3.9×10⁻⁴ (2 tailed) was considered for each individual test. This adjustment takes into account the full correlation matrix of metabolites and uses the mean correlation among the metabolites in the formula, where the new α-level for the kth hypothesis test (k=1,2,…,K) is readjusted for each individual metabolites according to the following:

\[
\alpha_k = 1 - (1 - \alpha)^{1/m_k},
\]

where \(m_k = K^{-1}\sum_{j=1}^{K} r_{jk}\), \(r_{jk}\) is the correlation coefficient between the jth and kth metabolites. When the average of the correlation coefficients is zero, this adjustment is according to the Bonferroni procedure, and when it is 1, the adjusted and the unadjusted P values are the same.

Metabolites are expected to be correlated in complex ways. Thus, a principal components analysis (PCA) was used to group the 187 metabolites into metabolomic patterns. The 3 patterns retained were selected on the basis of 3 criteria: (1) the Kaiser criterion (eigenvalues >1), (2) inflection point of the scree plot, and (3) the interpretability of the patterns.20.21 A factor score for each study participant was calculated from the sum of the levels from all the 187 metabolites, multiplied by their respective factor loadings. Metabolomic patterns were named according to the metabolite groups loading highest on each of the 3 factor patterns: sex steroids, α amino acids, and branch-chain amino acids (Table S2). The scores for each PCA-derived pattern were entered separately into the Weibull parametric models. HR of developing incident hypertension for the highest versus lowest quintile of these 3 patterns and the corresponding robust test for trend across quintiles were calculated in all analyses. For the PCA analyses, a significance level of 0.05 (2-tailed) was used.

All statistical analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC).

Results

During the ≈10-year follow-up period, 38.4% of the 896 normotensives at baseline (n=344; 39.3% of the female and 36.9% of the male normotensives) developed hypertension (incidence rate, 63.94 per 1000 person-years; median, 7.67 years to first diagnosis of hypertension), and 209 reported having begun anti-hypertensive medications. Participants who developed hypertension were more likely to weigh more, have diabetes mellitus, and have higher systolic and diastolic blood pressure at the baseline examination.
Table 1. Distribution of Baseline Risk Factors by Incident Hypertension Status Among Normotensives at Baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normotensive at Baseline (n=896)</th>
<th>Nonincident Hypertension (n=552)</th>
<th>Incident Hypertension (n=344)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>51.47±5.4</td>
<td>51.40±5.5</td>
<td>51.59±5.3</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>331 (36.9)</td>
<td>209 (37.9)</td>
<td>122 (35.5)</td>
</tr>
<tr>
<td>Leisure-time physical activity*</td>
<td>2.13±0.6</td>
<td>2.13±0.6</td>
<td>2.13±0.6</td>
</tr>
<tr>
<td>Current cigarette smoking, n (%)</td>
<td>258 (28.8)</td>
<td>165 (29.9)</td>
<td>93 (27.0)</td>
</tr>
<tr>
<td>Ethanol intake in g/wk, median (IQR)</td>
<td>0.0 (0.0–13.2)</td>
<td>0.0 (0.0–13.2)</td>
<td>0.0 (0.0–13.2)</td>
</tr>
<tr>
<td>BMI, kg/m²†</td>
<td>28.65±5.5</td>
<td>28.09±5.3</td>
<td>29.55±5.8</td>
</tr>
<tr>
<td>Prevalent diabetes mellitus, n (%)‡</td>
<td>97 (10.8)</td>
<td>47 (8.5)</td>
<td>50 (14.5)</td>
</tr>
<tr>
<td>eGFR, mL/min per 1.73 m²</td>
<td>107.35±15.6</td>
<td>107.46±15.4</td>
<td>107.18±15.9</td>
</tr>
<tr>
<td>SBP, mm Hg†</td>
<td>116.70±11.1</td>
<td>114.01±10.8</td>
<td>121.02±10.2</td>
</tr>
<tr>
<td>DBP, mm Hg†</td>
<td>74.70±7.8</td>
<td>73.38±7.8</td>
<td>76.83±7.2</td>
</tr>
</tbody>
</table>

For continuous variables except ethanol intake in g/wk, mean±SE are shown. Percentages for categorical variables are shown in parentheses. BMI indicates body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; IQR, interquartile range; and SBP, systolic blood pressure.

*2.13 was interpreted as moderate active on the basis of tertile distribution of the leisure-time physical activity score (low active, <2.00; moderate active, 2.00–2.49; high active, ≥2.50) among the current study participants.

†P<0.001; ‡0.001<P<0.01.

examination (Table 1). The median and interquartile range for the percentage of BDL/missing values for the 204 metabolites included in our analysis were 2.6% and 20.4%, respectively.

The metabolite 4-hydroxyhippurate was consistently associated with incident hypertension across models (HR per SD, 1.17 and 1.18, respectively, in Models 1 and 2; P<3.9×10⁻⁴; Table 2), suggesting its association with hypertension may be independent of kidney function. The adjusted HR per SD for 4-hydroxyhippurate also remained significant after adjusting for baseline blood pressure (HR per SD, 1.18; 95% confidence interval, 1.08–1.29; P=3.8×10⁻⁴; Table 2). No other metabolite was significantly associated with incident hypertension (Table S1).

PCA yielded 3 biologically plausible metabolomic patterns: sex steroids, α amino acids, and branch-chain amino acids (Table 3). The first pattern, sex steroids, was statistically significantly associated with incident hypertension in both models and the other 2 patterns were uniformly not significant (Table 3). Stratified analysis suggested a consistent association of the sex steroids pattern with incident hypertension in both men and women (in men, highest versus lowest quintile HR, 1.93; in women, highest versus lowest quintile HR, 1.50). With further adjustment for baseline systolic diastolic blood pressure, the significance of the sex steroids pattern was slightly attenuated (highest versus lowest quintile HR, 1.40; 95% confidence interval, 0.84–2.32; P for trend in quintile number, 0.10; Figure). To assess whether the association ascribed to sex steroids pattern was driven by 1 or a few metabolites, individual metabolites composing the sex steroids pattern were assessed. Three metabolites, epiandrosterone sulfate, 5α-androstan-3β 17β-diol disulfate, and androsterone sulfate were nominal significant predictors of incident hypertension (Table 2). It is of note that the variance of the metabolites explained by the 3 PCA-derived patterns was relatively low (17%), which is believed to be a result of the pathway diversity of the human metabolome.

Discussion

We prospectively examined a sample of middle-aged black normotensives having serum metabolomic data. After adjustment for traditional risk factors and kidney function, each SD increment of baseline 4-hydroxyhippurate was associated with an 18% higher risk of hypertension, which remained significant after adjusting for baseline blood pressure. In addition, a sex steroids pattern derived from PCA was also associated with elevated risk of incident hypertension (highest versus lowest quintile HR, 1.72; P for trend in quintile number, 0.03). To our knowledge, our study is among the first to study human serum metabolomic antecedents of hypertension in a well-defined prospective cohort setting. Although there is limitation in our study, such as a limited sample size and lack of availability of an independent replication sample, our findings provide potential
novel biomarkers associated with incident disease independently of traditional risk factors, and hold promise for better defining the underlying pathophysiology of hypertension. 4-Hydroxyhippurate is an end product of benzoate metabolism from microbial fermentation of polyphenols. It may also originate in the oxidative breakdown of many exogenous benzenoid substances by detoxifying enzymes in the endoplasmic reticulum or microsomes. In the cardiovascular system, oxidative stress plays a critical physiological role in controlling endothelial function, vascular tone, and cardiac function in hypertension. Therefore, we speculate that its mechanism of action on blood pressure regulation may be through a multitude of pathways, such as gut microbial fermentation and oxidative stress.

A sex steroids pattern was positively and independently associated with incident hypertension after adjustment for traditional risk factors and kidney function. This pattern may reflect the catabolism of pregnenolone, and the subsequent metabolism of its estrogen and androgen derivatives. It is not prudent to promote firm conclusions on the role of progesterone in hypertension, although it may lead to hypertension via the body’s response to stress. Of note, stress may have a more important effect on hypertension in blacks than other groups. For estrogens and androgens, a balance between activation mechanisms of vasoconstriction and vasorelaxation determines the net effect on vascular tone and blood pressure. The mechanisms by which sex steroids affect blood pressure involve direct effects on vascular, renal, and heart function.

### Table 3. Principal Component Patterns

<table>
<thead>
<tr>
<th>Pattern (Consisting Metabolites)</th>
<th>Individual Named Components</th>
<th>Eigen-Value</th>
<th>Variance Explained</th>
<th>$P$ for Trend in Quintile Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern 1 (sex steroids, branched-chain amino acids, and other AAs)</td>
<td>4-Androsten-3β,17β-diol disulfate 2 Pregnenediol sulfate DHEA-S</td>
<td>15.88</td>
<td>0.08</td>
<td>1.58 1.72 ($P=0.05$) ($P=0.03$)</td>
</tr>
<tr>
<td>Pattern 2 (α-amino acids and their derivatives)</td>
<td>3-Methoxytyrosine Erythronate Cholesterol</td>
<td>8.81</td>
<td>0.05</td>
<td>0.79 0.80 ($P=0.19$) ($P=0.24$)</td>
</tr>
<tr>
<td>Pattern 3 (branched-chain amino acid, long chain fatty acid, other lipids, peptide)</td>
<td>Leucine Valine Leucylleucine Isoleucine Glycerol Phosphate HXGXA Cholesterol Dihomo-linolenate (20:3n3 or n6)</td>
<td>7.09</td>
<td>0.04</td>
<td>1.39 1.39 ($P=0.23$) ($P=0.24$)</td>
</tr>
</tbody>
</table>

Weibull parametric models adjusted for traditional risk factors (age, sex, leisure-time physical activity, alcohol intake, current cigarette smoking status, prevalent diabetes mellitus, and body mass index) in Model 1, traditional risk factors plus estimated glomerular filtration rate in Model 2. AA indicates amino acids; and HR, hazard ratio for highest vs lowest quintile.

Weibull parametric models adjusted for traditional risk factors (age, sex, leisure-time physical activity, alcohol intake, current cigarette smoking status, prevalent diabetes mellitus, and body mass index) in Model 1, traditional risk factors plus estimated glomerular filtration rate in Model 2. AA indicates amino acids; and HR, hazard ratio for highest vs lowest quintile.

Figure. Hazard ratios and 95% confidence intervals of incident hypertension by quintiles of the sex steroids pattern at baseline from Weibull parametric models. Model 2 was adjusted for age, sex, leisure-time physical activity, current cigarette smoking status, alcohol intake, prevalent diabetes mellitus, body mass index, and estimated glomerular filtration rate. Compared with the quintile of the lowest scores for the sex steroids pattern, only the quintile of the highest scores had a significant higher risk of incident hypertension, but the trend of associations across quintiles was statistically significant ($P<0.05$). In the Model 2+BP model, baseline systolic BP and diastolic BP were added, and the effects of the sex steroid pattern were attenuated. BP indicates blood pressure.
cells, indirect effects mediated by humoral factors, as well as modifying aldosterone, renin, and aldosterone to renin ratio. Our findings provide further insight into important questions on the role of sex hormones in hypertension, although further research is required.

Enhancing the ability to identify high-risk individuals for developing hypertension is particularly important, because proven, preventive therapies exist, end-organ complications accrue over time, and the whole process can be delayed. Our prospective cohort study detected biomarkers of hypertension well before the onset of apparent clinical condition. The prospective cohort study detected biomarkers of hypertension well before the onset of apparent clinical condition. The strength of our study includes a population-based prospective cohort with detailed clinical characterization and the strict quality standards to ensure valid and reliable inference with a single measurement. The candidate metabolites of interest from this study should be measured in an independent replication sample of blacks before further application. The present study is among the largest to date and the first prospective cohort to explore serum metabolite profiles in hypertension. High-throughput metabolomics, like other omic technologies, bring a danger of generating false-positive associations because of multiple comparisons and overfitting. Application of traditional statistical approaches (eg, Bonferroni correction) without taking into account the correlations among metabolites may levy an insurmountable statistical penalty that can obscure biologically relevant associations (ie, false-negative results). The modified Bonferroni procedure used in this study considers the full correlation matrix among metabolites, which should preserve statistical power and minimize false-negative results. Clearly, the sources of variation underlying the human metabolome are varied, and the ability to predict incident hypertension after years of follow-up is influenced by many factors, including biological characteristics of the metabolites, study design, and laboratory factors.

Perspectives
Previous studies suggested that targeting high-risk, normotensive individuals for treatment may delay hypertension onset, allow earlier implementation of intervention measures, thereby possibly mitigating vascular complications. The extra information from metabolic studies may help target such individuals and potentially improve the sensitivity and specificity of the final algorithm for prediction of hypertension. The present study identified potential single metabolomic biomarker (ie, 4-hydroxyhippurate) and a metabolomic pattern (ie, sex steroids) independently pointing to dysregulated metabolic pathways underlying hypertension. The findings in this study may impact clinical care by allowing scarce resources to be concentrated on those at greatest risk of hypertension. In addition, an early indicator of hypertension may indicate earlier therapeutic interventions that could minimize the likelihood of serious complications.

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Disclosures
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References
Novelty and Significance

**What Is New?**

- One metabolite, 4-hydroxyhippurate, and a sex steroids pattern (consisting of pregnenolone and its estrogen and androgen derivatives) were identified as independent predictors of incident hypertension among blacks by untargeted high-through metabolomic profiling protocol.

**What Is Relevant?**

- Metabolomic studies may help target individuals at high risk for hypertension, and indicate earlier therapeutic interventions to them when necessary.

Summary

The present work is the first studying metabolomic antecedents of hypertension in blacks, who have the highest rates of hypertension among all races. It shows that metabolomic biomarkers of hypertension can be detected well before the onset of the clinical condition.
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