Original Article

# Gut Microbiota and Fecal Levels of Short-Chain Fatty Acids Differ Upon 24-Hour Blood Pressure Levels in Men

Justine Huart,* Justine Leenders,* Bernard Taminiau,* Julie Descy, Annie Saint-Remy, Georges Daube, Jean-Marie Krzesinski, Pierrette Melin, Pascal de Tullio,† François Jouret†

***Abstract***—Gut microbiota may influence blood pressure (BP), namely via end products of carbohydrate fermentation. After informed consent, male volunteers were prospectively categorized into 3 groups upon European Society of Hypertension criteria based on 24-hour ambulatory BP measurements: (1) hypertension, (2) borderline hypertension, and (3) normotension. Stool, urine and serum samples were collected in fasting conditions. Gut microbiota was characterized by 16S amplicon sequencing. Metabolomics, including quantification of short-chain fatty acids, was conducted using nuclear magnetic resonance. Two-way ANOVA combined with Tukey post hoc test, as well as multiple permutation test and Benjamini-Hochberg-Yekutieli false discovery rate procedure, was used. The cohort included 54 males: 38 hypertensive (including 21 under treatment), 7 borderline, and 9 normotensive. No significant difference was observed between groups concerning age, body mass index, smoking habits, and weekly alcohol consumption. The genus *Clostridium sensu stricto 1* positively correlated with BP levels in nontreated patients (n=33). This correlation was significant after multiple permutation tests but was not substantiated following false discovery rate adjustment. Short-chain fatty acid levels were significantly different among groups, with higher stool levels of acetate, butyrate, and propionate in hypertensive versus normotensive individuals. No difference was observed in serum and urine metabolomes. Correlation between stool metabolome and 24-hour BP levels was evidenced, with $R^2$ reaching 0.9. Our pilot study based on 24-hour ambulatory BP measurements, 16S amplicon sequencing, and metabolomics supports an association between gut microbiota and BP homeostasis, with changes in stool abundance of short-chain fatty acids. **(*Hypertension*. 2019;74:00-00. DOI: 10.1161/HYPERTENSIONAHA.118.12588.)** ● **Online Data Supplement**

**Key Words:** blood pressure ■ butyrate ■ gastrointestinal microbiome ■ humans ■ male



Arterial hypertension is a worldwide public health issue.[1] In most cases, hypertension is caused by a combination of genetic and environmental factors, with no unique pathogenesis. The pathophysiology of so-called primary hypertension remains largely unknown, which hampers the development of targeted therapies.[1] Recent observations in rodents and humans suggest an impact of gut microbiota (GM) on blood pressure (BP) regulation.[2–6] Indeed, GM directly or indirectly influences host physiology.[7,8] GM dysregulation has been linked to various chronic disorders,[2,9–11] including hypertension.[3–5,9] More specifically, GM-derived short-chain fatty acids (SCFAs) may be involved in BP homeostasis.[9,12] SCFAs correspond to end products of GM-mediated food-derived carbohydrate fermentation and mainly include acetate, propionate, and butyrate.[13] SCFAs act on GPCRs (G-protein–coupled receptors).[14]

On the basis of these observations, we designed a prospective collection of stool, urine, and serum samples from male adult individuals to characterize GM and quantify GM-derived SCFA on 24-hour BP levels. The 16S amplicon sequencing was used to characterize the microbial biodiversity among BP-based groups.[15] Nuclear magnetic resonance (NMR)–based metabolomics was performed on stool, urine, and serum to explore whether BP levels correlate with biofluid metabolomes (including SCFA abundance).

## Patients and Methods

Data and analytic methods are available to other researchers: all raw sequencing reads have been deposited at the National Center for Biotechnology Information and are available under the Bioproject PRJNA507937. Metabolomics data have been uploaded to MetaboLights. The present single-center prospective study was approved by the Institutional Review Board of the University of Liège (protocol No. B707201318600).

### Patients

Adult male individuals with or without known primary hypertension and with normal kidney and liver functions were recruited.

Received December 24, 2018; first decision January 12, 2019; revision accepted June 2, 2019.

From the Division of Nephrology, University of Liège Hospital (J.H., A.S.-R., J.-M.K., F.J.), Groupe Interdisciplinaire de Génoprotéomique Appliquée, Cardiovascular Sciences (J.H., J.-M.K., F.J.), Center for Interdisciplinary Research on Medicines, Metabolomics Group (J.L., P.d.T.), Department of Food Sciences, Faculty of Veterinary Medicine, Fundamental and Applied Research Center for Animal and Health (B.T., G.D.), and Clinical Microbiology, University of Liège Hospital (J.D., P.M.), University of Liège, Belgium.

*These authors contributed equally to this work.

†These authors share the senior authorship of this article.

**The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.118.12588.**

Correspondence to François Jouret, Division of Nephrology, Centre Hospitalier Universitaire de Liège, Ave Hippocrate, 13, B-4000 Liège, Belgium. Email francois.jouret@chuliege.be

Patients under antibiotics at the time of enrollment were excluded from the study. After signed informed consent, 24-hour ambulatory BP measurement (24-hour ABPM, Spacelabs 90207 device) was performed according to the European Society of Hypertension.[16] BP was measured every 20 minutes during the day and every 30 minutes during the night. Mean daytime and nighttime systolic BP (SBP), diastolic BP (DBP), and mean BP (MBP) levels were calculated on the basis of self-declared awake and asleep periods. MBP was calculated by Spacelabs at each BP measurement according to the following formula: MBP=DBP+1/3(SBP−DBP). Participants were divided into 3 groups based on the European Society of Hypertension criteria. Normotension was defined by mean 24-hour BP levels <130/80 mm Hg in untreated individuals. Borderline hypertension (bHT) was defined by mean 24-hour BP levels <130/80 mm Hg with either isolated daytime hypertension (≥135 mm Hg [systolic] and ≥85 mm Hg [diastolic]) or nocturnal hypertension (≥120 mm Hg [systolic] or ≥70 mm Hg [diastolic]). Hypertension was defined by mean 24-hour BP levels ≥130 mm Hg (systolic) or ≥80 mm Hg (diastolic) or in case of use of antihypertensive medications whatever the BP levels. A patient was categorized as dipper when his night-day BP ratio was ≤0.9 or nondipper when his night-day BP ratio was >0.9.[17]

## Samples

Feces were collected at home using stool collection tubes provided with the PSP Spin Stool DNA Plus Kit (ISOGEN Life Science) and Fecal Swab Collection tubes. The collectors for stool sampling were given at the time of ABPM onset (day 1) and were brought back the next day (day 2, when ABPM device was removed). Samples in stool collection tubes were immerged with stool DNA stabilizer solution[18] and stored at −80°C. Samples in Fecal Swab Collection tube were centrifuged for 10 minutes at 3000 rpm following a 10-s vortex and withdrawal of the swab from the cap. Supernatants were aliquoted and stored at −80°C. Serum and second urine were collected in fasting conditions on day 2, aliquoted and stored at −80°C.

## 16S Amplicon Sequencing

Total bacterial DNA was extracted using the PSP Spin Stool DNA Plus Kit following the manufacturer's recommendations. Polymerase chain reaction (PCR) amplification of the V1-V3 region of the 16S ribosomal DNA and library preparation were performed with the following primers (with Illumina overhand adapters), forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGGCAC-3′). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham) and diluted to 10 ng/µL. A final quantitative PCR quantification of each sample in the library was performed using the KAPA SYBR FAST quantitative PCR Kit (Kapa Biosystems, Wilmington) before normalization, pooling and sequencing on a MiSeq sequencer using v3 reagents (ILLUMINA). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run.

## Microbiota Profiling

Sequence read processing was used as described previously[19] using, respectively, MOTHUR software package v1.35[20] and UCHIME algorithm[21] for alignment, operational taxonomic unit clustering, and chimera detection. Clustering distance of 0.03 was used for operational taxonomic unit generation. 16S ribosomal DNA reference alignment and taxonomical assignation were based on the SILVA database (v1.28) of full-length 16S ribosomal DNA sequences.[22] Subsample datasets were obtained and used to evaluate ecological indicators, richness estimation (Chao1 estimator), microbial biodiversity (reciprocal Simpson index), and the population evenness (derived from Simpson index) using MOTHUR. Population structure and community membership were assessed with MOTHUR using distance matrice based on Bray-Curtis dissimilarity index (a measure of community structure which considers shared operational taxonomic units and their relative abundances).[23] Ordination analysis and 3-dimensional plots were performed with Vegan, Vegan3d, and rgl packages in R. Nonmetric dimensional scaling based on the Bray-Curtis dissimilarity matrix was applied to visualize the biodiversity between the groups using MOTHUR.[24]

## 1H-Nuclear Magnetic Resonance Data Acquisition

Six hundred microliters of stool supernatants were centrifuged for 6 minutes at 13.300 rpm (4°C) to eliminate membranes and cell residues. Four hundred microliters of supernatant was supplemented with 200 µL of deuterated phosphate buffer (pH 7.4), 100 µL of a 5-mmol/L solution of maleic acid, and 10 µL of a 10-mg/mL triméthylsilylpropanoate $D_2O$ solution. Serum (500 µL) and urine (500 µL) samples were supplemented with 100 µL of deuterated phosphate buffer (pH 7.4), 100 µL of a 35 mmol/L solution of maleic acid, and 30 (serum) or 10 (urine) µL of a 10-mg/mL triméthylsilylpropanoate D2O solution. All samples were recorded at 298K on a Bruker Avance spectrometer operating at 500.13 MHz for the proton signal acquisition. The instrument was equipped with a 5-mm TCI cryoprobe with a Z gradient. Maleic acid was used as internal standard for quantification and trimethylsilyl-3-propionic acid-*d*4 (triméthylsilylpropanoate) for the zero calibration. 1H-Nuclear magnetic resonance spectra were acquired using a 1D NOESY sequence with presaturation for urine samples and Carr-Purcell-Meiboom-Gill relaxation-editing sequence with presaturation for serum samples and stool supernatants. The nuclear overhauser effect spectroscopy presat experiment used a RD-90°-$T_1$-90°-$T_m$-90°-acquire sequence with a relaxation delay (RD) of 4 s, a mixing time ($T_m$) of 10 ms, and a fixed $T_{1/0}$ delay of 4 µs. Water suppression pulse was placed during the RD. Acquisition time is fixed to 3.2769001 s. The Carr-Purcell-Meiboom-Gill experiment used a RD-90-(t-180-t)n-sequence with an RD of 2 s, a spin echo delay (t) of 400 ms, and the number of loops (n) equal to 80. The water suppression pulse was placed during the RD. The acquisition time was set to 3.982555 s. For all samples, the number of transients was typically 64, and a quantity of 4 dummy scans was chosen. The data were processed with the Bruker Topspin 3. Phase and baseline corrections were performed manually over the entire range of the spectra, and the δ scale was calibrated to 0 ppm using the internal standard triméthylsilylpropanoate.

## Statistical Analysis

Continuous variables were expressed as mean±SD; frequencies of categorical variables were expressed as percentages. In the Table, the study compared the distribution of 6 continuous variables (age, body mass index, alcohol consumption, 24-hour SBP, 24-hour DBP, 24-hour MBP) in the 3 groups of BP status. For each variable, a Kruskal-Wallis test was used followed by 2 by 2 multiple comparisons of the groups according to the method described by Siegel and Castellan.[25] To maintain the overall statistical significance level α at the nominal level of 5%, a Bonferroni correction was applied, and each Kruskal-Wallis test was performed at the 0.0083 level. Tests were performed with Statistica software (v 13). Frequencies of categorical parameters were compared using a $\chi^2$ test of independence. For small frequencies, Fisher exact test was used. Statistics for bacterial biodiversity, richness, and evenness were assessed with 2-way ANOVA corrected for multitesting (Benjamini, Krieger, and Yekutieli) using PRISM 7 (Graphpad Software). Differences of population abundance between groups were assessed with ANOVA using STAMP software.[26] Statistical paired differences between treatment groups of specific bacterial populations were assessed by 2-way ANOVA and Tukey-Kramer post hoc test. Spearman correlation was tested after 1.000 permutations using WPERM packages in R software. Additionally, Benjamini-Hochberg-Yekutieli false discovery rate procedure was used (Psych package for R). For univariate analysis of SCFAs, spectral data of stool samples were used to provide a relative quantification of 3 preselected SCFA: acetate, butyrate, and propionate concentrations were obtained by

**Table. Clinical Characteristics of the Three 24-h Ambulatory BP Profiles**

| Clinical Characteristics | Reference to Mean 24-h BP | | | *P* Value* |
|---|---|---|---|---|
| | Normotension (1) | Borderline (2) | Hypertension (3) | |
| n | 9 | 7 | 38 | |
| Age, y | 46.2±11.4 | 50.3±13.3 | 52.5±8.2 | 0.40 |
| BMI, kg/m$^2$ | 24.3±1.1 | 23.9±3.5 | 27.2±3.5 | 0.021 |
| Smokers, % | 11 | 14 | 11 | 0.80 |
| Alcohol (glass/wk) | 5.7±4 | 3.8±3 | 5.5±5.4 | 0.74 |
| Family HT, % | 55 | 45 | 42 | 0.83 |
| Diabetes mellitus, % | 0 | 0 | 7.9 | 0.99 |
| CV history, % | 11 | 14 | 23.6 | 0.63 |
| GE history, % | 33 | 43 | 46 | 0.80 |
| Antibiotics during previous 3 mo, % | 22 | 43 | 13 | 0.18 |
| 24-h SBP, mm Hg | 118±6 | 124±5 | 137±14 | 0.000032 (1)<(3) 0.000063 |
| 24-h DBP, mm Hg | 73±5 | 77±1 | 85±11 | 0.000498 (1)<(3) 0.000909 |
| 24-h MBP, mm Hg | 87±5 | 92±2 | 102±11 | 0.000040 (1)<(3) 0.000074 |
| Non–anti-HT treatment, % | 100 | 100 | 45 | |
| Anti-HT treatment, % | 0 | 0 | 55 | |
| N anti-HT class (median) | | | 3 (1–6) | |
| Diuretics, % | | | 43 | |
| β-Blockers, % | | | 48 | |
| CCBs, % | | | 67 | |
| ACE inhibitors, % | | | 48 | |
| ARBs, % | | | 38 | |
| Central agents, % | | | 24 | |

ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; BP, blood pressure; CCB, calcium channel blocker; CV, cardiovascular; DBP, diastolic blood pressure; GE, gastroenterological; HT, hypertension; MBP, mean blood pressure; N anti-HT, number of anti-hypertension drug classes (median); and SBP, systolic blood pressure.

*For continuous variables, *P* value of the Kruskal-Wallis test with below the only pairwise comparisons that were significant at the *P* level ≤0.0083.

the integration of the signals at 1.93, 1.56, and 1.05 ppm, respectively, using maleic acid as internal standard (normalized to 2) and Topspin software (version 3.2; Bruker). Statistics between groups were done by a 1-way ANOVA. For multivariate analysis, optimized 1H-Nuclear magnetic resonance spectra were automatically baseline corrected and reduced to American standard code for information interchange files using AMIX software (version 3.9.14; Bruker). The spectral intensities were normalized to total intensities and reduced to integrated regions of equal width (0.04 ppm) corresponding to the 0.5- to 10.00-ppm region. Because of the residual signals of water and maleic acid, regions between 4.7 and 5 ppm (water signal) and 5.6 and 6.2 ppm (maleic acid signal) were removed before analysis. The reduced and normalized NMR spectral data were imported into SIMCA (version 13.0.3; Umetrics AB, Umea, Sweden). Pareto scaling was applied to bucket tables and discriminant analyses (DA), such as principal component analysis, partial least squares DA (PLS-DA), orthogonal PLS-DA, and partial least square regression, were performed. SIMCA was used to generate all principal component analysis, PLS, and PLS-DA models and plots. Principal component analysis was only used to detect possible outliers and determine intrinsic clusters within the data set, while PLS-DA maximized the separation. Metabolomics and 16S amplicon sequencing data were subjected to a Tukey test for multiple comparisons.

## Results

### Clinical Features of the Cohort

Our cohort initially included 28 hypertension patients of 28 patients with hypertension and 26 self-reported healthy volunteers. The European Society of Hypertension criteria for hypertension were applied to all 24-hour ABPM, which caused the reclassification of 17 healthy volunteers to hypertension (n=10) and bHT (n=7) groups. Therefore, our working cohort was based on 3 groups: 9 normotension, 7 bHT, and 38 hypertension patients (including 21 patients under antihypertensive medications, 55% of the hypertension; Figure 1). A median number of 3 different classes of antihypertensive drugs per day was reported. The clinical characteristics of the cohort are summarized in the Table. No difference was observed among groups concerning the age, body mass index, the diet, smoking habits, or weekly alcohol consumption. About half of the individuals had a family history of hypertension. Diabetes mellitus was present in 7.9% of the cohort and only concerns hypertension group. No difference was found among groups about the use of antibiotics during the 3-month period preceding study enrollment. Of note, the shorter period between the end of the antibiotherapy and stool collection was 15 days. By definition, 24-hour SBP, 24-hour DBP, and 24-hour MBP were significantly higher in the hypertension group when compared with normotension. When comparing hypertension to bHT subjects, BP differences did not reach statistical significance ($P$=0.061 for 24-hour SBP, $P$=0.073 for 24-hour MBP). Similar observations were made concerning daytime or nighttime BP.

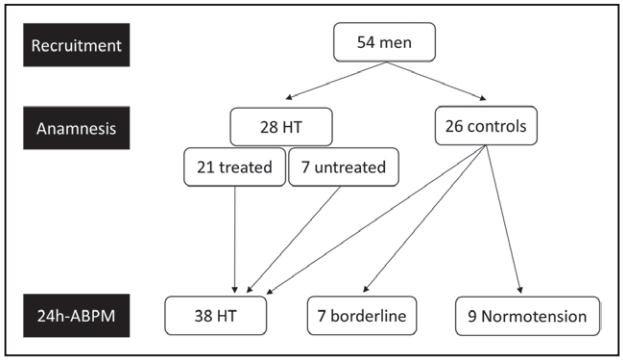


**Figure 1.** Flowchart of patient's recruitment and categorization. Fifty-four patients were enrolled. On basis of the anamnesis, 28 were hypertension (HT), including 21 under chronic antihypertensive medication. The remaining 26 self-reported healthy volunteers were tested for HT using a 24-hour ambulatory blood pressure measurement (ABPM; following the European Society of Hypertension criteria). The final cohort included 38 patients with HT, 7 patients with borderline hypertension, and 9 normotension individuals.

## Microbial Diversity in Patients Without Versus With Hypertension

The identity and relative abundance of dominant and subdominant microbial populations present in stool samples have been determined using sequencing of the V1-V3 hypervariable region of bacterial 16S ribosomal DNA. The 16S amplicon sequencing included 10.000 identifications by sample and was corrected for multiple tests. We focused on phylum, family, genus, and species. Only the bacterial populations with a median value different from zero among the entire cohort were considered. The most abundant phyla in the global cohort included *Firmicutes* and *Bacteroidetes*. Concerning families, 4 dominant populations were identified: *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, and *Prevotellaceae*. Two-way ANOVA followed by Tukey post hoc test failed to show statistically significant differences between bacterial phyla, families, genera, and species among the 3 groups. The Firmicutes/Bacteroidetes ratio has been calculated on the relative abundance of these phyla for each patient. No statistic difference between the mean ratios was found among groups. Still, a significant and positive correlation between the relative abundance of the genus *Clostridium sensu stricto 1* and 24-hour SBP, 24-hour DBP, and 24-hour MBP levels was found (Figure 2A). Significant negative correlations were observed between the relative abundance of 2 bacterial species (*Ruminococcaceae_ge_DQ807686* and *Clostridiales_ge_16S_OTU1343*) and 24-hour SBP, 24-hour DBP, and 24-hour MBP levels (Figure 2B). These correlations remained significant after multiple permutation tests but were not substantiated following false discovery rate adjustment (online-only Data Supplement). Patients under antihypertensive medications (n=21) were excluded from these correlations. Correlations between bacterial species or genus and BP levels were observed with 24-hour BP levels, as well as with nighttime BP levels or daytime BP levels, independently. Correlations between bacterial species or genera and only 1 or 2 of the 3 BP parameters (24-hour

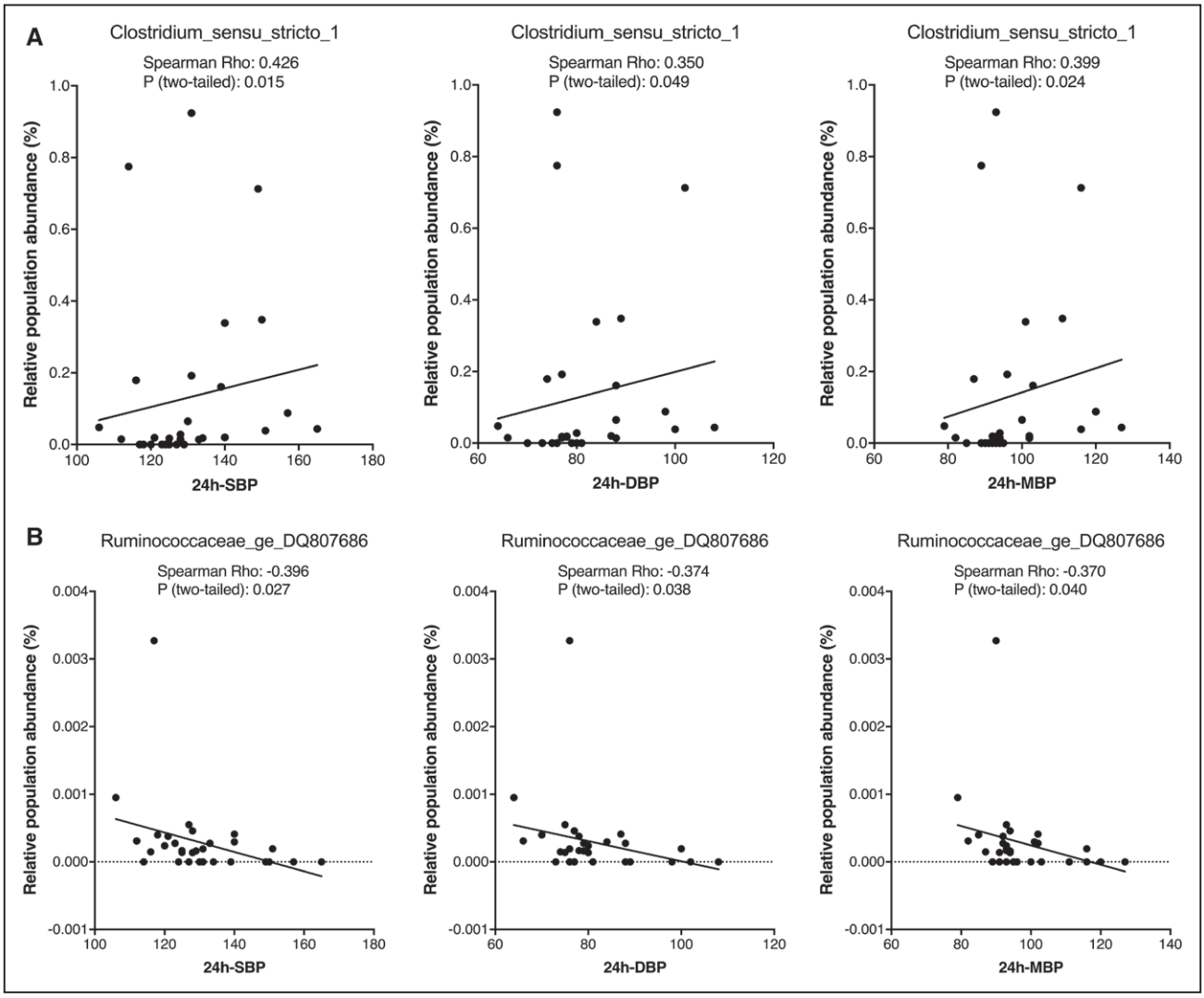


**Figure 2.** Correlation studies between bacterial genera/species and 24-hour blood pressure levels. **A**, Significant positive correlations between the relative abundance of the genus *Clostridium sensu stricto 1* in stools and 24-hour systolic blood pressure (SBP), 24-hour diastolic blood pressure (DBP), and 24-hour mean blood pressure (MBP) levels were observed in all patients without antihypertensive medication (n=33). **B**, Significant negative correlations between the relative abundance of the *Ruminococcaceae_ge_DQ807686* species in stools and 24-hour SBP, 24-hour-DBP and 24-hour-MBP levels were observed in all patients without antihypertensive medication (n=33).

SBP, 24-hour DBP, and 24-hour MBP) were rejected. No difference was found between dipper and nondipper patients regarding GM composition.

## Stool, Serum, and Urine Metabolomes in Patients Without Versus With Hypertension

### *Stool Metabolome*

NMR metabolomics analyses performed on stool samples did not statistically discriminate normotension versus bHT versus hypertension groups, even after using supervised methods ($R^2$ at 0.096 and $Q^2$ at −0.207). Note that aberrant and uninterpretable spectral data were removed from the analyses. Partial least square regression lines for stool metabolomes of all individuals with no antihypertensive medications (n=27) highlighted correlations with 24-hour MBP, 24-hour SBP, and 24-hour DBP levels (Figure 3). Stool metabolomes from untreated hypertension (n=14), bHT (n=7), and normotension (n=6) patients, respectively, correlated with 24-hour MBP levels, with $R^2$ coefficients of 0.84, 0.83, and 0.93. Partial least square regression including the 27 patients showed $R^2$ coefficient of correlation with 24-hour MBP of 0.40. Focusing on fecal SCFA levels, ANOVA between groups found statistically significant differences in acetate, butyrate, and propionate abundance (Figure 4). Stool levels of acetate reached 60.4±4.1 in hypertension patients (including both treated and nontreated patients, n=30), compared with 44.3±6.3 and 16.0±4.7 in bHT (n=6) and normotension individuals (n=8), respectively. Those quantifications are relative to maleic acid concentration. No significant difference of stool levels of acetate, butyrate, and propionate was found between treated and untreated hypertension patients. Similarly, fecal levels of butyrate reached 16.1±1.9 in hypertension patients, compared with 11.0±2.1 and 4.4±1.4 in bHT and normotension individuals, respectively. Fecal abundance of propionate tended to be increased in hypertension patients (14.5±1.4) in comparison to bHT (10.4±1.5) and normotension (3.6±1.3). No correlation was found between the stool levels of acetate, butyrate, and propionate, and the 24-hour BP levels.

### *Urine and Serum Metabolomes*

Global DA of serum and urine metabolomes did not find statistically significant distinctions among groups. Correlations performed with serum and urine metabolomes did not detect associations with 24-hour MBP, 24-hour SBP, or 24-hour DBP levels. SCFA levels could not be quantified in urine and serum spectral data because of their weak abundance in these biofluids. Urine pH considerably varied between individuals, which caused uninterpretable shifts of NMR spectral peaks.

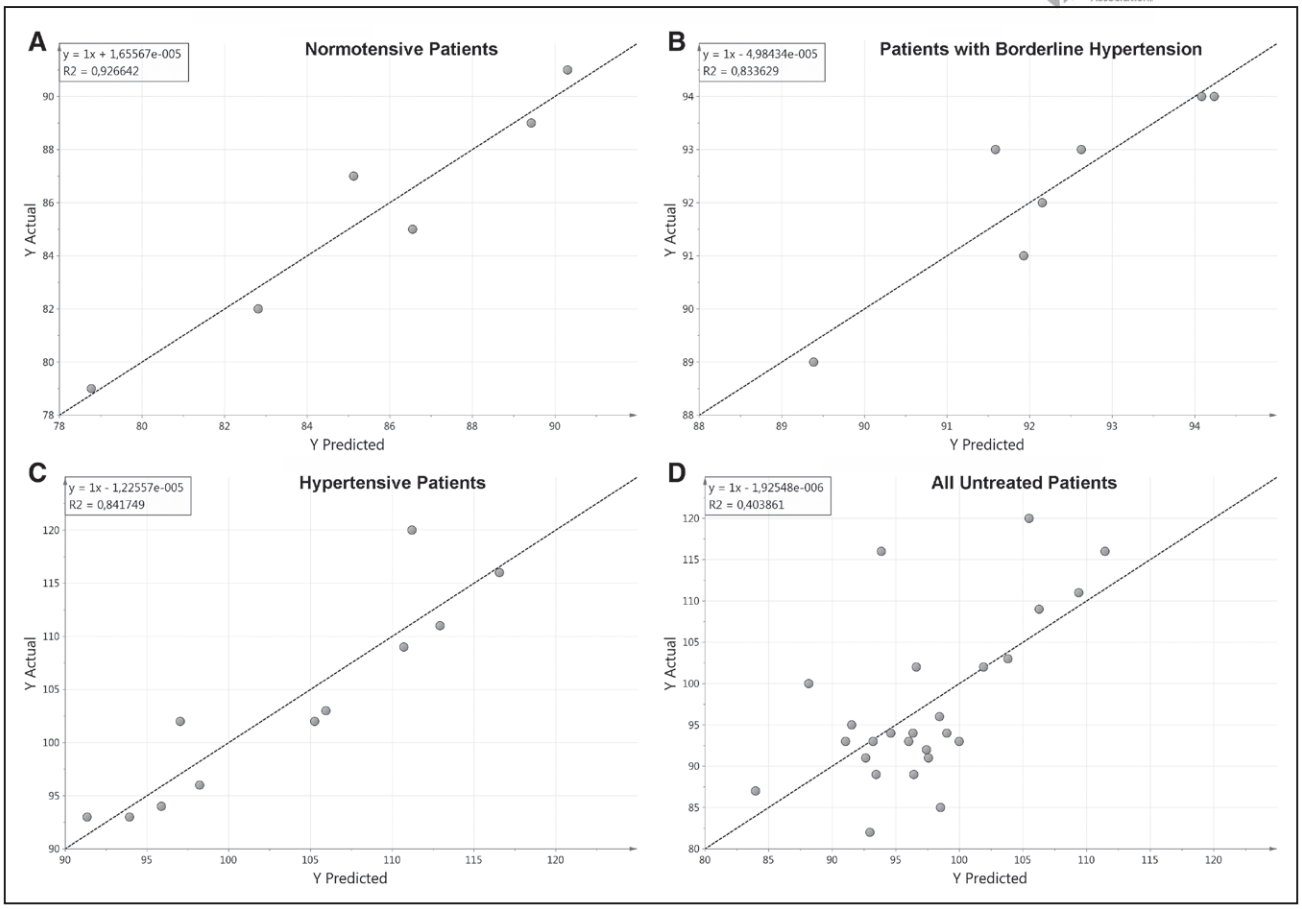


**Figure 3.** Transversal correlation studies between stool metabolome and 24-hour mean blood pressure (MBP) levels. Partial least square regressions were used to assess the correlations between the 24-hour MBP levels (Y actual values) and the stool metabolome (depicted as Y predicted) in controls (**A**; n=6, $R^2$=0.93), in borderline hypertension (bHT) patients (**B**; n=7, $R^2$=0.83), and in patients with untreated hypertension (HT; **C**; n=14, $R^2$=0.84). Transversal study of 24-hour MBP levels with stool metabolome of all untreated individuals (untreated HT+bHT+normotension) is represented in **D** (n=27, $R^2$=0.40).

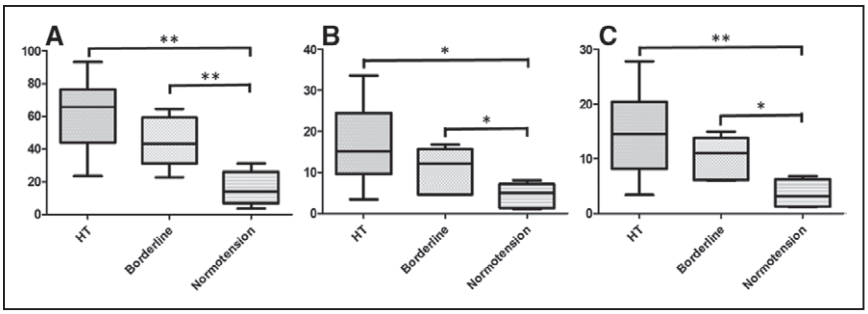


**Figure 4.** Short-chain fatty acid (SCFA) quantifications in stool samples. Acetate (**A**), butyrate (**B**), and propionate (**C**) were quantified on the basis of 1H-Nuclear magnetic resonance spectra of stool samples. SCFA abundance in hypertension (HT) patients (including both treated and untreated, n=30) was higher in comparison to individuals with borderline hypertension (n=6) or normotension (n=8). *0.01>*P*<0.05, **0.001>*P*<0.01.

## Discussion

In the present cohort, the genus *Clostridium sensu stricto 1* positively correlated with BP levels in nontreated individuals. This correlation remained significant after multiple permutation tests but was not substantiated following false discovery rate adjustment. Furthermore, a correlation between stool metabolome and 24-hour BP levels was evidenced, with increased fecal levels of acetate, propionate, and butyrate levels in hypertension patients. These observations support an association between GM composition and BP levels, possibly via stool abundance of SCFAs. The limitations of our monocentric study include the small number of patients. The size of our control group was significantly reduced by the a posteriori verification of the 24-hour BP levels following European Society of Hypertension criteria. These unanticipated pitfalls emphasize the unrecognition of hypertension in the general population based only on office BP. The strengths of our work actually rely on such a well-standardized 24-hour ABPM to subcategorize our cohort, as well as the focus on one single sex to limit the putative hormonal influence on BP levels and GM composition. To the best of our knowledge, our present study is the first one based on 24-hour ABPM in the GM field. Note that GM is regarded as stable over time in adults.[15] Antibiotics are known to modify GM, but the perturbations tend to disappear rapidly after treatment.[27] Moreover, several nonantibiotic drugs may influence the human GM.[28,29] Our study was designed before these reports. We provide the exhaustive list of nonantibiotic drugs taken by the subjects of our cohort at the time of sample collection. More specifically, 5 patients were under inhibitors of proton pump and 4 patients under metformin. After excluding these patients, our observations remained statistically significant (online-only Data Supplement). Note that no difference in stool abundance of SCFAs was observed in hypertension patients with versus without antihypertensive drugs.

Several preclinical and clinical models have suggested that gut dysbiosis influences BP homeostasis.[3–6,30] In hypertension rats, oral minocycline restored GM, reduced the *Firmicutes/Bacteroidetes* ratio, and attenuated BP levels.[4] In mice, fecal transplantation from hypertension human donors to germ-free animals causes hypertension, thereby suggesting that hypertension is transferrable through GM.[30] In a small cohort of patients with hypertension, an increased ratio of *Firmicutes/Bacteroidetes* phyla has been reported.[4] Similarly, Yan et al[3] have recently reported on GM dysbiosis in 60 hypertension patients compared with 60 healthy individuals, with increased abundance of *Klebsiella*, *Streptococcus*, and *Parabacteroides* species.[3] Compared with 41 healthy controls, Li et al[30] found distinct metagenomic composition in 99 hypertension patients and 55 bHT, with overgrowth of *Prevotella* and *Klebsiella*.

Changes in GM on BP levels have been linked to the inflammatory status of gut wall, with structural changes (increased fibrosis, decreased length of the villi, etc) and functional impacts (increased intestinal permeability).[2,31] These observations have highlighted putative links between (1) gut, (2) food-derived GM metabolites, and (3) kidneys as principal actors in BP homeostasis.[2,3,32] More specifically, SCFAs are end products of the fermentation of food polysaccharides by gut bacteria. SCFA most commonly refers to the straight-chain 2–4 carbon variety: acetate, propionate, and butyrate. Although acetate is generally reported to be the most abundant SCFA in the mammalian gut, the precise ratios of acetate versus butyrate versus propionate are variable on diet manipulations.[33,34] SCFA concentrations in the mammalian colon are about 100 mmol/L.[9,13] It depends on the GM composition, which is, in turn, influenced by SCFAs.[34] SCFAs diffuse through the intestinal mucosa, enter the bloodstream via the portal vein, and act by binding to GPCRs, which are broadly distributed in mammalian organisms.[9,14] Samuel et al[35] found that the intestinal absorption of SCFA seems to be influenced by the GPCR, GPR41. Indeed, GPR41-deficient mice show reduced intestinal absorption and delivery of SCFA, which was linked to a necessary interaction between SCFA and GPR41 to increase circulating levels of hormones that, in turn, increase SCFA absorption by reducing gut motility.[35] Hence, SCFAs participate to the regulation of adipose tissues[35] and the immune system,[36] thereby linking nutrition, GM, and host physiology and pathology.[34] Previous ex vivo observations using human samples showed that SCFAs induce arterial vasodilatation.[37] In a recent murine model, propionate and acetate modulated BP levels in opposite ways depending on the type of activated GPCR, that is, Olfr78 versus Gpr41.[14] Activated Gpr41 causes hypotension, whereas activated Olfr78 causes an increase in BP.[9,14] Of note, 2 additional SCFA GPCRs are currently under investigation: Gpr109A (butyrate) and Gpr43 (acetate, propionate, and butyrate).[9,38] The increased *Firmicutes/Bacteroidetes* ratio in hypertension rats and patients was associated with a decreased abundance of acetate- and butyrate-producing bacteria.[4] By contrast, the *Roseburia* and *Faecalibacterium prausnitzii* species, which are both regarded as SCFA producers, were more prevalent in healthy

normotensive individuals.[3] Similarly, epidemiological studies reported on a significant association between the rate of urinary SCFAs (particularly, formate) and BP levels.[39] Kim et al[31] have found an altered production of butyrate in stools of hypertension patients. Meta-analyses concluded that the use of probiotics (which induces an increased production of SCFAs) is associated with lower BP levels.[9,40] However, higher levels of plasma acetate were found in hypertension rats following microbial transplant, whereas higher levels of stool acetate and propionate were found in hypertension rats following high-salt diet[6,41]

In the present study, we observe a significant association between GM and BP levels, with a positive correlation between the relative abundance of the genus *Clostridium sensu stricto 1* in stool samples of untreated individuals and 24-hour BP levels. Significant negative correlations were also found between the relative fecal abundance of 2 bacterial species and 24-hour BP levels. These are part of the *Firmicutes* phylum in the *Clostridia* class and in the *Clostridial* order. They belong to 2 different families (*Ruminococcaceae* and *Clostridiales*) and differ by their genus. The *Rhuminococcaceae* are known to be butyrate producers,[42] which may be consistent with the results of Yan et al.[3] Interestingly enough, Sun et al[43] have recently reported in the CARDIA study (Coronary Artery Risk Development in Young Adults) involving 529 participants on a similar inverse association between *Ruminococcaceae* and BP levels. Furthermore, our observed positive correlation between the genus *Clostridium sensu stricto 1* and BP levels is original and goes in line with the positive association between hypertension and *Clostridium IV* genus found in the CARDIA study.[43]

Multivariate analysis of serum and urine metabolomes failed to detect significant discriminations, probably because of the limited number of controls. However, significant positive correlations were found between stool metabolome and BP levels in untreated individuals. The nature and number of metabolites contained in the corresponding 1H-Nuclear magnetic resonance spectra include SCFAs, methanol, and amino acids, as described in Lin et al.[44] More specifically, higher amounts of acetate, butyrate, and propionate were found in stools of hypertension patients (both treated and untreated) in comparison to controls. Our observations are similar to the ones of Bier et al[41] quantifying by metabolomics the SCFAs in the stools of hypertension rats. Conversely, they may appear contradictory to other reports in the field.[3,4,31] Most of the currently available reports did not directly measure stool levels of SCFAs in patients or animals but only showed a reduction in SCFA-producing enzymes and bacteria in the gut of hypertension animals and patients. Other studies correlated higher blood and urine levels of SCFAs with lower BP levels,[39] although Mell et al[6] found higher plasma levels of acetate in hypertension rats. Note that higher amounts of SCFAs in stools of hypertension patients may also be secondary to decreased intestinal absorption of SCFAs, as suggested by Samuel et al.[14,35]

The definition of hypertension was not conventional in previous reports, which questions the validity of patient categorization and data interpretation. In the study of Yan et al,[3] patients were enrolled according to an isolated BP measurement. In the study of Yang et al,[4] patients were categorized as hypertension if they were under antihypertensive medications and controls if they were untreated. Office BP measurements do not allow to discriminate white coat and masked hypertension. The ABPM, as performed in our present study, has been recently validated as a strong predictor of all-cause and cardiovascular mortality.[45] Finally, the impact of GM on BP homeostasis is far from being resolved. One given SCFA differently modulates BP according to the type of activated GPCR.[14] In addition, SCFA-independent mechanisms also contribute to GM impact on the development of hypertension.[2,46,47] Ethnic disparities, including different diet habits, and individual genetic susceptibility may also influence the pathophysiological connection between GM and hypertension.[2]

## Perspectives

Evidence accumulates regarding the implication of GM on BP homeostasis. This is a rapidly evolving field of research, which requires additional observational and interventional studies including well-categorized individuals. Particularly, modulating the GM in humans by antibiotic administration, ingestion of probiotics, or fecal transplantation may help tease apart the reciprocal interactions between the microbiota and the host. Assessing the impact of the sex (at various ages and hormonal status), the genetic background and the diet on GM composition and, in turn, on BP regulation is another challenge in the field. Also, given the controversial results published in the literature, it might be interesting to compare GM composition on 24-hour ABPM versus office BP to better discriminate GM influence on white coat and masked hypertension. These investigations may eventually lead to groundbreaking implications in hypertension prevention and treatment.

## Acknowledgments

We cordially thank patients and volunteers for joining the present study, as well as all members of the Divisions of Nephrology and Microbiology Laboratory and Pharmaceutical Chemistry Laboratory at the University of Liège Hospital in Liège, Belgium. The 16S amplicon sequencing analyses were performed par le Genalyse Partner sa company.

## Sources of Funding

P. de Tullio, J. Huart, and F. Jouret are Fellows of the Fonds National de la Recherche Scientifique (FNRS) and received support from the FNRS (Research Credit 2013/2015), from the University of Liège (Fonds Spéciaux à la Recherche–Fonds Léon Fredericq), as well as from the University of Liège Hospital (Fonds d'Investissement pour la Recherche Scientifique 2013/2014).

## Disclosures

None.

## References

1. Genest J. Progress in hypertension research: 1900-2000. *Hypertension.* 2001;38:E13–E18.
2. Raizada MK, Joe B, Bryan NS, et al. Report of the national heart, lung, and blood institute working group on the role of microbiota in blood pressure regulation. *Hypertension.* 2017;70:479–485.

3. Yan Q, Gu Y, Li X, et al. Alterations of the gut microbiome in hypertension. *Front Cell Infect Microbiol.* 2017;7:381. doi: 10.3389/fcimb.2017.00381
4. Yang T, Santisteban MM, Rodriguez V, Li E, Ahmari N, Carvajal JM, Zadeh M, Gong M, Qi Y, Zubcevic J, Sahay B, Pepine CJ, Raizada MK, Mohamadzadeh M. Gut dysbiosis is linked to hypertension. *Hypertension.* 2015;65:1331–1340. doi: 10.1161/HYPERTENSIONAHA.115.05315
5. Adnan S, Nelson JW, Ajami NJ, Venna VR, Petrosino JF, Bryan RM Jr, Durgan DJ. Alterations in the gut microbiota can elicit hypertension in rats. *Physiol Genomics.* 2017;49:96–104. doi: 10.1152/physiolgenomics.00081.2016
6. Mell B, Jala VR, Mathew AV, Byun J, Waghulde H, Zhang Y, Haribabu B, Vijay-Kumar M, Pennathur S, Joe B. Evidence for a link between gut microbiota and hypertension in the Dahl rat. *Physiol Genomics.* 2015;47:187–197. doi: 10.1152/physiolgenomics.00136.2014
7. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature.* 2011;474:327–336. doi: 10.1038/nature10213
8. Pluznick JL, Caplan MJ. Chemical and physical sensors in the regulation of renal function. *Clin J Am Soc Nephrol.* 2015;10:1626–1635. doi: 10.2215/CJN.00730114
9. Meijers B, Jouret F, Evenepoel P. Linking gut microbiota to cardiovascular disease and hypertension: lessons from chronic kidney disease. *Pharmacol Res.* 2018;133:101–107. doi: 10.1016/j.phrs.2018.04.023
10. Butel MJ. Probiotics, gut microbiota and health. *Med Mal Infect.* 2014;44:1–8. doi: 10.1016/j.medmal.2013.10.002
11. Robles Alonso V, Guarner F. Linking the gut microbiota to human health. *Br J Nutr.* 2013;109(suppl 2):S21–S26. doi: 10.1017/S0007114512005235
12. Miyamoto J, Kasubuchi M, Nakajima A, Irie J, Itoh H, Kimura I. The role of short-chain fatty acid on blood pressure regulation. *Curr Opin Nephrol Hypertens.* 2016;25:379–383. doi: 10.1097/MNH.0000000000000246
13. Bugaut M. Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. *Comp Biochem Physiol B.* 1987;86:439–472.
14. Pluznick JL, Protzko RJ, Gevorgyan H, et al. Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. *Proc Natl Acad Sci USA.* 2013;110:4410–4415. doi: 10.1073/pnas.1215927110
15. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA.* 2011;108(suppl 1):4516–4522. doi: 10.1073/pnas.1000080107
16. Mancia G, Fagard R, Narkiewicz K, et al. 2013 ESH/ESC guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J.* 2013;34:2159–2219. doi: 10.1093/eurheartj/eht151
17. Fagard RH. Dipping pattern of nocturnal blood pressure in patients with hypertension. *Expert Rev Cardiovasc Ther.* 2009;7:599–605. doi: 10.1586/erc.09.35
18. Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Dore J, Azpiroz F, Roca J, Guarner F, Manichanh C. Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiol.* 2012;12:158. doi: 10.1186/1471-2180-12-158
19. Rodriguez C, Taminiau B, Korsak N, Avesani V, Van Broeck J, Brach P, Delmée M, Daube G. Longitudinal survey of clostridium difficile presence and gut microbiota composition in a belgian nursing home. *BMC Microbiol.* 2016;16:229. doi: 10.1186/s12866-016-0848-7
20. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* 2009;75:7537–7541. doi: 10.1128/AEM.01541-09
21. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 2011;27:2194–2200. doi: 10.1093/bioinformatics/btr381
22. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 2013;41(Database issue):D590–D596. doi: 10.1093/nar/gks1219
23. Bray JR, Curtis JT. An ordination of the upland forest communities of southern wisconsin. *Ecol Monogr.* 1957;27:325–349.
24. Martin AP. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Appl Environ Microbiol.* 2002;68:3673–3682. doi: 10.1128/aem.68.8.3673-3682.2002
25. Siegel S, Castellan NJ. *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill; 1988.
26. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics.* 2010;26:715–721. doi: 10.1093/bioinformatics/btq041
27. De La Cochetière MF, Durand T, Lepage P, Bourreille A, Galmiche JP, Doré J. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *J Clin Microbiol* 2005;43:5588–92.
28. Forslund K, Hildebrand F, Nielsen T, et al; MetaHIT consortium. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature.* 2015;528:262–266. doi: 10.1038/nature15766
29. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, Brochado AR, Fernandez KC, Dose H, Mori H, Patil KR, Bork P, Typas A. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature.* 2018;555:623–628. doi: 10.1038/nature25979
30. Li J, Zhao F, Wang Y, et al. Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome.* 2017;5:14. doi: 10.1186/s40168-016-0222-x
31. Kim S, Goel R, Kumar A, Qi Y, Lobaton G, Hosaka K, Mohammed M, Handberg EM, Richards EM, Pepine CJ, Raizada MK. Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clin Sci (Lond).* 2018;132:701–718. doi: 10.1042/CS20180087
32. Marques FZ, Mackay CR, Kaye DM. Beyond gut feelings: how the gut microbiota regulates blood pressure. *Nat Rev Cardiol.* 2018;15:20–32. doi: 10.1038/nrcardio.2017.120
33. Pluznick JL. Gut microbiota in renal physiology: focus on short-chain fatty acids and their receptors. *Kidney Int.* 2016;90:1191–1198. doi: 10.1016/j.kint.2016.06.033
34. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilán CG, Salazar N. Intestinal short chain fatty acids and their link with diet and human health. *Front Microbiol.* 2016;7:185. doi: 10.3389/fmicb.2016.00185
35. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci USA.* 2008;105:16767–16772. doi: 10.1073/pnas.0808567105
36. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature.* 2009;461:1282–1286. doi: 10.1038/nature08530
37. Mortensen FV, Nielsen H, Mulvany MJ, Hessov I. Short chain fatty acids dilate isolated human colonic resistance arteries. *Gut.* 1990;31:1391–1394. doi: 10.1136/gut.31.12.1391
38. Peti-Peterdi J, Kishore BK, Pluznick JL. Regulation of vascular and renal function by metabolite receptors. *Annu Rev Physiol.* 2016;78:391–414. doi: 10.1146/annurev-physiol-021115-105403
39. Holmes E, Loo RL, Stamler J, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature.* 2008;453:396–400. doi: 10.1038/nature06882
40. Khalesi S, Sun J, Buys N, Jayasinghe R. Effect of probiotics on blood pressure: a systematic review and meta-analysis of randomized, controlled trials. *Hypertension.* 2014;64:897–903. doi: 10.1161/HYPERTENSIONAHA.114.03469
41. Bier A, Braun T, Khasbab R, Di Segni A, Grossman E, Haberman Y, Leibowitz A. A high salt diet modulates the gut microbiota and short chain fatty acids production in a salt-sensitive hypertension rat model. *Nutrients* 2018;10:E1154. doi: 10.3390/nu10091154s
42. Vital M, Howe AC, Tiedje JM. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *MBio.* 2014;5:e00889. doi: 10.1128/mBio.00889-14
43. Sun S, Lulla A, Sioda M, Winglee K, Wu MC, Jacobs DR Jr, Shikany JM, Lloyd-Jones DM, Launer LJ, Fodor AA, Meyer KA. Gut microbiota composition and blood pressure. *Hypertension.* 2019;73:998–1006. doi: 10.1161/HYPERTENSIONAHA.118.12109
44. Lin Y, Ma C, Liu C, Wang Z, Yang J, Liu X, Shen Z, Wu R. NMR-based fecal metabolomics fingerprinting as predictors of earlier diagnosis in patients with colorectal cancer. *Oncotarget.* 2016;7:29454–29464. doi: 10.18632/oncotarget.8762

45. Banegas JR, Ruilope LM, de la Sierra A, Vinyoles E, Gorostidi M, de la Cruz JJ, Ruiz-Hurtado G, Segura J, Rodríguez-Artalejo F, Williams B. Relationship between clinic and ambulatory blood-pressure measurements and mortality. *N Engl J Med.* 2018;378:1509–1520. doi: 10.1056/NEJMoa1712231
46. Antza C, Stabouli S, Kotsis V. Gut microbiota in kidney disease and hypertension. *Pharmacol Res.* 2018;130:198–203. doi: 10.1016/j.phrs.2018.02.028
47. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med.* 2013;19:576–585. doi: 10.1038/nm.3145s

## Novelty and Significance

### What Is New?

- Our study is the first one in the field based on 24-hour ambulatory blood pressure (BP) measurement following European Society of Hypertension criteria, including the dipping pattern. The categorization of patients according to 24-hour ambulatory BP measurement is highly relevant because (1) office BP measurement does not allow to distinguish white coat from masked hypertension and (2) 24-hour ambulatory BP measurement is a strong predictor of all-cause and cardiovascular mortality.

### What Is Relevant?

- Nuclear magnetic resonance–based metabolomics was used to quantify short-chain fatty acid, that is, acetate, butyrate and propionate, directly in feces.

### Summary

Evidences based on 24-hour ambulatory BP measurement, 16S amplicon sequencing, and metabolomics are provided regarding the association between gut microbiota and BP regulation.