



UNIVERSITY  
OF TURKU

# METABOLOMICS AND METAGENOMICS IN HYPERTENSION

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## ABSTRACT

While hypertension has been linked to various modifiable and fixed risk factors, the exact mechanisms behind blood pressure (BP) regulation and hypertension onset remain elusive. The rapid development in scientific branches studying genome, proteome, metabolome, and metagenome, coined collectively as ‘omics’, offer robust methods for studying complex biological phenomena in large population samples.

The motivation of the current study was to assess the relation of the metabolome and metagenome with high BP in large Finnish cohorts. The specific aims were to estimate the association between gut microbiota and hypertension, to study the plasma metabolic profile of hypertension, and to elucidate if a family of circulating polyunsaturated fatty acid derived small molecule regulators of systemic inflammation, eicosanoids, are associated with BP.

This and previous population studies demonstrate that gut microbiota is associated with human hypertension. Biologically reasonable pathophysiological mechanisms, including ones related to sodium intake, have been proposed to explain the phenomenon. However, the significance of these effects on population BP and health remains unclear and warrants further research.

In this thesis, we demonstrate a strong association between circulating eicosanoids and BP in humans. We also use conventional statistical methods and multivariable machine learning models to define a metabolic profile of hypertension and blood pressure change using high abundance serum metabolic measures. Our results suggest that particularly serum lipids, and particularly low density lipoprotein-derived and very low density lipoprotein-derived cholesterol measures, and glucose metabolism abnormalities are associated with hypertension onset.

Our studies improve the current knowledge on the associations of gut microbiota and circulating metabolites with BP. Metabolomics and metagenomics offer novel approaches to improve hypertension risk prediction and to discover potential targets for therapeutic intervention of elevated BP.

KEYWORDS: blood pressure, dietary salt, epidemiology, hypertension, lactobacillus, LC–MS, metabolomics, metagenomics, NMR, shotgun sequencing

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## TIVISTELMÄ

Verenpainetaudin ilmaantuminen on yhteydessä lukuisiin riskitekijöihin, joista osaan voimme vaikuttaa elintavoilla ja joista osa on synnynnäisiä. Tästä huolimatta ymmärryksemme verenpainetaudin synnystä on vielä puutteellinen. Viime vuosina tutkijoiden käyttöön on tullut laaja joukko uusia biomolekyylien tutkimusmenetelmiä, joita kutsutaan yhteisnimellä 'omiikat'. Tämän kehityksen myötä myös suurten kansallisten tutkimusaineistojen analysointi on muuttunut kustannustehokkaaksi.

Väitöskirjani keskeisenä tutkimuskysymykseniä oli selvittää, tarjoavatko nämä biomolekyylien tutkimusmenetelmät uutta tietoa kohonneesta verenpaineesta suurissa suomalaisissa väestöaineistoissa. Väitöskirjani ensimmäisessä osassa tutkimme suolistobakteerien yhteyttä verenpainetautiin ja ravinnon suolaan. Väitöskirjani toisessa osassa tutkimme verenkierton aineenvaihduntatuotteiden yhteyttä verenpaine-tautiin.

Tuloksemme yhdessä aikaisemman tutkimustiedon kanssa tukee käsitystä, että verenpaineen ja suolistobakteerien välillä on yhteys. Kykenemme myös jo kertyneen tiedon valossa esittämään hypoteeseja yhteyden tautiopillisesta perustasta. Erityisesti ravinnon suola vaikuttaa isäntälajin lisäksi myös suolistobakteerien elinolosuhteisiin. Emme kuitenkaan vielä kykene arvioimaan, onko suolistobakteerien ja verenpaineen välisellä yhteydellä kansanterveystieteellistä merkitystä, mutta odotamme tulevaisuuden tutkimusten vielä tuovan tähän kysymykseen vastauksen.

Väitöskirjani jälkimmäinen puoli tukee verenkierrossa kulkevien pienten rasvaliukoisten tulehdusvälittäjääineiden, eikosanoidien, yhteyttä verenpainetautiin. Tutkimme myös sekä perinteisiä tilastollisia menetelmiä että koneoppimismalleja käytetäen seerumin aineenvaihduntatuotteiden profilia verenpainetaudissa. Tulostenme perusteella seerumin rasva- ja sokeriaineenvaihdunnan häiriöt, erityisesti korkea LDL- ja VLDL-kolesteroli, kytkeytyvät verenpainetaudin ilmaantuvuuteen.

Väitöskirjani tuotti uutta tieteellistä tietoa suolistobakteerien ja verenkierton aineenvaihduntatuotteiden yhteydestä verenpainetautiin. Metabolomiikka ja metagenomiikka tarjoavat joukon tehokkaita uusia menetelmiä tutkimukselle, jonka tavoitteena on korkean verenpaineen ehkäiseminen tai hoitaminen.

**AVAINSANAT:** epidemiologia, laktobasilli, LC-MS, metabolomiikka, metagenomiikka, NMR, suola, shotgun sequencing, verenpaine, verenpainetauti

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# Abbreviations

AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ADMA	Asymmetric dimethylarginine
Apo	Apolipoprotein
ATC	Anatomical Therapeutic Chemical classification
ATR	Angiotensin II type 1 receptor
BM	Bone marrow
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CI	Confidence interval
COX	Cyclooxygenase
CRP	C-reactive protein
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DILGOM	Dietary, Lifestyle and Genetic determinants of Obesity and Metabolic syndrome study
DNA	Deoxyribonucleic acid
EC <sub>50</sub>	Half maximal effective concentration
EET	Epoxy-eicosatrienoic acid
FDR	False discovery rate
FHS	Framingham Heart Study
FIMM	Institute of Molecular Medicine Finland
FITC	Fluorescein isothiocyanate-dextran
FMT	Fecal microbiota transplantation
GPCR	G-protein-coupled receptor
Gper1	G-protein coupled estrogen receptor
Gpr41	G protein-coupled receptor 41
GWAS	Genome-wide association study
HDL	High density lipoprotein
HETE	Hydroxyeicosatetraenoic acid

hs-CRP	High-sensitivity C-reactive protein
ICD	International classification of diseases
IDL	Intermediate-density lipoprotein
IL	Interleukin
IL-6R	Interleukin-6 receptor
KO	Kyoto Encyclopedia of Genes and Genomes Orthology group
LA	Linoleic acid
LC-MS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoprotein
LOX	Lipoxygenase
LT	Leukotriene
m/z	Mass-to-charge ratio
MAP	Mean arterial pressure
MDS	Multidimensional scaling
MLN	Mesenteric lymph nodes
MR	Mendelian randomization
MUFA	Monounsaturated fatty acids
NADPH	Nicotinamide adenosine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
Olfr78	Olfactory receptor 78
OR	Odds ratio
OTU	Operational taxonomic units
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PG	Prostaglandins
PGI <sub>2</sub>	Prostaglandin I <sub>2</sub> , prostacyclin
PERMANOVA	Permutational multivariate analysis of variance
PUFA	Polyunsaturated fatty acids
PVN	Paraventricular nucleus of hypothalamus
PWV	Pulse wave velocity
RAG	Recombinase-activating gene
RAS	Renin-angiotensin system
RMSE	Root-mean-square error
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SFA	Saturated fatty acids
sICAM-1	Intercellular adhesion molecule-1
SHR	Spontaneously hypertensive rat

SNP	Single-nucleotide polymorphisms
SNS	Sympathetic nervous system
T <sub>H</sub>	T helper cell
TG	Triglycerides
THL	Finnish Institute for Health and Welfare
TMA	Trimethylamine
TMAO	Trimethylamine-oxide
TNF $\alpha$	Tumor necrosis factor alpha
Treg	Regulatory T cell
TXA <sub>2</sub>	Thromboxane A2
VLDL	Very-low-density lipoprotein
WKY	Wistar Kyoto rat
WGS	Whole genome shotgun sequencing

# List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Palmu J, Lahti L, Niiranen TJ. Targeting Gut Microbiota to Treat Hypertension: A Systematic Review. *International Journal of Environmental Research and Public Health*, 2021, 18: 1248.
- II Palmu J, Salosensaari A, Havulinna AS, Cheng S, Inouye M, Jain M, Salido RA, Sanders JG, Brennan C, Humphrey GC, Sanders JG, Vartiainen E, Laatikainen T, Jousilahti P, Salomaa V, Knight R, Lahti L, Niiranen TJ. Association Between the Gut Microbiota and Blood Pressure in a Population Cohort of 6953 Individuals. *Journal of the American Heart Association*, 2020, 9: e016641.
- III Palmu J, Watrous JD, Mercader K, Havulinna AS, Lagerborg KA, Salosensaari A, Inouye M, Larson MG, Rong J, Vasan RS, Lahti L, Allen A, Cheng S, Jousilahti P, Salomaa V, Jain M, Niiranen TJ. Eicosanoid Inflammatory Mediators Are Robustly Associated With Blood Pressure in the General Population. *Journal of the American Heart Association*, 2020, 9: e017598.
- IV Palmu J, Tikkanen E, Havulinna AS, Vartiainen E, Lundqvist A, Ruuskanen MO, Perola M, Ala-Korpela M, Jousilahti P, Würtz P, Salomaa V, Lahti L, Niiranen T. Comprehensive biomarker profiling of hypertension in 36 985 Finnish individuals. *Journal of Hypertension*, 2021.

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# 1 Introduction

Hypertension is the leading modifiable cause of cardiovascular disease, disease-adjusted life years, and premature death worldwide (GBD 2017 Risk Factor Collaborators, 2018). Hypertension is a prevalent and inadequately treated condition observed approximately in one of third adults globally while current treatment and control rates are generally unsatisfactory regardless of the income level of the distinct countries (Zhou et al., 2021). While hypertension has been linked to various modifiable and fixed risk factors, the exact mechanisms behind blood pressure (BP) regulation and hypertension onset remain elusive. Our incomplete understanding of the pathogenesis of hypertension is also reflected in drug development where the focus over the last decade has been in fixed-dose combinations of the established antihypertensive medications rather than in the discovery of novel treatment options (Ali et al., 2017; Saklayen & Deshpande, 2016).

The rapid development in scientific branches studying genome, proteome, metabolome, and metagenome, coined collectively as ‘omics’, offer robust methods for studying complex biological phenomena in large population samples. The research protocols of the Finnish cohort studies FINRISK 1997–2012, Health 2000/2011, and FinHealth 2017 included the collection of wide range of biological samples (plasma, serum, urine, and stool) that were stored in foresight with the future development of the research methodology. Today, linking the data acquired from these biological samples with information from health questionnaires, physical measurements, and longitudinal register-based follow-up, enables the researcher to execute cutting-edge epidemiological studies in well-phenotyped, large-scale human cohorts.

Changes in gut microbiota have recently been linked to various chronic diseases such as obesity (Petriz, 2014), metabolic syndrome (Fändriks, 2017), diabetes mellitus (Qin et al., 2012), cardiovascular disease (Wang et al., 2011), and even 15-year mortality risk (Salosensaari et al., 2021). Pioneering animal models implementing fecal microbiota transplants and studying genetic deletions and direct metagenomics analyses have suggested that gut microbiota is linked with host BP (Vijay-Kumar et al., 2010; Yang Tao et al., 2015; Adnan et al., 2016). Intriguingly, high salt intake, a classic risk factor for both hypertension and cardiovascular

disease, has been demonstrated to modulate mural gut microbiota particularly depleting *Lactobacillus* species (Wilck et al., 2017). Consistently, oral administration of *L. murinus*, the most strongly modulated *Lactobacillus* strain isolated from the mural feces, prevented the development of salt-sensitive hypertension in mice (Wilck et al., 2017). In Study I of this thesis, we performed a systematic literature review to summarize the current knowledge of the link between gut microbiota and BP. In Study II, we analyzed the cross-sectional associations between gut microbiota, dietary sodium intake, and BP in FINRISK 2002.

Metabolites are the intermediate and end products of the numerous biological processes occurring in living organisms. In addition to host metabolism, gut microbiota derived metabolites such as short-chain fatty acids (SCFAs) have the ability to enter the host circulation affecting the host homeostasis (Al Khodor et al., 2017; Pluznick, 2017). The measurement of circulating metabolites and metabolic biomarkers provides functional information about the host physiology offering a natural method to study BP regulation and hypertension onset. To date, most studies reporting results for the link between the circulating or urine metabolome and BP have been performed in small cohorts and with a small number of metabolites (Islam, 2017). Recent development in high-throughput nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography mass spectrometry (LC–MS) offer two cost-effective methods with distinct strengths and weaknesses to study large human cohorts (Nikolic et al., 2014). In Study III, we analyzed the cross-sectional associations between BP and comprehensive panel of >500 distinct high-quality upstream eicosanoids and related oxylipin mediators in FINRISK 2002 using LC–MS. In Study IV, we analyzed the cross-sectional and longitudinal metabolic profile of hypertension in the FINRISK 1997–2012, Health 2000/2011, and FinHealth 2017 cohort studies using NMR measured high abundance serum biomarkers.

## 2 Literature Review

### 2.1 Gut microbiota and hypertension

Technical discoveries have foreshadowed our understanding of the microbes from microscopy in 1670, microbial cultivation in 1857, mass spectrometry in 1911, polymerase chain reaction (PCR) technique in 1983, next-generation sequencing in 2005, and to third-generation sequencing in 2008 (Berg et al., 2020). The scientific branches studying the genome, proteome, metabolome, and metagenome, coined collectively as ‘omics’, offer robust method for studying microbial communities (Figure 1). In particular, metagenomics offers information about the presence and abundance of microbes in biological sample (including microbes that would be difficult to culture) and metabolomics offers information about active functional processes in the microbial community. The collection of microbes in a biological system is called microbiota, and microbiota in context with the biological, physical, and chemical properties of the microbial habitats is called the microbiome (Berg et al., 2020). We summarize in following paragraphs the main tools used in metagenomics and review the studies reporting associations between gut microbiota and BP.

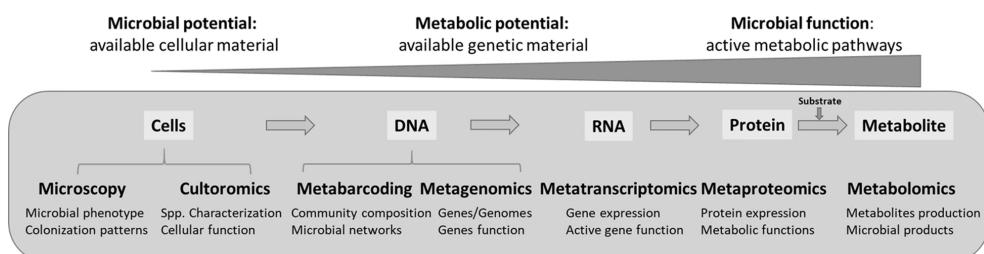


Figure 1. The description of different methods available to study microbial communities\*

\* Released under Creative Commons Attribution license (Berg et al., 2020).

### 2.1.1 Sequencing gut microbiota

Gut microbiota is composed of bacteria, viruses, bacterial viruses, and fungi that reside in the gastrointestinal tract. Rectal swapping and collecting stool samples are noninvasive and convenient methods that allow large scale and longitudinal gut microbial study designs (Claesson et al., 2017; Knight et al., 2018; Quince et al., 2017). While these two collection methods offer limited control over sampling sites, contain dead bacteria, include bacteria from unspecified sites, and may underrepresent important microbial colonies, both methods are regarded as an acceptable proxy for distal gut microbiota (Claesson et al., 2017).

Appropriate delivery and storage protocol for stool samples is required to preserve the microbial deoxyribonucleic acid (DNA). Sample deterioration can be prevented using commercially available preservation kits. The main benefit of a kit is extended ambient temperature storage and reduced number of freezing-thawing cycles (Anderson et al., 2016). For studies, in particular, performed without preservation kits, uniform storage protocols, the ambient temperature exposure, and freezing conditions should be reported and evaluated (Vogtmann et al., 2017).

Extracting microbial DNA from stool samples is complicated by the presence of host DNA and various substances present in the gastrointestinal tract. Most commercial applications are based on chemical and mechanical cell lysis techniques performed in buffers that protect the liberated nucleic acids (Persson et al., 2011). The extracted and cleaned DNA can be sequenced using either 16S ribosomal RNA (rRNA) sequencing or shotgun metagenomic sequencing.

#### 2.1.1.1 16S rRNA sequencing

Ribosomes are cellular particles that translate the messenger ribonucleic acid (RNA) into sequence of amino acids complying the central dogma of molecular biology: DNA makes RNA, and RNA makes protein. The 16S rRNA is a component of the prokaryotic ribosome and therefore ubiquitous in bacteria (Claesson et al., 2017). The 16S rRNA gene consists of nine hypervariable regions flanked by more conserved sequences (Martinez-Porcha et al., 2017). PCR primers can be targeted to the conserved regions of the 16S rRNA gene while the bacteria can be identified reading the sequence of the hypervariable region (Figure 2). PCR primers can also be labeled with specific barcode sequences that identify different samples (hosts).

The sequence reads can be clustered into operational taxonomic units (OTUs). The usual clustering threshold is 97% similarity between sequences (Claesson et al., 2017). The nucleotide sequences of different OTUs can be compared with reference databases of previously sequenced bacteria to infer likely taxonomic classification (Johnson et al., 2019). The conventional 16S rRNA is often accurate at the genus level while full-length 16S rRNA sequence data could potentially provide taxonomic

resolution of bacterial species and strain level (Johnson et al., 2019). However, amplicon sequence variants could replace OTUs in future offering finer sequencing resolution (Callahan et al., 2016). Because viruses and fungi do not possess 16S rRNA gene, the choice of this particular marker gene limits the observed results to bacteria and archaea.

### 2.1.1.2 Shotgun metagenomic sequencing

Instead of selecting specific marker genes for PCR, in whole genome shotgun sequencing (WGS) the complete extracted DNA is randomly sheered into desired fragment sizes for processing (Claesson et al., 2017). The short sequence reads of 150–400 base pairs (in second generation sequencing) are often assembled to longer sequences using reference genomes or de novo methods (Figure 2). The assembled DNA sequences are grouped by their likely host genomes and assigned taxonomy using reference databases (Claesson et al., 2017). Using databases with genes annotated with, for instance, KEGG Orthology groups, allows calculating functional profiles for potential molecular functions present in microbiota (Hillmann et al., 2018).

Compared to 16S rRNA, WGS is more costly and requires more fine-tuned quality control in addition to excluding the host DNA from analyses (Claesson et al., 2017). However, incorporating barcodes in primers in WGS allows simultaneous high-throughput sequencing of multiple samples; barcoded DNA fragments allow simultaneous sequencing of multiple samples in process coined shallow WGS which makes the method cost-effective alternative for 16S rRNA in large studies (Hillmann et al., 2018). Nevertheless, the benefits of WGS compared to 16S rRNA that include greater microbial resolution and detection of non-bacterial microbes, require the contribution from a skilled bioinformatician and ability to perform computationally intensive calculations in cluster computing environment (Claesson et al., 2017).

## 2.1.1 Gut microbiota data analysis

### 2.1.1.1 Microbiota data are compositional

High-throughput sequencing produces a random sample of the relative abundances of the observed microbes because the total microbial load (number of microbes) is unknown (Gloor et al., 2017). Therefore, microbial data are inherently compositional and changes in few species can alter the observed counts for all species and changes in total microbial load make different scenarios indistinguishable (**Error! Reference source not found.**). Relative abundance data can be studied using appropriate

statistical methods or estimating the microbial load using additional biochemical analyses (Morton et al., 2019).

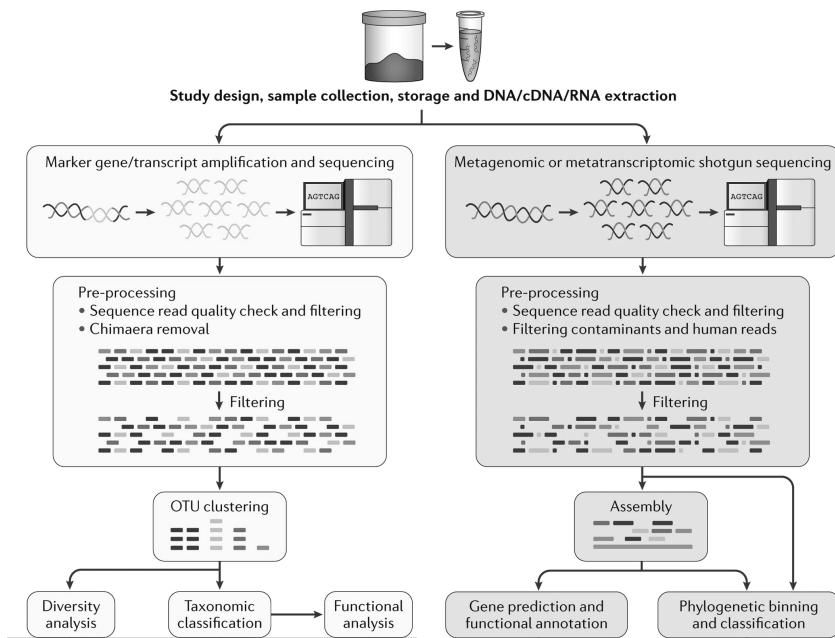


Figure 2. The two main sequencing methods in the metagenomics\*

\*Adapted by permission from Springer Nature (Claesson et al., 2017).

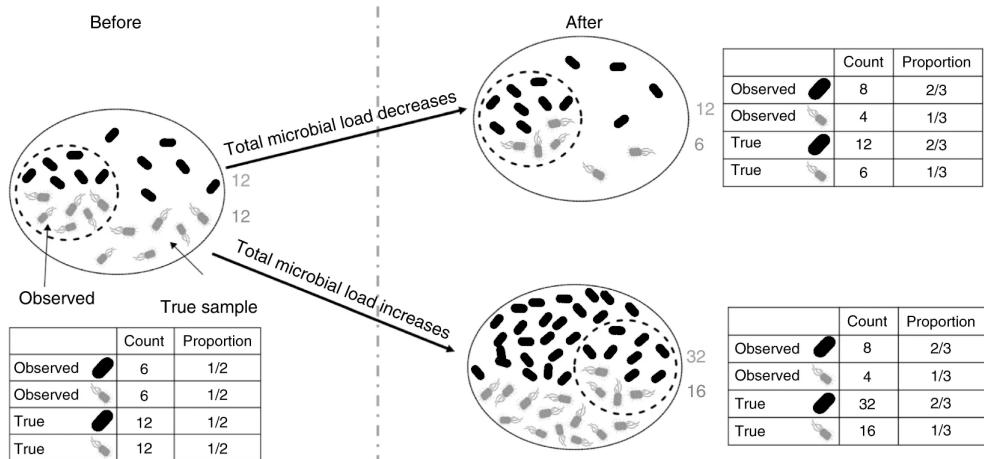


Figure 3. Microbial data is inherently compositional\*

### 2.1.1.2 Taxonomic diversity

While diversity is straightforward concept, formulating its mathematical definition has proved difficult in practical applications (Daly et al., 2018). Natural communities can be described using two levels of diversity: within-sample variance called alpha diversity and between-sample variance called beta diversity (Whittaker, 1960). These ecological concepts, originally intended to characterize vegetation in large geological areas, can also be applied in metagenomics.

The straightforward definition for alpha diversity is the number of distinct taxa present in a sample, information that is independent from the properties of all other samples. A more sophisticated and popular choice for alpha diversity is the Shannon's index (Daly et al., 2018). Shannon's index was originally developed to measure information content in data. In metagenomics, the index quantifies the difficulty to identify randomly picked taxon from all taxa in sample. The index is low in samples with few taxa and high in samples with large number of taxa with varying abundances; observation from limited sample may also underestimate the diversity.

Beta diversity describes one-on-one differences between samples. Two straightforward definitions for beta diversity are Manhattan distance and Euclidian distance: respectively the sum of the absolute and squared differences between number of each taxon in the two studied samples (Legendre & De Cáceres, 2013). Bray-Curtis dissimilarity, commonly used indices for beta-diversity, scales the Manhattan distances by the total number of taxa observed in the two compared

\*Licensed under Creative Commons Attribution license (Morton et al., 2019).

samples (Legendre & De Cáceres, 2013). The dissimilarity between two samples increases as the differences between proportions of shared taxa increase ranging zero to one (Qian et al., 2020).

### 2.1.1.3 Comparing microbial abundances

There are multiple benchmarked and consistent methods to study differences between distinct microbial abundances (Schurch et al., 2016; Weiss et al., 2017). The small number of replicates and large number of low prevalence taxa often observed in metagenomics, require sophisticated methodology to reach adequate statistical power in analyses and to avoid biases.

DESeq2 is commonly used library in metagenomics to study differences in fold-changes, the ratios between observed counts, between studied groups or conditions (Love et al., 2014). DESeq2 performs internal normalization for microbial counts which accounts for varying microbial load. DESeq2 also uses Bayesian dispersion estimate to reliably estimate the variances in low-abundance taxa (Figure 4). This shrinkage procedure reduces the chance of false positives in taxa with abnormally low dispersion and false negatives in taxa with moderately high dispersion (Love et al., 2014). To reduce the variance in fold-changes of low count taxa, DESeq2 uses zero biased Bayesian method to shrink fold-changes based on the amount of information that was present in the model. The Bayesian method also provides standard errors for the observed associations. DESeq2 uses Wald test for significance testing.

For a more detailed explanation, DESeq2 models sequenced count data  $K_{ij}$  using negative binomial distribution  $K_{ij} \sim NB(\mu_{ij}, \alpha_i)$  for taxon  $i$  and sample  $j$ , where  $\mu_{ij}$  represents fitted means and  $\alpha_i$  the dispersion parameters. Fitted means  $\mu_{ij} = s_j q_{ij}$  are scaled using size factors  $s_j$  that is based on median of the geometric mean of the microbial counts. Linear predictors are mapped to model variable using link function  $\log_2 q_{ij} = x_j \beta_i$  where  $x_j$  are model covariates and  $\beta_i$  the estimated parameters for covariates. DESeq2 performs analyzes in four steps: first size factors  $s_j$  are estimated using geometric means, second dispersions  $\alpha_i$  are estimated using Bayesian model, third negative binomial generalized linear model is fitted for  $\beta_j$ , and finally significance is tested using Wald statistics with false discovery rate (FDR) correction. FDR correction is a method to compensate for performing large number of statistical tests; using uncorrected  $P$ -values would inflate the number of false positive associations observed at  $P<0.05$ .

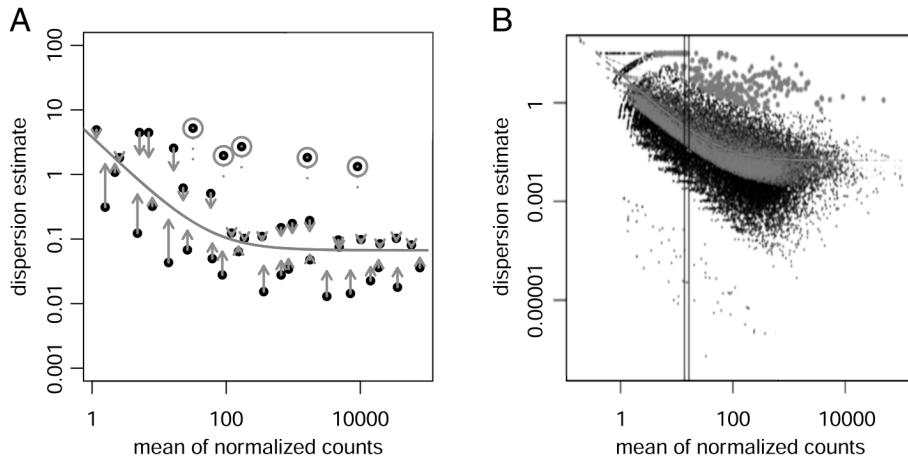


Figure 4. DESeq2 uses shrinkage estimation for dispersion to reduce model biases\*

The choice of negative binomial distribution in DESeq2 is reasonable (Y. Chen et al., 2014). While the read counts could be modeled using binomial distribution  $\text{Bin}(n, p)$ , the calculations would be straining due to large number of sequencing reads. Poisson distribution  $\text{Pois}(\lambda)$  with  $\lambda = np$  could be used to approximate  $\text{Bin}(n, p)$  when  $n \rightarrow \infty$ . However, due to single parameter fixing both mean and variance, Poisson distribution can only estimate technical variation of repeated measures from single sample. Estimating the  $\lambda$  parameter with Gamma distribution, a family of positive valued continuous probability distributions, allows the model to account for the biological variation in Poisson parameters between samples. Calculating compound probability distribution of Poisson distribution with Gamma distribution produces the negative binomial distribution. The choice of negative binomial distribution can also be justified studying the mean-variance relationship of the sequence data (Y. Chen et al., 2014).

## 2.1.2 Animal studies

### 2.1.2.1 Fecal microbiota transplantation

The first metagenomics study analyzing gut microbiota and BP was a cecal transplantation in Dahl rats reaching family level resolution using 16S rRNA (Mell et al., 2015). Salt-sensitive (S) and salt-resistant (R) Dahl rats were fed high-salt diet, gavaged antibiotics to ablate the native microbiota, and transplanted with S or R rat

\*Licensed under Creative Commons Attribution license (Love et al., 2014).

cecal contents. R to S rats had higher BP and shorter life-span compared to S to S rats after single bolus of gavaged cecal content. No differences were observed between S to R and R to R groups. To study whether the effect was introduced by antibiotic treatment or fecal microbiota transplantation (FMT), the authors compared the BP between S rats with and without antibiotic treatment. In this study, the BP in two groups did not differ indicating that BP change in FMT observed in S rats was not due to the loss of the original gut microbial composition. However, R to S had higher circulating acetate and reduced urinary sodium excretion compared to S to S giving possible mechanism for host-microbial interactions in salt-sensitive hypertension.

In another rodent study, FMT from spontaneously hypertensive rats (SHR) to normotensive Wistar Kyoto rats (WKY) increased BP compared to FMT from WKY to WKY (Adnan et al., 2016). In another study, FMT from SHR to SKY increased T cell activation in mesenteric lymph nodes (MLN), circulating T cells, aortic T cell infiltration, and impaired endothelial function in addition to previously reported increase in BP (Toral, Robles-Vera, de la Visitación, Romero, Sánchez, et al., 2019). FMT from WKY to SHR also reduced the production of reactive oxygen species production and proinflammatory cytokines in the paraventricular nucleus (PVN) of the hypothalamus indicating that gut microbiota may modulate sympathetic response of the host (Toral, Robles-Vera, de la Visitación, Romero, Yang, et al., 2019). Notably, a cross-species FMT from hypertensive individuals to mice resulted to higher BP compared to FMT from a normotensive individual at 10 weeks post-transplantation (Li et al., 2017). Previous FMTs also induced changes in gut microbiota of the rodents, as would be reasonable to expect.

#### 2.1.2.2 Gut wall permeability

Chronic angiotensin II infusion in SHRs has been demonstrated to induce changes in gut wall permeability (Santisteban et al., 2017). Hypertensive rodents had reduced number of goblet cells and tight junction proteins, and stunted villi. Intestinal permeability was assessed measuring plasma levels of gavaged fluorescein isothiocyanate-dextran; SHRs had increased fluorescent marker levels compared to normotensive WKYs indicating increased functional permeability in hypertension that are consistent with the histopathological changes. The decreased level of tight junction protein in young prehypertensive SHRs compared to normotensive age-controls suggests that gut wall pathology could precede or at least have close temporal connection to the onset of hypertension.

In addition to previous histopathological changes, SHRs have reduced levels of mucin and increased levels of circulating endotoxins compared to normotensive WKYs (Robles-Vera et al., 2020). Mucin is a barrier protein produced in goblet cells

that protects gut wall against pathogens. Reduced gut wall permeability could lead increased levels of bacterial wall components such as lipopolysaccharides (endotoxins) to enter host circulation where they can activate toll-like receptors promoting oxidative stress and vascular inflammation (Liang et al., 2013).

In SHRs, candesartan treatment increased both the expression of genes encoding tight junction proteins and ameliorated depletion of *Lactobacillus* species induced by hypertension (Wu et al., 2019). Another renin-angiotensin system (RAS) inhibitor, captopril, also demonstrated attenuated gut wall pathology and dampened posterior pituitary neuronal activity in SHRs maintained over prolonged withdrawal (T. Yang et al., 2019). Vasopressin is released from the posterior pituitary.

### 2.1.2.3 Short-chain fatty acids

SCFAs are gut microbial fermentation products of dietary fibers that can be absorbed in the host circulation where they can potentially modulating host homeostasis (Pluznick, 2017). While the gut microbiota may not be the only contributor to host SCFA production, germ-free mice have been reported to possess negligible endogenous production of the three primary SCFAs, acetate, propionate, and butyrate (Perry et al., 2016). The plasma concentration of the three primary SCFAs were 30–400  $\mu\text{mol/l}$  and dry weight fecal molality 10–300  $\mu\text{mol/g}$  in mice (Perry et al., 2016). The serum levels of SCFAs have consistently been positively associated with dietary fiber levels in mice (Trompette et al., 2014). In summary, gut microbiota contributes to SCFA production and only proportion of SCFAs enter circulation.

Recognizing the ligand profile of a family of renin release and glomerular filtration rate modulating G-protein-coupled receptor (GPCRs) led to the discovery that SCFAs act as ligands for GPCRs (Pluznick, 2017). Olfactory receptor 78 (Olfr78) mediates renin secretion in juxtaglomerular apparatus (Pluznick et al., 2013). Both Olfr78 and G protein-coupled receptor 41 (Gpr41) are expressed in the muscle cells of resistance vessels (Pluznick et al., 2013). In knock-out animal models, Gpr41<sup>-/-</sup> knockout mice developed hypertension and Olfr78<sup>-/-</sup> knockout mice hypotension compared to wild type mice (Pluznick et al., 2013; Natarajan et al., 2016). Notably, Gpr41 has a lower half maximal effective concentration than Olfr78, and, therefore, Gpr41 could be active at basal concentrations, whereas the activation of Olfr78 could help to balance the effect (hypotension) at high concentrations (Pluznick, 2017). OR51E2 is human ortholog with murine Olfr78 (Pluznick, 2017). In summary, SCFAs modulate host homeostasis binding to GPCRs (**Error! Reference source not found.**).

Table 1. G-protein-coupled receptors binding SCFAs associated with hypertension

	G protein-coupled receptor 41 (Gpr41)	Olfactory receptor 78 (Olfr78)	Olfactory receptor 51E2 (OR51E2)
Species	Humans, mice	Mice	Humans
Ligands	Acetate, butyrate, propionate	Acetate, propionate, lactate	Acetate, propionate
Location	Vascular epithelium	Renal afferent arteriole, vascular smooth muscle cells	Gastrointestinal tract
Null mice	Gpr41 <sup>-/-</sup> mice are hypertensive	Olfr78 <sup>-/-</sup> mice are hypotensive	
References	(Natarajan et al., 2016)	(Pluznick et al., 2013)	(Pluznick, 2017) (R. Muralitharan & Marques, 2021)

Hypertension has been linked with reduced numbers of SFCA producing bacteria and decreased circulating SCFAs (Robles-Vera et al., 2018). Treatment with *Bifidobacterium* and *Lactobacillus* probiotics has been linked with increased butyrate-producing bacteria while direct treatment with acetate and butyrate alleviated the gut-wall pathology in SHRs compared to normotensive WKYs (Robles-Vera et al., 2020). Acetate supplementation has also been associated with reduced cardiac fibrosis, left ventricular hypertrophy, and hypertension in mineralocorticoid-excess treated mice compared to mice fed control diet (Marques et al., 2017). High dietary salt was associated with increased fecal acetate, propionate, and isobutyrate levels in rats (Bier et al., 2018). Intervention with candesartan, an angiotensin II type 1 receptor blocker, was associated with improved gut wall permeability, as well as increased fecal acetate, propionate, and butyrate levels in SHRs compared to controls (Wu et al., 2019).

#### 2.1.2.4 Gut modulated sympathetic activity

A seminal work in mice with genetic deletion of the recombinase-activating gene (RAG-1<sup>-/-</sup>) demonstrated that T cell activation has a role in hypertension onset (Guzik et al., 2007). RAG-1<sup>-/-</sup> mice lack T and B cells and have a blunted response to angiotensin II infusion. However, adoptive transfer of T but not B cells restored the hypertensive response to angiotensin II. In particular, angiotensin II also increased the T cell tissue homing into the perivascular adipose tissue and increased the nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase activity in wild-type mice.

Like other hematopoietic cells, T cells are created in the bone marrow. In a rodent study, ablation of the bone marrow (BM) in normotensive WKYs followed by reconstructing of the BM from SHR promoted neuroinflammation and hypertension in chimeric rats (Santisteban et al., 2015). BM transplantation also increased the number of circulating pro-inflammatory and increased microglial activity in the PVN of the hypothalamus. Consistently, oral administration of minocycline, an inhibitor of microglial activation, reduced blood pressure in SHRs compared to SHR controls. The effect was later demonstrated to be anti-inflammatory rather than antimicrobial using chemically modified tetracycline-3 that is deficient in antibacterial properties but retains minocycline's anti-inflammatory properties (Sharma et al., 2019).

An *in situ* decerebrated artificially-perfused rat preparation model of phrenic nerve activity patterns revealed elevated sympathetic sensitivity in SHRs compared to normotensive WKYs (Santisteban et al., 2017). Using pseudorabies virus expressing green fluorescent protein retrograde labeling applied in the colon and small intestine of rats demonstrated significant fluorescent labeling of neurons in the PVN of hypothalamus in of SHRs and angiotensin II infused WKYs but not WKY controls. SHRs had also increased levels of tyrosine hydroxylase immunoreactivity in small intestine compared to WKYs indicating increased norepinephrine generation in SHRs.

#### 2.1.2.5 Inflammation and dietary sodium

Table 2 summarizes the role of T cells in hypertension (Wenzel et al., 2016). The high dietary salt was recently associated with changes in gut microbiota and host inflammation response, in addition to, previously well-documented deleterious effect on cardiovascular health (Wilck et al., 2017).

In vitro, the half maximal growth inhibition for *Lactobacillus* species was 0.6 mol/L sodium at 37 °C under aerobic conditions (Wilck et al., 2017). In vivo, high dietary sodium (4% dietary and 1% drinking water salt) resulted in colonic sodium concentration of 0.3 mol/L and normal dietary salt (0.5% dietary salt) of 0.1 mol/L. Consistently, high dietary salt was associated with changes in gut microbiota and particularly depleting *L. murinus* in mice compared to controls fed normal dietary salt. However, *L. murinus* treatment prevented the development of salt-induced hypertension and increase in IL-17A producing CD4<sup>+</sup> T<sub>H</sub>17 in gastrointestinal lymphocytes (Wilck et al., 2017).

The changes in gut microbiota in hypertension have been associated with increased T helper 17 (T<sub>H</sub>17) to regulatory T (Treg) ratio in mesenteric lymph nodes and aorta (Toral, Robles-Vera, de la Visitación, Romero, Sánchez, et al., 2019). Similarly, salt-induced hypertension has been demonstrated to increase the number of immune cells in mesenteric arterial arcade and FMT from hypertensive mice to

germ-free mice resulted to increased circulating interleukin (IL)-6 and IL-17 levels (Ferguson et al., 2019). The prohypertensive effects transmitted in FMTs appear to be connected to B7-dependant activation of T cells and the effect is at least partially governed by the TH17/Treg ratio, the ratio of pro- and anti-inflammatory cells (Toral, Robles-Vera, de la Visitación, Romero, Sánchez, et al., 2019).

Table 2. Summary for the role of different T cells in hypertension\*

Variable	T helper cell 1 (TH1)	T helper cell 17 (TH17)	Regulatory T cell (Treg)
Function	Cell mediated autoimmunity	Cell mediated autoimmunity	Downregulation of the immune response
Experimental hypertension models	Increased IFN-γ production in Ang II-induced hypertension	Increased IL-17a production in Ang II-induced hypertension. Increased number of TH17 cells in kidney of hypertensive mice.	Treg correlates with the amount of injury
Effect of knockout	IFN-γ has either no effect or reduced BP	L-17a and IL-6 deficiency reduces BP	Knockout is lethal
Effect of overexpression	IFN-γ induces vascular dysfunction	IL-17a induces hypertension	
Effect of administration		IL-17 increases BP	Treg decreases BP

### 2.1.3 Human studies

Previous animal studies have suggested that gut dysbiosis and hypertension could be causally related. In animal models, both hypertension and gut microbiota were linked to gut wall permeability regulation. Gut microbiota has in experimental models also been demonstrated to modulate host sympathetic activity and produce bioactive metabolites that can enter host circulation. In particular, *Lactobacillus* species were associated with both salt-induced hypertension and host inflammatory response. However, even optimally performed preclinical trials may fail to replicate in humans (Worp et al., 2010). These translation problems can be partially explained by the differences in disease manifestation, pharmacokinetics, pharmacodynamics, and immune response in different species (Pound & Ram, 2020). Therefore, the previously discussed preclinical results still require observational and interventional human studies to validate the role of gut microbiota in human hypertension. The gold standard for causality in clinical studies are randomized controlled trials while

\* Adapted by permission from American Society of Nephrology (Wenzel et al., 2016).

observational studies generally produce only information about associations. However, epidemiological studies can also provide information about causality in observational data using Mendelian randomization where genetic variants fixed at conception mimic the randomization performed in interventional studies (Sekula et al., 2016; Davies et al., 2018).

#### 2.1.3.1 Gut microbiota and human hypertension

While numerous scientific studies have addressed the association between gut microbiota and hypertension, only few studies have been performed in a representative sample of >500 individuals (Jackson et al., 2018; Sun et al., 2019; Verhaar, Collard, et al., 2020). However, in small study of Chinese individuals ( $N = 129$ ), genus level differences were observed between hypertension, isolated systolic and diastolic hypertension, pre-hypertension, and normotension (Dan et al., 2019). A small American pilot study ( $N = 52$ ) suggested that gut microbiota may contribute the differences observed in hypertension between black and white individuals (Walejko et al., 2018). Hypertension in black patients may be accompanied by increased oxidative stress and insulin resistance compared to white individuals (Walejko et al., 2018). In summary, different subtypes of hypertension may have accompanying differences in gut microbiota and the genomics of the host may influence the metagenomics of the gut.

Three large cross-sectional studies had reported associations between gut microbiota and human hypertension excluding Study II. In the TwinsUK ( $N = 2,737$ ; 89% women, age  $60 \pm 12$ ; 16S rRNA) study, the researchers explored the connection between gut microbiota and 38 common diseases and 51 medications (Jackson et al., 2018). However, self-declared hypertension was not associated with 68 microbiota markers (Jackson et al., 2018). In the Coronary Artery Risk Development in Young Adults (CARDIA;  $N = 529$ ; 54% women, age  $55 \pm 3$ ; 16S rRNA) study, microbial alpha diversity and *Robinsoniella*-genus were negatively associated with systolic BP (Sun et al., 2019). In the Healthy Life In an Urban Setting (HELIUS;  $N = 4,672$ ; 52% women, age  $50 \pm 12$ ; 16S rRNA) study, authors used gradient boosting machine learning model and self-defined formula for the proportion of variance to estimate the overall effect between gut microbiota and hypertension (Verhaar, Collard, et al., 2020). Gut microbiota explained 4.4% of the overall unadjusted systolic BP variance and 2.2% of the residual systolic BP adjusted for age, sex, and BMI. However, in stratified analyses, only residual BP had consistent  $R^2$  within the six ethnicities.

Observational studies are susceptible to various biases including confounding and reverse causation. In metagenomics studies medications can independently influence both gut microbiota and host pathophysiology (Jackson et al., 2018). While statistical models should be adjusted for relevant covariates, non-adherence to

antihypertensive medication, in particular, is common and can potentially result in misclassification of study participants (Tomaszewski et al., 2014). Access to register based information of regular drug purchases or direct measurement of drug metabolites in biofluids such as urine could potentially improve quality of observational studies. Mendelian randomization models would also be less likely affected by the conventional biases of the observational studies and provide information about causality (Davies et al., 2018).

### 2.1.3.2 Dietary salt in human hypertension

The previously discussed study reporting results for dietary salt and *Lactobacillus* species in mice included a human pilot study (Wilck et al., 2017). In a moderate salt challenge, 12 healthy men received 6 g slow-releasing sodium chloride in addition to their accustomed diets for total daily salt intake  $13.8 \pm 2.6$  g. The challenge was linked with nocturnal BP increase from baseline 106/60 mmHg to 111/67 mmHg and 1.8-fold increase in circulating CD4 $^{+}$ IL-17A $^{+}$ TNF- $\alpha$  $^{+}$  T<sub>H</sub>17 cells. Participants also had reduced survival of *Lactobacillus* species (Figure 5) compared to published time course data from 121 individuals not undergoing any intervention.

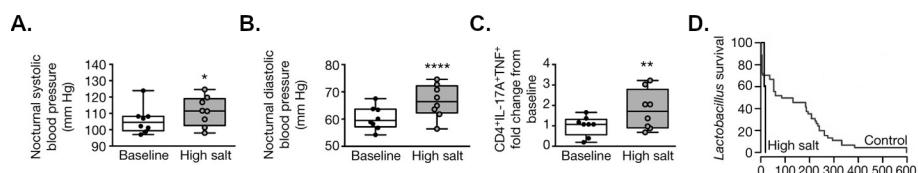


Figure 5. The effect of dietary sodium intervention to BP, T<sub>H</sub>17 cells and *Lactobacillus* survival\*

### 2.1.3.3 SCFAs are altered in human hypertension

Gut microbial fermentation of the indigestible foods in cecum and ascending colon as well as hepatic de novo synthesis are the main sources of SCFAs in humans (Overby & Ferguson, 2021). While multiple bacterial species produce SCFAs (Table 3), our understanding about SCFA production and pathophysiology is still partial. Hypertension has been associated with increased levels of fecal SCFAs in Spanish ( $N = 61$ ), Colombians ( $N = 441$ ), Belgians ( $N = 54$ ), and Dutch ( $N = 200$ ) individuals (Calderón-Pérez et al., 2020; de la Cuesta-Zuluaga et al., 2018; Huart et al., 2019; Verhaar, Collard, et al., 2020). Abundances of multiple SCFA producing bacteria such as *Ruminococcaceae*, *Roseburia*, and *Faecalibacterium* have been negatively

\*Reprinted by permission from Springer Nature (Wilck et al., 2017).

associated with human hypertension (Verhaar, Prodan, et al., 2020). Few studies have reported on the links between hypertension and circulating SCFA levels; however, negative associations were observed between BP and plasma SCFAs levels in Spanish (N = 61) and American (N = 40) individuals (Calderón-Pérez et al., 2020; Kim et al., 2018).

In a moderately sized randomized controlled cross-over trial (N = 145, 34% women), sodium reduction was associated with increased circulating levels of eight SCFAs, including butyrate (L. Chen et al., 2020). Notably, subgroup-analysis revealed that results were sex-differentiated and significant SCFA increases in response to sodium reduction were observed in women only. In summary, hypertension and high dietary sodium have been associated with changes in 1) gut microbiota, including in SCFA producing bacteria; 2) gastrointestinal tract function; and 3) fecal and circulating SCFA levels.

Table 3. The main SCFA producing bacterial genera present in human gut microbiota

SCFA	Main producers	Source
Acetate	<i>Bifidobacteria, Lactobacillus, Prevotella, Ruminococcus</i>	(Baxter et al., 2019; Franke & Deppenmeier, 2018; Moens et al., 2017)
Propionate	<i>Akkermansia, Alistipes, Bacteroides, Blautia, Coprococcus, Dialister, Eubacterium, Phascolarctobacterium, Prevotella, Roseburia</i>	(Louis & Flint, 2017)
Butyrate	<i>Anaerostipes, Clostridium, Coprococcus, Eubacterium, Faecalibacterium, Roseburia, Subdoligranulum</i>	(Louis & Flint, 2017; Parada Venegas et al., 2019)

#### 2.1.3.4 Host inflammatory response

Human hypertension has been linked to increased endotoxemia and increased expression of peripheral blood T<sub>H</sub>17 cells (Wilck et al., 2017; Kim et al., 2018). Immunohistochemical analysis on tissue samples from a biobank revealed that hypertensive individuals have also increased infiltration of T cells and macrophages in the intestinal wall compared to normotensive controls (Ferguson et al., 2019). Hypertension may therefore be modulated by immune-mediated signals that are present in both gastrointestinal tract and peripheral blood. SCFAs can similarly induce changes in host homeostasis: for example, they can locally modulate

gastrointestinal permeability and remotely bind to SCFA receptors. However, the translation of the results from animal studies to humans is still ongoing.

## 2.2 Circulating metabolites and hypertension

### 2.2.1 Introduction to the circulating metabolome

Metabolites are low-molecular weight components observed within various cells, tissues, and biofluids. Unlike amino acids in proteins or nucleotides in DNA, metabolites do not carry information encoded using repeating subunits, but the information is rather confined in the thousands of the metabolites themselves (Nikolic et al., 2014). The varying chemical properties and concentrations of metabolites make their identification and quantification difficult.

Metabolomics studies are usually classified to targeted and untargeted analysis designs. Targeted analysis focuses on known sets of metabolites and untargeted analysis aims to identify new molecular compounds. In the following section, we will review common analytical methods used in metabolomics and review the literature for metabolites associated with hypertension.

### 2.2.2 Analytical technologies

#### 2.2.2.1 Mass spectrometry

The modern mass spectrometric device consists of multiple components that are varied to meet the needs of the study at hand (Pitt, 2009; Alsaleh et al., 2019). We focus our review on the liquid chromatography mass spectrometry system (LC–MS), because solvent phase is particularly suitable in studying plasma and urine samples.

A liquid chromatograph is used to separate aqueous biological samples into separate components (Figure 6). First, samples are injected into a moving stream of liquid solvent called mobile phase. Then, analytes are transported into a liquid chromatographic column that is packed with granular solid material called stationary phase. The differences in the affinity of the distinct analytes in the studied sample with the mobile and stationary phases results in varying travel times along the column. Different columns are used in different applications, and in some columns, a pneumatic pump is used to maintain the flow along the column constant instead of relying on gravity to sustain it. The choice of phase materials affects the travel time and should preferably be reported to allow the replication of the analyses.

Notably, different analytes arrive to MS device at different times from LC. The analytes are ionized and transformed into gaseous state to allow further spectroscopic

analysis using high precision electromagnetic fields. In biological samples, soft ionization methods that produce adduct ions without causing fragmentation, are preferred. After ionization, the analytes pass through a vacuum interface into a mass analyzer.

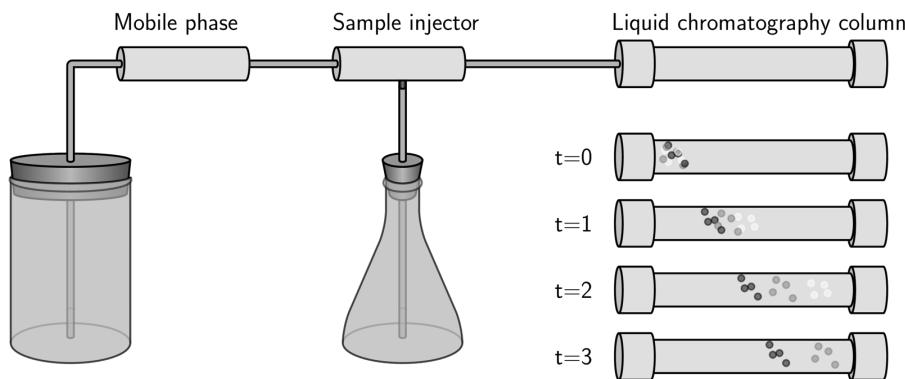


Figure 6. Liquid chromatography column\*

Electric quadrupoles are typically used to filter charged analytes based on their mass-to-charge ( $m/z$ ) ratio (Figure 7). Different trajectories near specific  $m/z$  values can be stabilized in the time-varying quadrupolar field by adjusting constant voltages and varying radio frequency voltages applied to the quadrupole rods. Finally, information about retention time in LC and  $m/z$  in MS can be linked with the measured abundance of analytes reaching detector.

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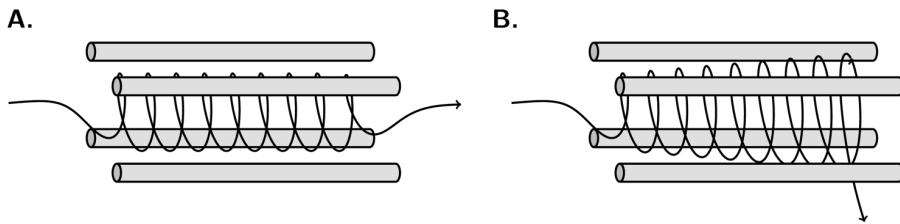


Figure 7. Left panel illustrates stable trajectory for analytes with specific mass-to-charge ratio compared to instable trajectory in the right panel\*

The liquid chromatography mass spectrometer analysis has multiple steps and sources of batch-to-batch variation making reproducible and quantitative determination of absolute responses difficult (Pitt, 2009). Calibration standards with distinguishable chemical properties can be used in spectroscopy to link the measured abundances with known standard concentrations. Internal standards are mixed within the study samples and external standards are studied separate from the samples. In brief, internal standards can be used to estimate molar concentrations for analytes and external standards to correct for the batch-to-batch variation. If systematic signal drifts and patch effects are not properly compensated with peak alignment and quality control, the measurement data is unreliable (Figure 8).

While quality control steps can be often automated, the identification of analytes is a common bottleneck of LC–MS. Biofluids contain large number of distinct metabolites and a single metabolite can produce multiple distinct spectral peaks due to differences in ionization. When studying few analytes using targeted analysis design, authentic standards could be used in identification. However, in an untargeted approach, the analytes are not known *a priori* and even with strong hypotheses, the use of authentic standards is limited by feasibility, cost, and commercial availability. Unknown analytes can be characterized using information about their spectroscopic features such as retention time and mass-to-charge ratio.

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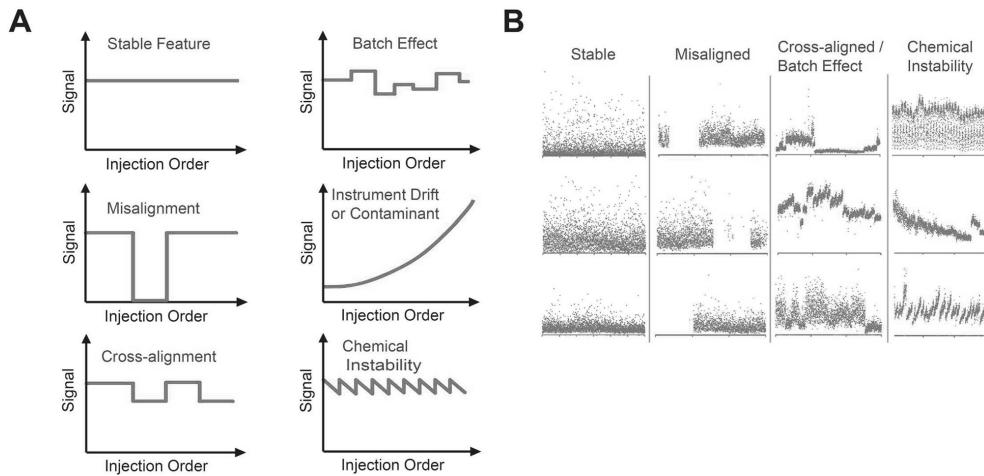


Figure 8. Liquid chromatography mass spectrometry analysis has multiple error sources\*

### 2.2.2.2 Nuclear magnetic resonance spectrometry

Nuclear magnetic resonance (NMR) is a physical phenomenon that offers two medical applications: NMR imaging and NMR spectroscopy (Tognarelli et al., 2015). A nucleus with an odd mass number has a half-integer nuclear spin, and a nucleus with an even mass number has an integer spin. Nuclei with half-integer (non-zero) spin have a non-zero magnetic moment that interacts with external magnetic fields.

Hydrogen-1, the most abundant atom in biological molecules, has a half-integer nuclear spin. When a biological sample is placed in a constant external magnetic field, the degenerate energy levels of hydrogen nuclei (protons) split. This means the nuclei will be left with differing energy levels depending on whether the spin is parallel or antiparallel with the external magnetic field (Figure 9). However, each hydrogen nucleus within the biological sample experiences perturbations in the applied magnetic field from chemical bonds close to the nucleus. Therefore, the energy levels of hydrogen nuclei will also contain information about its local chemical structure. We summarize in the following paragraphs the principles of  $^1\text{H}$ -NMR spectrometry.

\*Adapted with permission from American Chemical Society (Watrous et al., 2017).

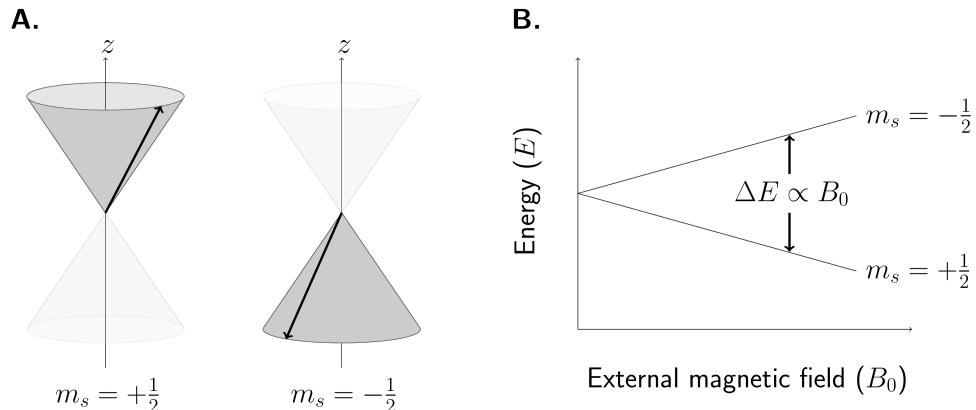


Figure 9. The nuclear energy states of hydrogen split in magnetic field\*

In an NMR spectroscopy device, the sample is placed inside a strong magnetic field and a short, intense radio frequency pulse is imposed to excite some of the nuclei to their higher energy state (Figure 10). When the radio frequency field is turned off, the nuclei tend to return to the low energy state, emitting photons in process. This free induction decay is the superimposition of all signals in the sample and is captured using detector coil. The chemical shifts imposed by the local environment of each hydrogen atom gives rise to a spectrum that contains information about chemical structures observed in the sample. NMR spectral profiles have been well categorized, making metabolite identification easy and reproducibility high.

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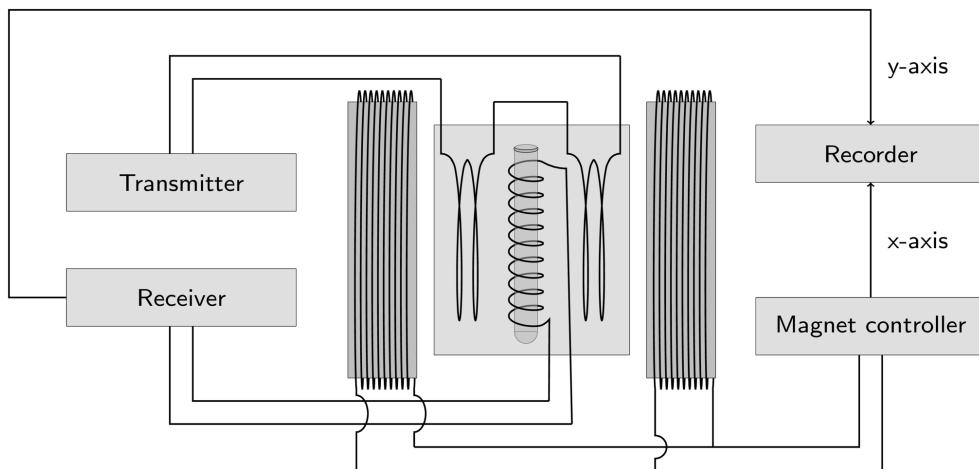


Figure 10. Schematic for nuclear magnetic resonance spectroscopy\*

Notably, the NMR spectroscopy neither degrades the samples nor do the samples leave residue in the device because the applied radiation is non-ionizing and no physical contact is required between the analyte and the device. Inclusion of gradient magnetic field (field with varying strength) across the sample would permit the location of the emission, information that is used in NMR imaging.

In summary, LC–MS and NMR can be used to study various biological samples and the two methods have distinct strengths and weaknesses (Nikolic et al., 2014). NMR has low sensitivity of <100 analytes per run but high reproducibility and easy analyte identification while untargeted LC–MS has high sensitivity of >1000 metabolites per run but is laborious and requires non-trivial analyte identification. Research groups performing spectroscopy analyses should include skilled analytical chemist to allow, in particular, proper quality control of the results and analyte identification.

### 2.2.3 Human lipidome

Lipids are biomolecules that are soluble in nonpolar solvents (Quehenberger & Dennis, 2011). Because plasma is mostly composed out of water, the circulating lipids are solubilized and dispersed in carrier proteins such as albumin and lipoprotein particles. Lipids are the largest group of biological molecules in plasma and different lipid molecules have large structural diversity (Figure 11). We will review in following sections the current literature of human lipidome, the collection

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of all lipids in organism, and focus in more detail on a small group of bioactive signaling molecules, eicosanoids.

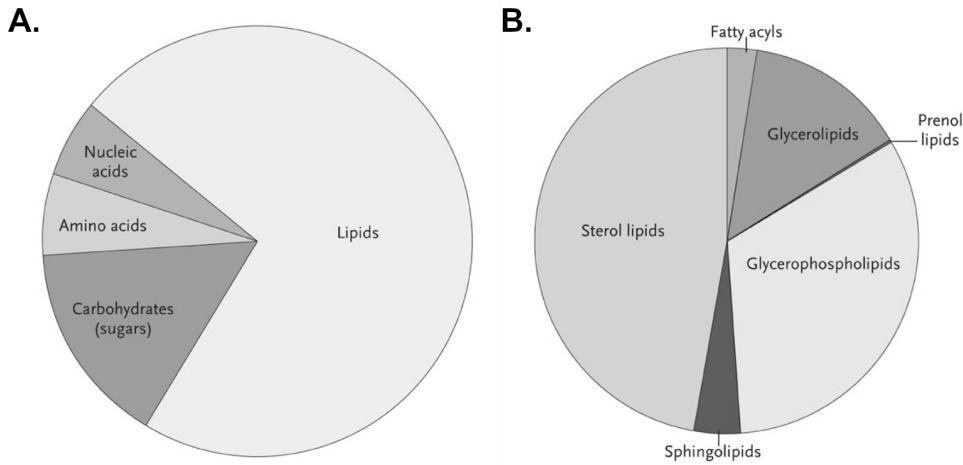


Figure 11. The distribution of biological molecules (A; g/dl) and lipids (B; mol) in human plasma\*

### 2.2.3.1 Lipoprotein particles

While LDL cholesterol has log-linear association with coronary artery disease (CAD), nearly half (49.5%) of the patients hospitalized for CAD in large cross-sectional study had LDL cholesterol level under recommended levels <100mg/dl or <2.6 mmol/l (Sachdeva et al., 2009). A meta-analysis of individuals under statin therapy in seven placebo controlled trials, revealed that risk reduction of statin therapy was more closely related to reduction of apolipoprotein B compared to low-density lipoprotein (LDL) cholesterol (Thanassoulis et al., 2014). While one copy of apolipoprotein B is found on multiple lipoprotein particles including LDL, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and lipoprotein(a), most (>90%) apolipoprotein B in plasma is associated with LDL due to particles long plasma residence time (Varvel et al., 2015). The LDL particle number and apolipoprotein B concentrations are therefore highly correlated ( $R^2 = 0.79$ ) and both measures offer stronger predictor for future cardiovascular events than the current lipid therapy treatment target, LDL cholesterol concentration (Varvel et al., 2015).

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A few large cohort studies report associations between circulating lipid measures and the development of hypertension. In the Women's Health Study ( $N = 17,527$ ; aged  $48.2\text{--}59.6$ ), the average VLDL particle size, apolipoprotein B and total triglycerides were positively and the average LDL particle size negatively associated with incident hypertension (Paynter et al., 2011). Apolipoprotein B and total triglycerides had positive association with the development of hypertension (Paynter et al., 2011). In the Brisighella Heart Study ( $N = 1,864$ ; 49.1% women, aged  $50.8 \pm 11.4$ ), baseline LDL cholesterol level was positively related to rate of hypertension onset (Cicero et al., 2014). In a study of non-hypertensive Japanese men ( $N = 14,215$ , aged  $38 \pm 9$ ), total cholesterol, LDL cholesterol (calculated using Friedewald formula), and non-HDL cholesterol were positively associated with development of hypertension (Otsuka et al., 2016).

In the Women's Health Study, high-density lipoprotein (HDL) cholesterol, large HDL particle concentration, and average HDL particle size were negatively associated with incident hypertension (Paynter et al., 2011). In contrast, in cross-sectional study of healthy Japanese ( $N = 2,953$ ; 38.9% women, aged  $49.7 \pm 9.0$ ), HDL cholesterol was positively associated with hypertension (Oda & Kawai, 2011). U-shaped association between HDL cholesterol and hypertension onset was observed in 14 215 (aged  $38 \pm 9$ ) normotensive men (Otsuka et al., 2016). However, several disease processes (including diabetes, coronary artery disease, and chronic kidney disease) may lead to HDL particle dysfunction that promotes impaired endothelial repair, increased proinflammatory activation, and increased BP (Lüscher et al., 2014; Shimizu et al., 2017). In a cross-sectional study of elderly Japanese men ( $N = 477$ , aged  $65.4 \pm 2.6$ ), positive association between HDL cholesterol and hypertension was observed only in a subsample of participants with high levels of CD34-positive circulating endothelial progenitor cells (Shimizu et al., 2017). While the exact role between CD34-positive cells and HDL cholesterol is still unclear, high level of CD34 cells could indicate reduced endothelial anti-inflammatory ability of HDL particles.

### 2.2.3.2 Free fatty acids

Fatty acids are aliphatic chains of carbon atoms with carboxyl group (R-COOH). Free fatty acids are used to build more complex lipids such as sphingolipids, phospholipids, and glycerolipids such as triglycerides (Figure 11). In a cross-sectional study of Chinese individuals ( $N = 2,447$ , 52.9% women, aged 35–79), polyunsaturated fatty acids (PUFA) and omega-3 PUFAs were negatively and saturated fatty acids (SFA) positively associated with hypertension (B. Yang et al., 2016). In two small studies of Japanese men ( $N = 315$ , 69.7% women, aged  $52.2 \pm 7.3$ ) and of South Africans ( $N = 300$ , aged  $53.1 \pm 9.8$ ), a small number of omega-6

PUFAs, including linolenic acid, were negatively associated with hypertension (Tsukamoto & Sugawara, 2018; Zec et al., 2019).

### 2.2.3.3 Eicosanoids

Eicosanoids are fatty acids with 20 carbon atoms that are enzymatically generated from  $\omega$ -3 and  $\omega$ -6 PUFAs (Khanapure et al., 2007). Compared to circulating free fatty acids, eicosanoids are paracrine signaling molecules that act locally in nanomolar concentrations rather than conventional fatty acid substrates of anabolism and catabolism (Capra et al., 2015). Arachidonic acid (AA) is a 20:4( $\omega$ -6) PUFA abundantly located in the membrane phospholipids that serves as an essential substrate of eicosanoid synthesis (Capra et al., 2015). Notably, the calcium dependent activation of phospholipase A<sub>2</sub> and following release of AA from membrane phospholipids is the rate limiting factor in eicosanoid production (Mitchell et al., 2021).

#### 2.2.3.3.1 Biosynthesis of eicosanoids

Eicosanoids are produced from AA and other PUFAs following three major enzymatic pathways (Figure 12). Cyclooxygenase (COX) pathway produces prostaglandins (PG) that are named with letter denoting the components of the five-member prostane ring (A-K) and subscript denoting the number of double bonds in the PG (Buczynski et al., 2009). COX enzyme has two functional isoforms that have minor differences in activity sites that, in particular, allows COX-2 metabolize dihomo- $\gamma$ -linolenic and eicosapentaenoic acid in addition to AA. COX-1 is constitutively expressed in most cells while COX-2 is constitutively expressed only in selected locations in brains, gut, thymus, lungs, and kidneys (Mitchell et al., 2021). However, COX-2 is rapidly induced in sites of inflammation or cancer (Mitchell et al., 2021). The catalytic activity of COX on AA produces highly unstable PGH<sub>2</sub> (Capra et al., 2015). Specific synthase enzymes convert PGH<sub>2</sub> to prostacyclin (PGI<sub>2</sub>), thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and other bioactive PGs (Capra et al., 2015). Circulating PGs interact with transmembrane GPCRs (Capra et al., 2015).

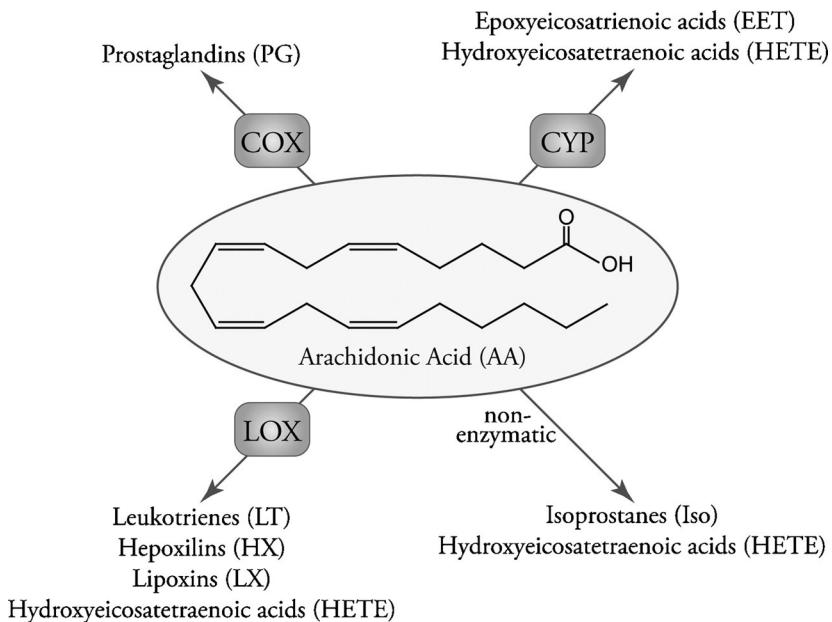


Figure 12. Three enzymatic pathways of eicosanoid synthesis\*

Lipoxygenase (LOX) pathway produces another large family of eicosanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETE), and lipoxins (Figure 12). 5-lipoxygenase (5-LOX) is expressed in myeloid cells, where it catalyzes the formation of leukotriene (LT) A<sub>4</sub> from AA (Buczynski et al., 2009). Leukotrienes follow similar nomenclature as PGs: letter indicates the structure and subscript the number of double bonds in the molecule (Samuelsson & Hammarström, 1980). LTA<sub>4</sub> is further catalyzed into more stable LTB<sub>4</sub>, LTC<sub>4</sub>, and LXA<sub>4</sub>. 12-LOX pathway produces, in particular, HETEs and, in concert with 5-LOX, hepxolinis (Buczynski et al., 2009).

Cytochrome P450 (CYP) pathway catalyzes AA to hydroxyeicosatetraenoic acids (HETEs) and epoxy-eicosatrienoic (EET) acids (Figure 12). While CYP enzymes are present in all forms of life, the function of the enzyme is highly varied under single amino acid mutations resulting unique set of CYP enzymes in each species (Buczynski et al., 2009). Remarkably, the catalyzation of unstable intermediates of eicosanoid synthesis (LTA<sub>4</sub> and PGH<sub>2</sub>) into final compounds can result from transcellular biosynthesis from nearby cells (Capra et al., 2015).

\*Published under Creative Commons Attribution license (Buczynski et al., 2009).

### 2.2.3.3.2 Pathophysiological roles of eicosanoids

Prostaglandins promote and suppress platelet activation. PGI<sub>2</sub> is produced in endothelial blood vessels and released PGI<sub>2</sub> binds to platelet receptors suppressing platelet adhesion, aggregation, and granule secretion (Crescente et al., 2019). Another prostaglandin, TXA<sub>2</sub> produced in activated platelets promotes thrombogenesis; TXA<sub>2</sub> amplify local platelet activation, TXA<sub>2</sub> formation, and platelet aggregation (Crescente et al., 2019). TXA<sub>2</sub> also induces proliferation and vasoconstriction in vascular smooth muscle cell (Crescente et al., 2019). Constitutive COX-2 activity regulates kidney homeostasis, including renal hemodynamics, sodium excretion, and angiotensin II formation (Mitchell et al., 2021). Notably, COX-2 enzyme is the treatment target of the non-steroidal anti-inflammatory drugs and COX-2 inhibition has been linked with increased BP (Mitchell & Kirkby, 2019). Current hypothesis suggest that inhibition of COX-2 pathway removes the inhibition of methylarginine pathway and results in increased production of potent nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA); a knockout rat model indicated PGI<sub>2</sub> as possible end product of the constitutive COX-2 synthesis that limits ADMA production (Mitchell & Kirkby, 2019).

CYP pathway derived 20-HETE and EETs modulate BP homeostasis. EETs are produced in vascular endothelial cells where they have anti-inflammatory effect and promote vasodilation (Crescente et al., 2019). 20-HETE increasing renal vascular resistance inducing contraction in the afferent arterioles and inhibits sodium reabsorption in proximal tubule and thick ascending loop of Henle (Elshenawy et al., 2017). The afferent vasoconstriction is partly mediated by the COX enzymes that catalyze 20-HETE to PGs (Elshenawy et al., 2017). CYP pathway derived EETs are produced in endothelial cells, kidney, and heart (Imig, 2015). EETs dilate preglomerular afferent arterioles and inhibit sodium transport in the proximal tubule and the cortical collecting duct (Imig, 2015). Remarkably, mutation in the CYP4A11 and CYP4F2 enzymes has been linked to elevated BP in humans (Fan et al., 2015). Consistently, deficiency in the formation of 20-HETE and EETs have been linked with salt-sensitive hypertension (Fan et al., 2015).

5-LOX pathway derived LTs promote bronchoconstriction and leucocyte recruitment during inflammation, asthma, and allergy (Dennis & Norris, 2015). LTs, and particularly LTB<sub>4</sub>, have been linked with pulmonary arterial hypertension offering potential treatment option using anti-LT therapy (Tian et al., 2014).

Finally, the biosynthesis of eicosanoids is markedly increased in response to inflammatory stimuli (Dennis & Norris, 2015). PGs and LTs allow the innate immune reaction against bacteria while bacterial pathogens are able to release virulence factors altering the expression of eicosanoid biosynthesis enzymes (Sheppe & Edelmann, 2021). PGE<sub>2</sub>, in particular, enhances inflammasome activation, secretion of distinct interleukins, and formation of insoluble components

that are able to trap bacteria inside macrophages (Sheppe & Edelmann, 2021). Eicosanoids have been reported to express both pro- and anti-inflammatory functions (Dennis & Norris, 2015).

#### 2.2.4 Amino acids

A few recent studies have reported on the associations between circulating amino acids and hypertension. In the Dutch Prevention of Renal and Vascular End-stage Disease study ( $N = 4,169$ ; 54.4% women; aged  $49.2 \pm 10.3$ ) and in a Japanese study ( $N = 5,541$ ; 51.9% women), circulating branched amino acids, isoleucine, leucine, and valine, were positively associated with hypertension (Flores-Guerrero et al., 2019; Mahbub et al., 2020). In another cross-sectional study of Japanese ( $N=8,115$ ; 52.7% women; aged  $56.5 \pm 15.1$ ) circulating glycine, glutamine, and histidine were negatively and alanine positively associated with hypertension (Yamaguchi et al., 2017).

#### 2.2.5 Energy metabolism-related measures

In healthy individuals, postprandial insulin secretion promotes changes in circulating metabolites reflecting switch from catabolism to anabolism (Shaham et al., 2008). Increased lactate indicates increases glycolysis, decreased glycerol indicates decreased lipolysis, decreases beta-hydroxybutyrate indicates decreased ketogenesis, and decreased amino acids indicate decreases proteolysis. In the Bogalusa Heart Study ( $N=1,249$ ; 58.8 women; aged  $48.2 \pm 5.3$ ), fasting glucose was positively associated with systolic BP (He et al., 2020). In an American study ( $N=5,554$ ; 54.9% women; aged  $61.9 \pm 5.5$ ), lactate was positively associated with hypertension onset in women only (Juraschek, 2015).

#### 2.2.6 Fluid balance related-metabolic measures

Albumin increases vascular colloid-osmotic pressure and transports hormones, drugs, amino acids, and free fatty acids (Høstmark et al., 2005). In the Oslo Health Study ( $N=5,171$ ; 61.9% women; aged 30–75) and in the Neuroprotective Model for Healthy Longevity among the Malaysian Elderly study ( $N=2,322$ ; 52.0% women), albumin was positively associated with BP (Eshkoor et al., 2016; Høstmark et al., 2005). However, in a retrospective study of normotensive Japanese ( $N=2,240$ ; 38.2% women, aged  $49.8 \pm 8.7$ ), albumin was negatively associated with hypertension onset (Oda, 2014). The cross-sectional positive association could possibly indicate greater vascular volume and negative longitudinal association indicate other properties albumin imposes or transmits (Oda, 2014).

### 2.2.7 Inflammation markers

Hypertension has been linked with chronic inflammation in animal and human studies (Harrison et al., 2011; Barrows et al., 2019). Indirect immunofluorescence technique and high-resolution confocal microscopy revealed existence of C-reactive protein (CRP) deposits associated in atherosclerotic plaques in human coronary artery sections from 68 autopsies (Y. X. Zhang et al., 1999). In the cross-sectional Physicians' Health Study (N=508), two inflammation markers, intercellular adhesion molecule-1 and IL-6, were positively associated with BP adjusted for cardiac risk factors in men (Chae et al., 2001). In the Women's Health Study (N=20,525; median follow-up 7.8 years), baseline CRP was positively associated with incident hypertension adjusted for baseline coronary risk factors in women (Sesso et al., 2003). In moderate sized study, high-sensitivity CRP (hs-CRP) was independent predictor of pulse wave velocity, the gold-standard measure of arterial stiffness (Mahmud & Feely, 2005).

Mendelian randomization meta-analysis of 47 epidemiological studies (N= 194,418) examined the causal relationship between circulating CRP concentration and coronary heart disease (Collaboration (CCGC), 2011). Information was available on four SNPs (rs3093077, rs1205, rs1130864, rs1800947) that were associated with CRP and were unrelated to traditional cardiovascular risk factors. In conclusion, individual SNPs and genetically predicted CRP level were not significantly associated with coronary heart disease. Therefore, the association between CRP and CAD appears to be non-causal and because hypertension is a well-established risk factor for CAD, the association between CRP and BP is probably likewise non-causal.

Nonsynonymous mutation of amino acid position Asp358Ala on the main cleavage site of IL-6 receptor (IL-6R) affects IL-6 signaling (Revez et al., 2013). Minor allele of the IL-6R gene (rs2228145, A>C) has been demonstrated to increase the expression of soluble isoform of IL-6R and reduce the classical IL-6 signaling (Stephens et al., 2012). Meta-analysis of 82 studies (N>200000) demonstrated that each 358Ala copy inherited was associated with increased soluble IL-6R, reduced CRP, and reduced CAD risk independent of the classical risk factors (IL6R Genetics Consortium Emerging Risk Factors Collaboration et al., 2012). Therefore, while CRP is not causally associated with coronary artery disease, previous study supports causal IL-6 mediated inflammation hypothesis in cardiovascular disease.

Harrison et al. proposed a 2-step inflammation hypothesis where the early pre-hypertensive elevation of BP caused by dietary habits and other factors brings about an inflammatory response causing sustained hypertension (Harrison et al., 2011). The inflammatory response recruits T cells and macrophages into the perivascular fat and kidney where the interplay of released cytokines, catecholamines, reactive

oxygen species, and angiotensin II promote vasoconstriction, vascular remodeling, and sodium retention (Harrison et al., 2011).

### 2.2.8 Other metabolites associated with hypertension

The metabolites in human circulation have three origins: they can be host derived, microbiota derived, or host-microbiota derived. Alterations in carbohydrate, lipid, amino acid, tri-carboxylic acid, and ketone metabolism have been linked with hypertension (Islam, 2017; Chakraborty et al., 2020). Microbiota can produce in addition to SCFAs other metabolites that are also produced in human metabolism (Chakraborty et al., 2020).

Bile acids and trimethylamine (TMA) regulate BP and are produced in interplay between host and microbiota (Chakraborty et al., 2020). The microbial TMA is oxidized in liver to trimethylamine-oxide (TMAO) that has been positively linked with stroke and CAD events (Nie et al., 2018; Jaworska et al., 2019). Meta-analysis of 11750 individuals revealed positive association between TMAO and BP (Ge et al., 2020). However, the significance of the roles between TMA and TMAO is not currently fully understood (Jaworska et al., 2019). Hydrogen sulfide ( $H_2S$ ) is endogenously produced gaseous signaling molecule (van Goor et al., 2016). In animal models, inhibition of enzymes in  $H_2S$  production has been reported to promote hypertension and administration of  $H_2S$  has resulted to decreased BP (van Goor et al., 2016).

## 2.3 Summary

Metagenomics and metabolomics offer practical and cost-effective methods to analyze samples from large human cohorts to study the pathophysiology and correlates of chronic diseases. We reviewed in this chapter the basic methodologies of metagenomics and metabolomics and synthetized the relevant literature related to hypertension in this domain.

Animal models have built weight of evidence linking gut microbiota with host hypertension. Working hypotheses have also been formulated for the observed findings. Gut microbiota could affect BP, in particular, by modulating sympathetic activity, gut wall permeability, and producing signal molecules in circulation. Dietary sodium, a classic risk factor for hypertension, could also affect gut microbiota potentially by depleting beneficial microbial species. However, much of our knowledge is still based solely on animal models and only few large-scale human studies have been published to date. In particular, the clinical significance of the observed associations to human hypertension are unclear.

The circulating human lipidome is complex. A large number of prior studies have focused on lipoprotein particles. While the results for atherogenic lipoprotein particles, such as LDL, are mostly consistent, the role of HDL cholesterol to CAD and hypertension is currently not fully understood. There also exists a large family of bioactive fatty acids, eicosanoids, that have been linked to thrombogenesis and BP modulation in mainly small animal and studies. In addition to lipids, other metabolites and metabolic biomarkers, including amino acids, glycolysis-related metabolites, and inflammation markers, have been associated with hypertension.

While our current knowledge is based on promising results from animal studies, the utilization of third generation sequencing and study designs combining data from multiple omics fields have potential to transition the research focus from the experimental models to human studies in near future. Emergence of openly available data, modeling software, and analysis code, and the shift to open-access publishing has potential to increase the availability, quantity, and quality of future omics studies.

### **3 Aims**

This thesis was designed to study novel correlates of hypertension using contemporary metagenomics and metabolomics.

The specific aims were:

1. To review the current knowledge on the association between gut microbiota and hypertension in animal and human studies (I).
2. To study the links between gut metagenome, dietary salt, and BP (II).
3. To investigate the associations between eicosanoids, the PUFA-derived small molecule activators and suppressors of systemic inflammation, and BP (III).
4. To investigate the cross-sectional and prospective associations between high abundance metabolite biomarkers and BP (IV).

# 4 Materials and Methods

## 4.1 Systematic literature review

We performed a systematic literature review for original research articles using three scientific databases: Medical Literature Analysis and Retrieval System Online (MEDLINE), Excerpta Medica database EMBASE, and Cochrane Library. Our search terms aimed to capture the intersection of studies reporting results for essential hypertension and metagenomics (Table 4). While our study focused on gut microbiota, the exclusion of unrelated metagenomics studies was left to screening.

Table 4. The search terms used in three scientific databases

Database	Search terms
MEDLINE	("Blood Pressure"[MeSH] OR "Hypertension"[MESH] OR "blood pressure"[TI] OR "hypertension"[TI] OR "blood pressure"[OT] OR "hypertension"[OT]) AND ("Gastrointestinal Microbiome"[MeSH] OR "microbiota"[tiab] OR "microbiome"[tiab] OR "metagenomics"[tiab])
EMBASE	('blood pressure':ti OR 'hypertension':ti OR 'blood pressure':kw OR 'hypertension':kw) AND ('intestine flora':exp OR 'microbiota':ti,ab OR 'microbiome':ti,ab OR 'metagenomics':ti,ab)
Cochrane Library	((blood pressure):ti,kw OR (hypertension):ti,kw) AND ((microbiome):ti,ab,kw OR (metagenomics):ti,ab,kw OR (microbiota):ti,ab,kw)

MEDLINE, Medical Literature Analysis and Retrieval System Online; EMBASE, Excerpta Medica database.

Our literature review followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses guidelines (Liberati et al., 2009). We included in the review original research articles reporting results for essential hypertension and sequenced gut microbiota. Interventional studies were limited to stool transfers, orally administrated probiotics, dietary sodium, antihypertensive medications, and short chain fatty acids, and genomic knockout models. Duplicate search results were automatically detected using Digital Object Identifiers. Manual screening was performed by single author using first titles, second abstracts, and finally full-text.

## 4.2 Study samples

### 4.2.1 FINRISK (II-IV)

The Finnish Institute for Health and Welfare has performed population surveys every five years since 1972 to monitor the development of cardiovascular risk factors in the Finnish population (Borodulin et al., 2018). The FINRISK 1997–2012 study samples consist of participants randomly drawn from the national population register from up to six geographical areas stratified by sex, region and 10-year age group. The six areas are Helsinki and Vantaa, Turku and Loimaa, North Karelia, Northern Savo, Northern Pohjanmaa and Kainuu, and Lapland. Lapland was included in FINRISK 2002 with health examination and in FINRISK 2007 with self-reporting questionnaire. In recent FINRISK studies the age range of invited individuals has broadened to ages 25–74 in all areas, while only individuals aged 25–64 were invited in Northern Savo, Turku and Loimaa, and Oulu areas in FINRISK 1997 and 2002. The participation rates to health examination in FINRISK 1997–2012 was 56–72%, the mean age of the participants was 45.2–45.7 years, and the proportion of women was 52.6–54.0%. In FINRISK 2002, the study protocol included the collection of stool samples and urinary sodium samples (Study II) in addition to venous blood samples (studies II–IV). The FINRISK study was approved by the Ethics Committee of the Hospital District of Southwest Finland.

The participants of FINRISK 2007 were invited to participate in the Dietary, Lifestyle and Genetic determinants of Obesity and Metabolic syndrome (DILGOM) study (Konttinen et al., 2018). All DILGOM 2007 participants alive at the end of 2013 were invited to participate in DILGOM 2014. In 2014, the participants from the two southern study areas were invited to health examination while the participant (Study IV) from the three northern study areas provided self-reported physical measurements. The flowchart of the DILGOM study is presented in Figure 13.

### 4.2.2 Health 2000-2011 (IV)

Health 2000-2011 is a multidisciplinary epidemiological survey of individuals aged  $\geq 30$  years living in mainland Finland (Heistaro, 2008; Lundqvist & Mäki-Opas, 2016). The study was carried out by the Finnish Institute for Health and Welfare in collaboration with multiple research and funding agencies and the main aim of the study was to gather information about major health problems in population  $\geq 30$  years, health service needs, and working capacity. Study population was stratified by five university hospital districts: Helsinki, Turku, Tampere, Kuopio, and Oulu. From each university hospital regions, 16 health care districts were sampled. All living participants were invited to follow-up examination in 2011 (Figure 14). The

studies were approved by the Coordinating Ethics Committee of the Helsinki University Hospital District.

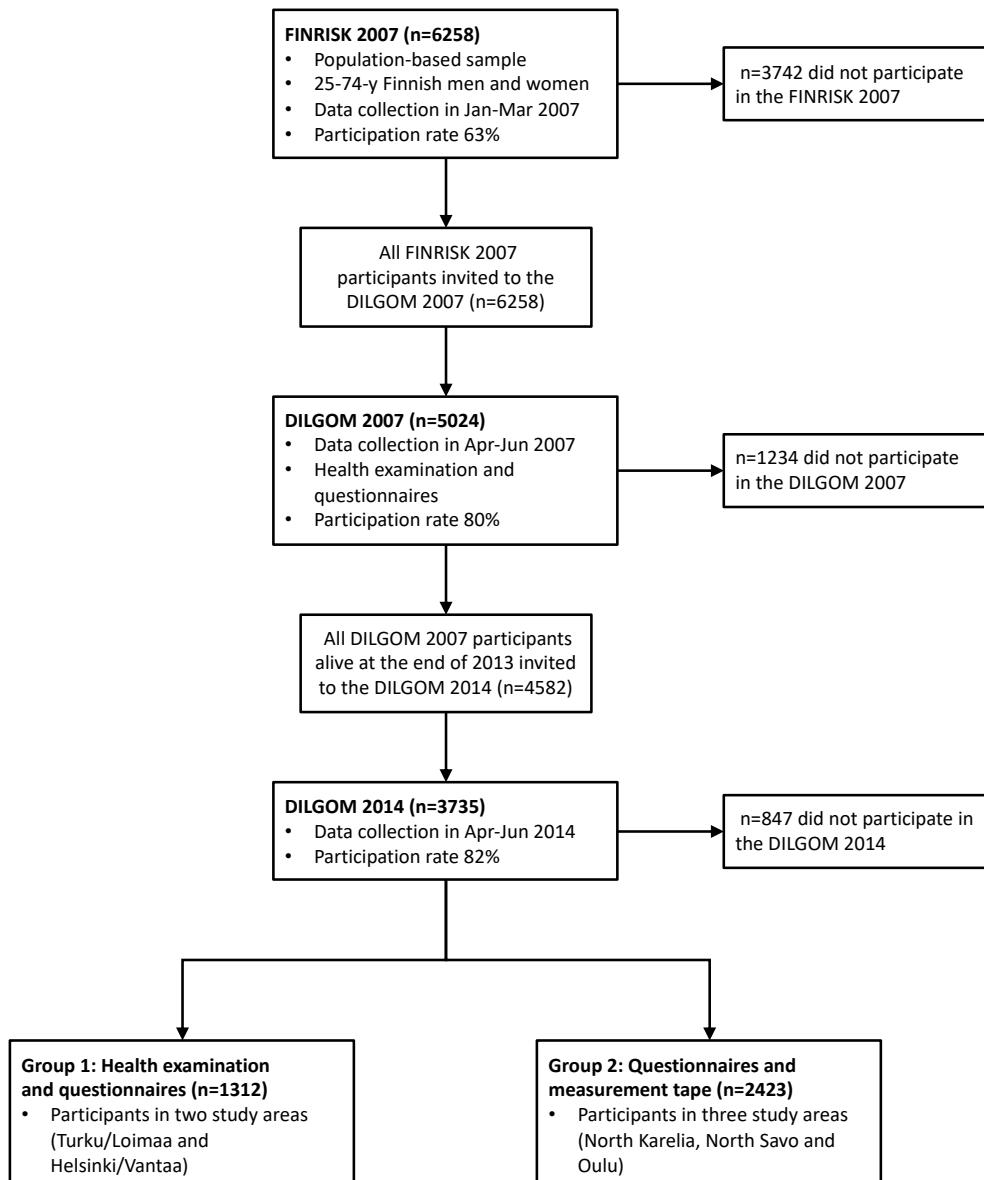


Figure 13. Participant flow chart of the DILGOM study\*

\*Reprinted by permission from Springer Nature (Konttinen et al., 2018).

#### 4.2.3 FinHealth 2017 (IV)

After FINRISK 2012, the Finnish Institute for Health and Welfare merged the National FINRISK study and Health 2000-2011 survey protocols to National FinHealth Study (Borodulin & Sääksjärvi, 2019). The aim of FinHealth is to obtain information on health, functional capacity and well-being of adults (aged  $\geq 18$ ) residing in Finland. In FinHealth 2017 study, mainland Finland was stratified in 50 health centre districts: 15 largest cities and seven randomly selected health centre districts from each university hospital regions. The participation rate in the health examination was 58%. The FinHealth 2017 study was approved by Coordinating Ethics Committee of the Helsinki University Hospital District.

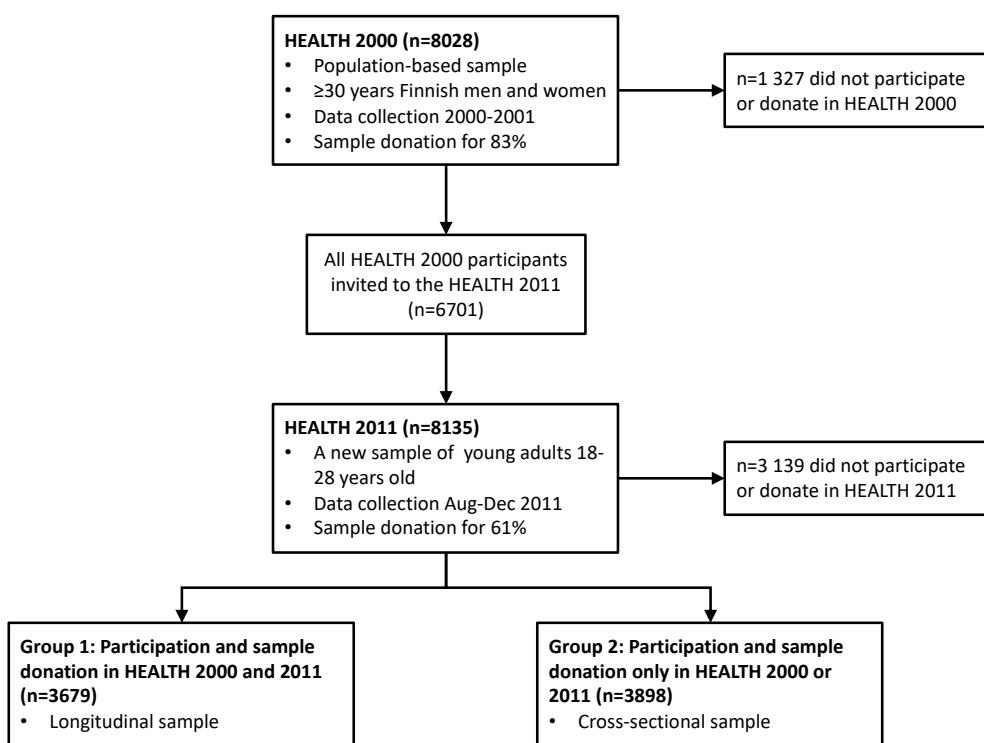


Figure 14. Participant flow chart of the Health 2000-2011 study

#### 4.2.4 Framingham Heart Study (III)

The first-generation (i.e. the ‘Original’) cohort of the Framingham Heart Study (FHS) included a random sample of two thirds of the adult population of Framingham, MA who were enrolled in a longitudinal community-based cohort study in 1948. The children and children’s spouses of the couples of first-generation

FHS were invited to participate in FHS Offspring study (Kannel et al., 1979). The objective of FHS Offspring study is to obtain information about cardiovascular disease risk factors and family patterns of cardiovascular disease. The participants of the FHS Offspring study have been re-examined every four-to-eight years since the first examination in 1971. In the eighth examination cycle of the study in 2005–2008, serum samples were collected in addition to physical examination. FHS Offspring was approved by Boston University Medical Center's Institutional Review Board.

## 4.3 Study flow

In the FINRISK and FinHealth 2017 studies (Borodulin et al., 2018; Borodulin & Sääksjärvi, 2019), after filling in a questionnaire on sociodemographic information, lifestyles, medications, and medical history at home, the participants attended a physical examination at a local study site. The participants underwent measurements for height and weight and blood samples were drawn mainly after a minimum of 4 hours of fasting.

In the Health 2000–2011 study (Heistaro, 2008; Lundqvist & Mäki-Opas, 2016), participants were interviewed by centrally trained interviewers on sociodemographic information, lifestyle, medications, and medical history 1–6 weeks before attending physical examination at local study sites. The participants underwent measurements for height and weight. Overnight fasting blood samples were drawn.

According to FHS Offspring Cycle 8 study protocol (Kannel et al., 1979), body weight was measured without shoes optionally wearing a gown and the height was measured barefoot or wearing thin socks using vertical mounted stadiometer rounding down to the nearest quarter inch. Technicians recorded participant's regular medication at on home visit using medication bottles or nursing home charts. Medical history, including diabetes status and smoking, was obtained using standardized interview forms.

### 4.3.1 Blood pressure measurement (II-IV)

The European Health Examination Surveys recommends that BP should be measured after five minutes of rest taking three measurements one minute apart (Tolonen, 2016). Study participants should fast for >4 hours and avoid smoking and vigorous exercise for one hour before examination (Tolonen, 2016). FINRISK, Health, FHS, and FinHealth studies performed sequential blood pressure measurements using a mercury column sphygmomanometer on seated participants but some difference remains due to contemporary guidelines (Table 5). In FINRISK and FinHealth 2017 studies, a study nurse measured sitting BP three times from the

right arm using a mercury sphygmomanometer with an appropriately sized cuff (Borodulin et al., 2018; Borodulin & Sääksjärvi, 2019). In Health 2000-2011 study, a study nurse measured sitting BP two times from the right arm using a mercury sphygmomanometer and a 15 x 43 cm sized cuff; a larger cuff was used when needed (Heistaro, 2008; Lundqvist & Mäki-Opas, 2016). In FHS study, blood pressure was measured by average of two measurements using manual mercury sphygmomanometer using with appropriate cuff size (Kannel et al., 1979).

Table 5. Blood pressure measurements in FINRISK, FHS, Health, and FinHealth

Study	N	Cuff size (proximal arm circumference)	Rest for first measurement	Rest between measurements
FINRISK 1997	2	13 cm x 42 cm	>5 min	
Health 2000*	2	12 cm x 35 cm for ≤35 cm 15 cm x 43 cm for >35 cm	>5 min	2 min
FINRISK 2002	3	14 cm x 36 cm	>5 min	>1 min
FHS OFFSPRING 8th cycle	2	Adult thigh cuff Adult large cuff Adult regular cuff Pediatric cuff	>5 min	>30 sec
FINRISK 2007	3	14 cm x 36 cm	>5 min	>1 min
Health 2011†	2	12 cm x 35 cm for ≤35 cm 15 cm x 43 cm for >35 cm	>5 min	>1 min
FINRISK 2012	3	14 cm x 36 cm	>5 min	>1 min
FinHealth 2017‡	3	small cuff for <24 cm medium cuff for 24–32 cm large cuff for 32–48 cm extra large cuff for >48 cm	>5 min	>1 min

Information is combined from reported FINRISK (Borodulin et al., 2018), Health 2000 (Heistaro, 2008), FHS (Kannel et al., 1979), Health 2011 (Lundqvist & Mäki-Opas, 2016), and FinHealth 2017 (Borodulin & Sääksjärvi, 2019) study protocols. The Finnish language study protocols were consulted with respect to rest between BP measurements (ISBN 951-740-073-X, ISBN 951-740-356-9, ISBN 978-951-740-911-7, ISBN 978-952-302-052-8).

\*Participants asked not to eat, smoke, and avoid physical exertion.

†Participants asked to avoid heavy exercise, cola drinks, coffee, tea, eating, and smoking before

‡Participant advised to refrain from heavy exercise, eating and drinking prior the examination.

### 4.3.2 Blood samples (II-IV)

In the FINRISK studies, blood samples were drawn after a minimum of 4 hours of fasting, the samples were centrifuged at the field surveys sites (Borodulin et al., 2018). In FINRISK 1997 and 2002, fresh samples were sent daily to THL and from FINRISK 2007, sera were frozen after separation at field surveys sites and

transported to THL once a week. Blood samples were stored at -70°C. In FHS, samples were drawn after 12-hour fast, centrifuged 22 min at 4°C, and separated plasma was stored at -80°C within 90 minutes of collection (Kannel et al., 1979). In the Health 2000 study, blood samples were drawn after a minimum of 4 hours of fasting, serum samples were frozen on site at -20°C within 90 minutes from sampling and stored at -70°C within 1-2 week after sampling (Heistaro, 2008). In the FinHealth 2017 study, blood samples were drawn after a minimum of 4 hours of fasting, the samples were frozen at -20°C within 120 minutes from sampling and stored at -70°C within 1-2 week after sampling (Borodulin & Sääksjärvi, 2019).

#### 4.3.3 Stool samples (II)

In the FINRISK 2002 study, stool samples were collected from all voluntary participants (Borodulin et al., 2018; L. Valsta, personal communication, 2019). At the final stage of the health examination, a research nurse informed participants about the stool sample protocol and provided stool sample kits. Participants were instructed to collect stool at home and send the stool samples to THL in 50 ml Falcon tubes using provided mailing packages. The samples were then frozen in -20°C and kept unthawed until 2017, when they were sent to the University of California San Diego for microbiome sequencing.

#### 4.3.4 Urine samples (II)

In the FINRISK 2002 study, 10-year age group and sex stratified subsample of 2240 individuals aged 25–64 years was drawn from North Karelia, southwestern Finland, and Helsinki area for 24-hour urine collection (Laatikainen et al., 2006). Participants were instructed to start the 24-hour urine collection on a Sunday morning and return the container the following day to the examination site. The purpose of the urine collection to assess the dietary salt measurement was not specifically mentioned to the participants. At the examination site, a study nurse mixed the sample, measured total urine volume and took a sample of urine to central laboratory. The samples were frozen at -20°C for storage and later analyzed using an ion-selective electrode (Optima analyzer, Thermo Electron Oy, Vantaa, Finland). Daily urine sodium excretion was calculated as the product of 24-hour urine sodium concentration and volume.

Of the 2240 invited individuals, 1564 participated and 919 returned the urine specimen. Of the 919 returned urine collections, 10 were deemed incomplete due to creatinine  $\leq 5.0$  mmol/day or creatinine  $\leq 6.0$  mmol/day with volume  $< 1000$  ml for final urinary sodium subsample of 909. Out of the 909 participants with completed

urinary collection, 63 were excluded due to missing stool collection, and 17 for missing relevant covariates for final urinary sodium subsample of 829 participants.

#### 4.4 Shotgun metagenomics (II)

Microbiota analyses for stool samples collected in FINRISK 2002 were performed at the University of California San Diego using whole genome untargeted shallow shotgun metagenomic sequencing following previously published protocol (Salosensaari et al., 2021). Illumina-compatible libraries were prepared from isolated DNA and normalized to 5 ng input per sample. DNA was ligated with iTru dual-indexing system allowing sample pooling (Glenn et al., 2019). Barcoded and amplified libraries were then pooled in approximately equimolar ratios and sequenced using Illumina Hi-Seq 4000 for paired-end 150 bp reads. Sequenced reads were mapped against taxonomy using SHOGUN v1.0.5 (Hillmann et al., 2018) against NCBI RefSeq database version 82 (O’Leary et al., 2016).

Functional profiling was obtained using Kyoto Encyclopedia of Genes and Genomes Orthology group (KO) annotations for RefSeq-derived genes following the default parameters of SHOGUN tool (Hillmann et al., 2018). To improve the accuracy of low-abundance genes, the KO profiles were also estimated using reference genomes to predict the presence of unsampled genes within the observed genome. The final KO table represents the average of directly observed and weighted predicted KO profiles.

Out of the 8799 individuals who took part in FINRISK 2002, we excluded 1568 participants who did not provide stool samples, 20 participants due to low sequencing depth (<50000 reads), and 258 participants due to missing relevant covariates for a final study sample of 6953 individuals who were included in the analysis. The average read count was approximately 900,000 reads per sample (Salosensaari et al., 2021).

#### 4.5 Genotyping (III)

The genome-wide single-nucleotide polymorphisms (SNP) genotyping and quality control have been previously described in detail (Abraham et al., 2016). The participants of FINRISK 2002 were genotyped in two batches together with FINRISK 1992-1997 participants: Illumina HumanHap610 platform was used in first and Illumina CoreExome genotyping array in second batch. Genotype calls were generated at the Institute of Molecular Medicine Finland (FIMM) and genotype imputation was performed using Sequencing Initiative Suomi v3 reference panel (Pärn, Fontarnau, et al., 2018; Pärn, Isokallio, et al., 2018).

## 4.6 Spectroscopy

### 4.6.1 Eicosanoid profiling (III)

We used high-throughput measure of bioactive lipids using directed non-targeted LC–MS approach described previously in detail (Fendt & Lunt, 2019; Watrous et al., 2019). Using a directed non-targeted LC–MS approach in conjunction with computational chemical networking of spectral fragmentation patterns, we identified 545 eicosanoids and related oxylipins in the FINRISK. Metabolite data were adjusted for technical variation in off-plate pooled plasma samples and in spike-in internal standards. We adjusted for patch variation normalizing each metabolite measurement in each plate using formula (raw peak intensity - plate median peak intensity)/(median absolute deviation of plate). Missing values were replaced with minimum value for each eicosanoid abundance. While observed metabolites were labeled using mass-to-charge ratios and retention times, we were prepared to manually match a subset of all metabolites between FINRISK and FHS comparing their LC–MS profiles, reference standards, and online databases.

After participants with missing relevant covariates were excluded, we had eicosanoid and related oxylipins profiles available for 8099 participants in FINRISK 2002 (discovery cohort) and 2859 participants in FHS (replication cohort). The group of signals we defined as eicosanoids, were highly consistent with known and putative eicosanoids and related oxylipins in human plasma (Watrous et al., 2019).

### 4.6.2 NMR metabolic measures (IV)

We had access to metabolomics analyses performed using <sup>1</sup>H-NMR spectroscopy on highly automated platform from serum samples by Nightingale Health Ltd, Helsinki, Finland (Soininen et al., 2015; Würtz et al., 2017). Frozen serum samples were thawed over night at +4°C. Automatic liquid handler inserted buffer to serum samples and moved the aliquots to 96-format racks. Three molecular windows were available using 500 MHz and 600 MHz spectrometers: broad signals arising from lipoproteins, low-molecular-weight metabolites, and lipids. The validity of this approach in detection of lipoprotein cholesterol, triglycerides, glucose, circulating fatty-acids, and beta-hydroxybutyrate has been demonstrated compared to routine clinical assays, gas chromatography, and enzymatic method (Würtz et al., 2017).

We included in our analyses 53 circulating biomarkers and 97 lipoprotein subclass measures for cross-sectional sample of 36985 and longitudinal sample of 4197 FINRISK 1997–2012, Health 2000-2011, and FinHealth 2017 participants.

## 4.7 Definitions

### 4.7.1 Blood pressure measurements

We used in all studies the mean of the first two BP measurements to define systolic and diastolic BP. We defined hypertension as systolic blood pressure  $\geq 140$  mmHg, diastolic blood pressure  $\geq 90$  mmHg or use of antihypertensive medication. Study II used register-based and studies III and IV self-reported information about antihypertensive medication use. We defined pulse pressure as systolic minus diastolic BP and mean arterial pressure as  $[(2 \times \text{diastolic BP}) + \text{systolic BP}] / 3$ .

### 4.7.2 Questionnaire based definitions

Smoking was defined by self-reported current daily smoking. Leisure time physical activity was self-reported from four options: (i) sedentary, (ii) light activity for over four hours per week, (iii) fitness training or other strenuous exercise for over three hours per week, and (iv) competitive sports. In Study IV, diabetes, antihypertensive medication use, and lipid medication use was self-reported.

### 4.7.3 Anthropomorphic measures

Body mass index (BMI) was defined as weight (kg) divided by the square of the body height (m).

### 4.7.4 Register and laboratory-value based definitions

The information about medication use was retrieved from Finnish national Drug Purchase Register using Anatomical Therapeutic Chemical classification (ATC) codes (The Social Insurance Institution of Finland, 2012; WHO Collaborating Centre for Drug Statistics, 2018). Finnish pharmacies fill prescriptions for a maximum of three months and medication use was determined as a drug purchase occurring within the four months preceding baseline. Information about different comorbidities was acquired from nationwide Care Register for Health Care using International Classification of Diseases (ICD) codes (Finnish Institute for Health and Welfare, 2021; Lääkintöhallitus, 1986).

In studies II, antihypertensive medication was defined using four ATC classes: diuretics (C03\*), beta blockers (C07\*), calcium channel blockers (C08\*), and renin-angiotensin system inhibitors (C09\*). In studies II and III, prevalent diabetes was defined as self-reported diabetes, previous diagnostic code (ICD-10 codes E10-E14 or ICD-8/9 code 250), a previous diabetes medication purchase (ATC code A10\*),

or special reimbursement code for diabetes medications in the Drug Reimbursement Register. However, in FHS Offspring, prevalent diabetes was defined as a fasting plasma glucose  $\geq 7.0$  mmol/l or self-reported use of glucose-lowering medications.

## 4.8 Statistical analyses

We used R version 3.6 for all statistical analyses and published the source code for the analyses under open license in open-access repository Zenodo.

### 4.8.1 Study I

The preliminary literature review did not reveal any consistent numerical features that could be used to perform meta-analysis.

### 4.8.2 Study II

Unless otherwise noted, we adjusted the analyses for the well-established correlates of hypertension: age, sex, BMI, smoking, exercise, diabetes mellitus, diuretic use, beta blocker use, calcium channel blocker use, and renin–angiotensin system inhibitor. We calculated alpha diversity using Shannon’s index on species level data. We used Bray-Curtis dissimilarity indices on compositional microbial species-level abundance for beta diversity and to perform Principal Coordinates Analysis (PCoA). We analyzed the proportion of variance BP explains about microbial beta diversity using multivariate analysis of variance (PERMANOVA) with 999 permutations. We defined common microbial genera to be prevalent in at least 1% of sample population with a relative abundance over 0.1%. We used DESeq2 with Benjamini-Hochberg correction to study the associations between microbial abundances and BP (Benjamini & Hochberg, 1995; Love et al., 2014). First, we studied the associations between common microbial genera and blood pressure indices. Second, we studied the associations between *Lactobacillus* species with (1) blood pressure indices and (2) 24-hour urinary sodium excretion. We used abundances from all available species to estimate size factors to be used in analyses with *Lactobacillus* species. We studied the association between log(x+1) transformed KO groups and systolic BP using linear regression with Benjamini-Hochberg correction.

### 4.8.3 Study III

Unless otherwise noted, we adjusted all analyses for age, sex, BMI, current smoking, diabetes, antihypertensive medication, and technical variable (mass spectrometry batch). We corrected for missing LC–MS data imputing missing values with

minimum observed values for each feature. All features were centered to zero and standardized to unit variance without other transformations. We used linear and logistic regression models to study the associations between eicosanoids and BP. We adjusted for multiple testing using Bonferroni correction to minimize the type I error. We studied the Spearman correlation between eicosanoid significantly associated with systolic BP in combination with hierarchical cluster analysis with complete linkage method. We studied the multivariable association between eicosanoids and systolic BP using forward stepwise regression with inclusion threshold of  $P=0.05/545$ . We defined eicosanoid risk score using effect sizes from forward selection model according to the formula  $\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \dots + \beta_n \cdot X_n$  with  $X_i$  denoting the standardized value for the  $i$ :th eicosanoid abundance, and  $\beta_i$  denoting the effect size in the forward selection model. Finally, we calculated the association between eicosanoid risk score and BP. We replicated these analyses in FHS Offspring using the eicosanoid abundances in FHS and the regression coefficients from FINRISK.

To analyze the causative role of the eicosanoid risk score, we performed Mendelian randomization (MR). To account for ordered patterns in genetic data, we calculated multidimensional scaling based on raw Hamming distances using PLINK version 1.9. We performed genome-wide association study (GWAS) for the continuous eicosanoid risk scores and the autosomes using SNPTEST version 2.5.2 adjusted for age, sex, batch, and first ten MDS-axes. In brief, we used FINRISK data to find SNPs associated with eicosanoid risk score and UK Biobank data to find the associations between SNPs and BP. We performed the MR using TwoSampleMR with SNPs that had Hardy–Weinberg equilibrium  $>1E-6$ ,  $P < 5E-8$ , and minor allele frequencies  $>0.01$ . We estimated the causative roles using five regression method.

#### 4.8.4 Study IV

Unless otherwise noted, we adjusted all analyses for age, sex, BMI, smoking, diabetes, leisure-time physical activity, antihypertensive medication, lipid medication, and cohort. Cohort was included to account for the time-dependent differences in the diagnosis and management of hypertension. Antihypertensive medication was not used in models where outcome was hypertension. All features were centered to zero and standardized to unit variance. We adjusted for multiple testing using Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). We studied the associations between systolic BP, diastolic BP, and hypertension in the cross-sectional sample using linear and logistic regression models. To study the age- and sex-related differences in metabolite-BP associations, we performed similar analyses in groups divided by median age (50.5 years) and sex. In the longitudinal sample, we defined systolic BP change as follow-up BP minus baseline BP. We

studied associations between the baseline metabolites and systolic BP change using linear models; we included baseline systolic BP among the covariates in the longitudinal model.

We used gradient boosting machine learning algorithm XGBoost to assess the multivariable associations of the 53 circulating biomarkers with BP (T. Chen & Guestrin, 2016). We used root-mean-square error (RMSE) to estimate model fit. We performed leave-one-out cross-validation in FINRISK 1997–2002 and Health 2011 and five-fold cross-validation for Health 2000–2011. We used FinHealth 2017 and FINRISK 2007–2014 for testing. We tuned the hyperparameters using Bayesian optimization using R package ‘mlrMBO’ (Bischl et al., 2017). We compared the model fit using three set of covariates: (1) clinical covariates, (2) metabolic measures, (3) the combination of clinical covariates and metabolic measures. We studied marginal associations between distinct metabolic measures and BP using partial dependency plots using pdp-package (Greenwell, 2017).

# 5 Results

## 5.1 Literature review for gut microbiota (I)

The systematic literature search for original research studies reporting results between gut microbiota and essential hypertension was performed on 4 September 2020 without language and publication date restrictions. The search utilized three medical databases and it identified 669 potential original research articles (Figure 15). In initial screening, we excluded 180 duplicate search results, 264 results based on title and 132 results based on abstract.

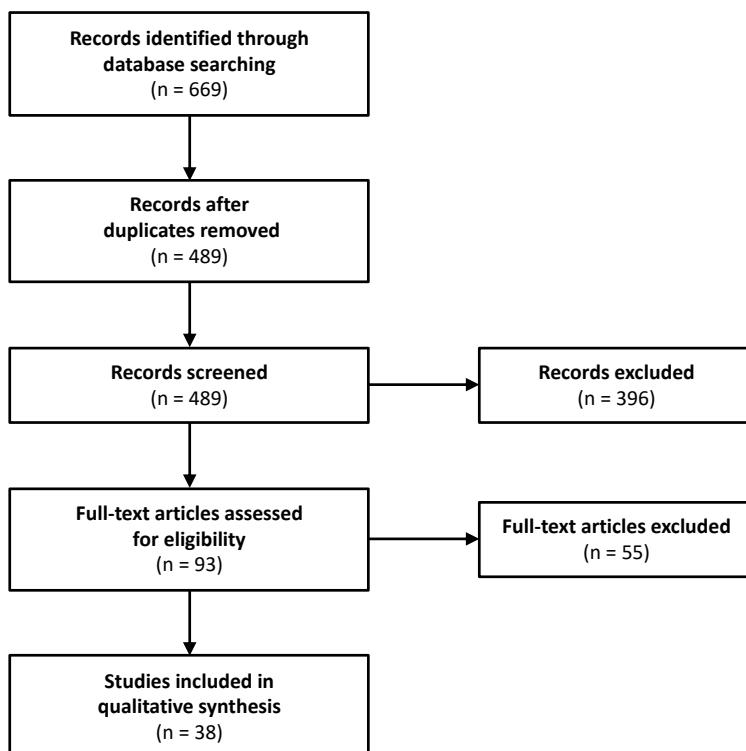


Figure 15. Flow chart for the original research articles identified the systematic literature review

In total, 93 manuscripts were assessed for eligibility in full-text screening. Four manuscripts were excluded as review articles. While reporting results, 20 manuscripts did not perform gut microbiota sequencing and 27 manuscripts performed interventions outside of the scope of our systematic literature review. The 38 original research articles included in our systematic literature review reported results for 22 animal studies, 13 small-scale human studies, four large-scale human studies, and one interventional pilot study in humans.

The animal studies contained comprehensive set of interventional research designs aimed to study the association between BP and gut microbiota in rodents. We summarized the animal studies in table identifying the studied animals, sequencing method, intervention type, the positive and negative associations the intervention had with circulating biomarkers, vasculature, organs significant in BP regulation, and the positive and negative associations the intervention had with gut microbiota and fecal SCFAs (Study I, Table 1).

In human studies, except one interventional pilot study, all original research articles reported cross-sectional associations between BP and hypertension. We summarized the human studies in the table identifying the study population, gut microbiota sequencing methods, the taxa with positive association with BP, the taxa with negative association with BP, the associations SCFAs had with hypertension, and the association between taxa and dietary salt (Study I, Table 2).

In summary, animal studies have suggested that gut microbiota and hypertension are linked. However, the evidence from human studies is still incomplete as only four large-scale human studies have been published to date. Future studies could be improved by 1) using more accurate methods of BP measurement, 2) performing deep metagenomic sequencing, and 3) utilizing interventional designs.

## 5.2 Gut microbiota and blood pressure (II)

The main sample of Study II included 6953 participants (mean age  $49.2 \pm 12.9$  years, 54.9% women) and 24-hour urinary sodium subsample 829 participants (mean age  $47.2 \pm 10.9$  years, 55.5% women). We observed 91 common microbial genera (4.7% of all available genera) and 134 *Lactobacillus* species.

### 5.2.1 Microbial diversity and BP

In models adjusted for age and sex, a standard deviation increase in microbiota alpha diversity was inversely associated with systolic blood pressure (effect size -0.54 mmHg; 95% confidence interval [CI], -0.96 to -0.12;  $P = 0.012$ ), diastolic blood pressure (-0.31 mmHg; 95% CI, -0.56 to -0.06;  $P = 0.016$ ), and hypertension (odds

ratio [OR] 0.91; 95% CI, 0.86 to 0.96;  $P<0.001$ ). However, alpha diversity was not significantly associated with BP in the fully adjusted models (Study II, Figure 1).

Consistently, we observed associations between beta diversity and BP indices in age- and sex-adjusted models ( $P\leq0.04$  for all). The coefficients of determination ( $R^2$ ) for the BP indices and beta diversity varied between 0.0002 and 0.0006. In multivariable-adjusted models, only diastolic blood pressure ( $R^2 = 0.0002$ ,  $P = 0.032$ ) was significantly related to beta diversity (Study II, Figure 1). The results for all diastolic BP model covariates are presented in Table 6. Due to the implementation of the PERMANOVA method, the covariate order affects the observed  $R^2$  favoring the first introduced covariates. The first three PCoA axes explained 31.3% of the variation in bacterial abundances (Study II, Figure 2).

Table 6. The proportion of gut microbial beta diversity explained by diastolic BP and model covariates.

<b>Model covariates</b>	<b>Age and sex adjusted model</b>		<b>Multivariable-adjusted model</b>	
	<b><math>R^2</math></b>	<b><math>P</math></b>	<b><math>R^2</math></b>	<b><math>P</math></b>
Age at baseline	0.49%	0.001	0.49%	0.001
Women	0.45%	0.001	0.45%	0.001
BMI			0.29%	0.001
Smoker			0.23%	0.001
Exercise			0.10%	0.001
Diabetes			0.06%	0.001
Diuretic			0.04%	0.003
Beta blocker			0.03%	0.002
Calcium channel blocker			0.02%	0.151
Agents acting on the RAS			0.03%	0.019
Diastolic BP	0.05%	0.001	0.02%	0.032
Residuals	99.00%		98.24%	

Multivariable-adjusted model was adjusted for age, sex, BMI, smoking, exercise, diuretics, beta blockers, calcium channel blockers, and renin-angiotensin system blockers. Analysis of variance for beta diversity was calculated using 999 permutations. BP, blood pressure; RAS, renin-angiotensin system;  $R^2$ , proportion of variation.

### 5.2.2 Common microbial genera and BP

We observed 122 significant associations between 45 distinct gut microbial genera and BP in fully adjusted model with FDR-corrected  $P < 0.05$  (Study II, Figure 3). A subset of genera that are significantly associated with both systolic BP and hypertension is presented in Table 7. We studied the number of significant associations for hypertension when third BMI was introduced to the age- and sex-adjusted model and observed that the number of significant associations was reduced from 39 to 23 (59%).

Table 7. Bacterial genera associated with both hypertension and systolic BP

	Systolic BP		Hypertension	
	Log2FC ± SE	P	Log2FC ± SE	P
<i>Anaerostipes</i>	0.06 ± 0.02	0.015	0.16 ± 0.04	<0.001
<i>Anaerotruncus</i>	-0.05 ± 0.01	<0.001	-0.06 ± 0.02	0.037
<i>Blautia</i>	0.04 ± 0.01	0.015	0.11 ± 0.03	<0.001
<i>Coprobacillus</i>	-0.12 ± 0.02	<0.001	-0.20 ± 0.04	<0.001
<i>Coprococcus</i>	0.05 ± 0.01	0.004	0.10 ± 0.03	0.012
<i>Dielma</i>	0.23 ± 0.03	<0.001	0.28 ± 0.07	0.001
<i>Enterobacter</i>	0.25 ± 0.04	<0.001	0.89 ± 0.09	<0.001
<i>Fournierella</i>	-0.02 ± 0.01	0.022	-0.07 ± 0.02	0.002
<i>Holdemania</i>	0.10 ± 0.02	<0.001	0.20 ± 0.04	<0.001
<i>Megasphaera</i>	0.19 ± 0.03	<0.001	0.23 ± 0.07	0.008
<i>Phascolarctobacterium</i>	0.10 ± 0.03	0.011	0.22 ± 0.07	0.007
<i>Ruthenibacterium</i>	0.07 ± 0.02	0.004	0.12 ± 0.05	0.034

Models are adjusted for age, sex, BMI, smoking, exercise, diuretics, beta blockers, calcium channel blockers, and renin–angiotensin system blockers. Log2FC, fold change in logarithmic scale to base 2; SE, standard error.

### 5.2.3 *Lactobacillus* species and BP

We observed 41 significant associations between 19 distinct *Lactobacillus* species and BP indices in the fully adjusted models with FDR-corrected  $P < 0.05$  (Study II, Figure 3). Of the 41 observed associations, 12 had positive and 29 negative association with BP indices. A subset of *Lactobacillus* species also associated with systolic BP is presented in Table 8. Notably, *Lactobacillus* genus was not significantly associated with BP in the fully adjusted models which could be explained by the presence of both positive and negative associations observed in

species-level analyses. In the age- and sex-adjusted model and in most three covariate models, *Lactobacillus* genus had a negative association with hypertension.

Table 8. *Lactobacillus* species associated with systolic BP

	Systolic BP		Hypertension	
	Log2FC ± SE	P	Log2FC ± SE	P
<i>L. aviarius</i>	-0.13 ± 0.03	0.001		
<i>L. farciminis</i>	-0.33 ± 0.05	<0.001	-0.60 ± 0.10	<0.001
<i>L. hominis</i>	-0.26 ± 0.04	<0.001	-0.42 ± 0.09	<0.001
<i>L. iners</i>	-0.18 ± 0.05	0.013	-0.45 ± 0.11	0.001
<i>L. kalixensis</i>	0.23 ± 0.07	0.013		
<i>L. kefiranofaciens</i>	0.20 ± 0.06	0.025		
<i>L. paracasei</i>	-0.15 ± 0.04	0.020		
<i>L. sakei</i>	0.15 ± 0.04	0.009		

Models are adjusted for age, sex, BMI, smoking, exercise, diuretics, beta blockers, calcium channel blockers, and renin–angiotensin system blockers. BP, blood pressure. Log2FC, fold change in logarithmic scale to base 2; SE, standard error.

### 5.2.4 *Lactobacillus* species, dietary salt, and BP

In 24-h urinary sodium subsample (N = 829), the mean sodium excretion was 142.3 ± 62.9 mmol. We observed 15.5% *Lactobacillus* prevalence at the detection limit of 0.1% relative abundance. 24-hour urinary sodium excretion was not associated with *Lactobacillus* genus. We, however, observed two significant associations between 24-hour urinary sodium excretion and species level *Lactobacillus* abundances (Study II, Figure 4). *L. paracasei* had a negative (Log2FC -0.018 ± 0.002, P<0.001) and *L. salivarius* (Log2FC 0.007 ± 0.002, P = 0.004) a positive association with urinary sodium excretion.

### 5.2.5 Functional analysis of gut microbiota

We observed 481 associations between KO groups and systolic BP with FDR-corrected P<0.05. The most prominent observed pathways were related to lipid metabolism, gluconeogenesis, and xenobiotic metabolism (Study II, Figure S3). The KO groups provide information about potential metabolic pathways available for the observed gut microbiota; the information about gut microbial gene expression and the presence of different metabolic end products could be measured using transcriptomics and metabolomics methods.

## 5.3 Eicosanoids and BP (III)

The main discovery sample of Study III included 8099 participants (FINRISK 2002, mean age  $48.0 \pm 13.1$  years, 53.1% women) and replication sample 2859 participants (FHS, mean age  $66.3 \pm 8.9$  years, 54.7% women). We observed 545 eicosanoids and related oxylipin mediators using a high-throughput directed non-targeted LC–MS approach (Watrous et al., 2019). Oxylipins are collection of oxygenated lipids and eicosanoids are an important subgroup of oxylipins in mammals (Noverr et al., 2003); we use in the following paragraphs the term eicosanoids while potentially referring also to other closely related non-eicosanoid PUFAs and PUFA derivates.

### 5.3.1 Association between eicosanoids and BP

We used systolic BP as our main outcome variable due to its well-established association with cardiovascular diseases and linear correlation with age. We observed 187 (34.3%) significant associations between distinct eicosanoids and systolic BP with  $P < 0.05/545$  (Study III, Figure 1). The majority of the observed features were positively ( $N = 175$ , 93.6%) associated with systolic BP. Spearman's rank correlations revealed strong overall correlations between eicosanoids but only minor clustering when ordered using hierarchical cluster analysis with complete linkage method (Study III, Figure 2).

### 5.3.2 Defining an eicosanoid risk score

We used a fully adjusted forward selection linear regression model with a Bonferroni-corrected inclusion threshold  $P < 0.05/545$  to find multivariable association between set of eicosanoids and systolic BP in the discovery cohort. The model resulted to six eicosanoids that were identified using reference standards and online databases (**Table 9**). We rounded the effect sizes to two decimals and defined eicosanoid risk score using the formula  $0.88 \cdot X_1 + 0.91 \cdot X_2 + 1.00 \cdot X_3 + 1.32 \cdot X_4 + 1.35 \cdot X_5 + 0.83 \cdot X_6$ , where  $X_i$  refers to the eicosanoid at  $i$ :th row or the **Table 9**.

The spectroscopic profile of the six eicosanoids from discovery cohort were matched in replication cohort with four eicosanoids. Two of the eicosanoids, 11-dehydro-2,3-dinor-TXB<sub>2</sub> and 295.2279/4.89, could not be detected in the plasma samples of the replication cohort. While the metabolite identification in LC–MS is non-trivial, the matching of features between cohorts was robust. The abundances of the missing eicosanoids were treated zero valued in replication cohort giving rise to effective eicosanoid risk formula  $0.91 \cdot X_2 + 1.32 \cdot X_4 + 1.35 \cdot X_5 + 0.83 \cdot X_6$  (**Table 9**).

Feature selection regression model and single eicosanoid models in both discovery and replication cohorts gave consistent results. Remarkably, all captured associations had positive association with systolic BP.

Table 9. Results for feature selection model and corresponding association of features with systolic BP in FINRISK and FHS

	Feature selection	Single eicosanoid model in FINRISK	Single eicosanoid model in FHS
Metabolite	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)
11-dehydro-2,3-dinor-TXB2	0.88 (0.47–1.30)	1.60 (1.20–2.01)	
12-HHTrE	0.91 (0.52–1.30)	1.12 (0.74–1.50)	1.38 (0.77–1.98)
295.2279/4.89*	1.00 (0.60–1.39)	1.96 (1.58–2.34)	
5,6-EET	1.32 (0.81–1.83)	2.74 (2.37–3.11)	1.42 (0.82–2.02)
Adrenic Acid	1.35 (0.81–1.89)	2.77 (2.37–3.16)	1.26 (0.63–1.88)
Tetranor-12(R)-HETE	0.83 (0.44–1.23)	1.43 (1.05–1.81)	1.89 (1.28–2.49)

All models were adjusted for age, sex, BMI, current smoking, diabetes mellitus, antihypertensive medication, and batch. Asterisk (\*) denotes putative eicosanoid. FHS, Framingham Heart Study; HHTrE, hydroxyhepta-decatrienoic acid; TXB2, thromboxane B2; HETE, hexadecatrienoic acid.

### 5.3.3 Eicosanoid risk score and systolic BP

Individuals at the highest quartile of risk score in discovery cohort had 9.0 mmHg (95% CI 8.0–10.1 mmHg) and in replication cohort 6.8 mmHg (95% CI 5.1–8.5 mmHg) higher systolic BP compared to individuals in the lowest quartile (Study III, Figure 4). A 1-SD increase in risk score was associated with 3.6 mmHg (95% CI 3.2–3.9,  $P<0.001$ ) and 2.2 mmHg (95% CI 1.6–2.8,  $P<0.001$ ) higher systolic BP in the discovery and replication cohorts, respectively.

### 5.3.4 Two-sample Mendelian randomization

In GWAS, 222 SNPs were associated with the eicosanoid risk score. 132 SNPs were located in chromosome 1 and 90 in chromosome 11. To account for nonrandom associations between alleles of different loci, the linkage-disequilibrium, only the SNP with lowest  $P$ -value was retained in each 10kb window (Table 10).

To increase statistical power, we used two-sample approach in Mendelian randomization where features are linked with SNPs in one sample and SNPs are linked with outcome in another sample. UK Biobank (Cardiff University, United Kingdom) reports association between GWAS results and systolic BP for 436419 individuals. However, two-sample Mendelian randomization between the three

SNPs in FINRISK 2002 and automated systolic BP measurement in UK Biobank did not produce significant results ( $P > 0.26$ ). Therefore, we were unable to provide evidence for the causality of the observed associations in the current study.

Table 10. The SNPs associated with eicosanoid risk score in genome-wide association study after adjusting for linkage-disequilibrium

SNP	Chromosome	Alleles	MAF	Effect size ± SE	P
rs72681939	1	A/G	13.6%	0.136 ± 0.025	3.5E-08
rs7523082	1	A/T	34.3%	0.116 ± 0.018	8.1E-11
rs174536	11	A/C	42.8%	-0.174 ± 0.017	2.6E-24

Models were adjusted for age, sex, batch. MAF, minor allele frequency; SE, standard error; SNP, Single-nucleotide polymorphisms.

## 5.4 Biomarker profile of hypertension (IV)

The cross-sectional sample of Study IV included 36985 participants (FINRISK 1997–2012, Health 2000, FinHealth 2017; mean age  $50.5 \pm 14.2$  years, 53.1% women) and longitudinal sample 4197 participants (FINRISK 2007–DILGOM 2014, Health 2000–2011; mean age  $49.4 \pm 11.8$  years, 55.3% women). We included in our core analyses 53 circulating metabolite biomarkers measures using high-throughput NMR. We also studied in more detail 97 lipoprotein measures related lipoprotein particle subclasses.

### 5.4.1 Cross-sectional associations

In the conventional linear regression models of the cross-sectional sample, only two amino acids, histidine, and valine, of all 53 circulating biomarkers included in our analysis were not associated with BP (Study IV Figure 1). We also performed sex- (Study IV Figure S3) and median age-stratified (Study IV Figure S4) analyses. Acetate was negatively associated with hypertension in women only and acetooacetate positively associated with hypertension in men only. The significant association between total cholesterol, LDL cholesterol, esterified cholesterol, and HDL cholesterol with hypertension was not observed in older than median age participants. Men compared to women and younger participants compared to older participants had in some cases slightly larger effect sizes. Large and extremely large HDL fractions were negatively and medium and small HDL fractions positively associated with hypertension (Study IV Figure S5). The cross-sectional associations were highly consistent across study cohorts supporting data pooling (Figure 16).

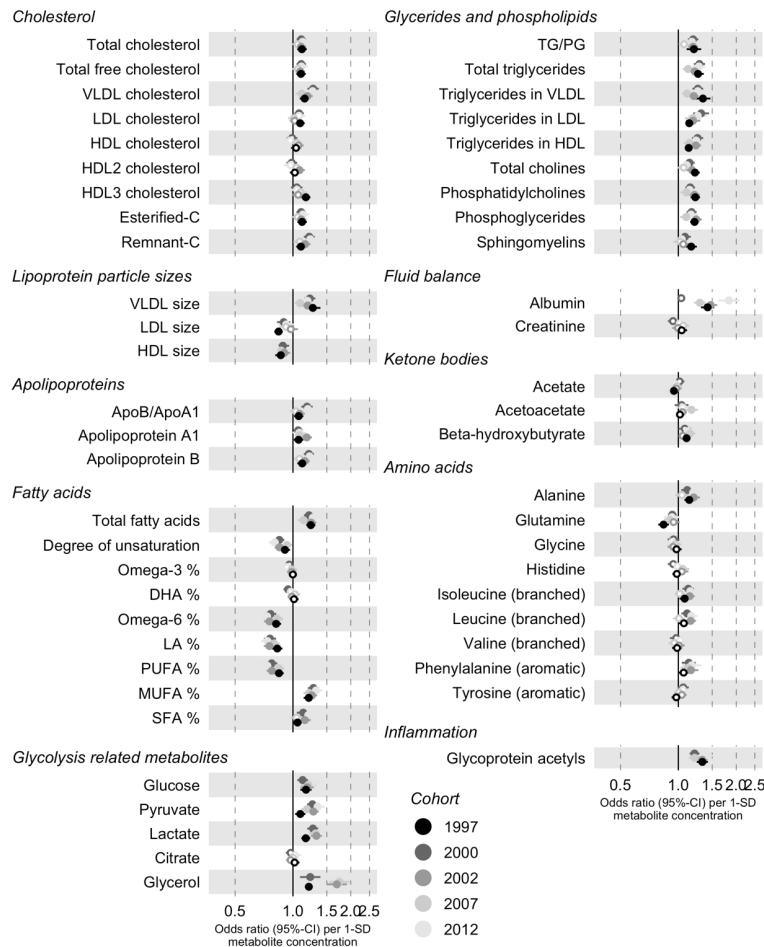


Figure 16. Cross-sectional associations between metabolic measures and hypertension performed separately in each study cohort\*

#### 5.4.2 Longitudinal associations

We studied the associations between baseline metabolic measures and systolic BP change between baseline and a follow-up of 7–11 years (Study IV, Figure 2). We observed that LDL cholesterol ( $\beta = 0.74$  mmHg per 1-SD metabolite concentration; 95% CI 0.28–1.20 mmHg;  $P = 0.01$ ), remnant cholesterol ( $\beta = 0.62$  mmHg; 95% CI 0.14–1.10 mmHg;  $P = 0.03$ ), apolipoprotein B ( $\beta = 0.63$  mmHg; 95% CI 0.14–1.11 mmHg;  $P = 0.03$ ), and acetate ( $\beta = 0.83$  mmHg; 95% CI 0.25–1.41 mmHg;  $P = 0.02$ ) were positively and average HDL particle size ( $\beta = -0.89$ ; 95% CI -1.46 to -0.32

\*Figure published under Creative Commons Attribution license (Palmu et al., 2021).

mmHg;  $P = 0.01$ ) negatively associated with systolic BP change between baseline and follow-up examinations. Large and extremely large HDL fractions were negatively and other lipoprotein fractions mostly positively associated with systolic BP change (Study IV, Figure S6).

### 5.4.3 Metabolic profile of hypertension

We used multivariable gradient boosting machine learning models to study the cross-sectional and longitudinal metabolic profile of hypertension. We compared the predictive accuracy between three set of model covariates: (1) clinical covariates, (2) metabolic measures, (3) the combination of clinical covariates and metabolic measures. We estimated the model performance in test models using FinHealth 2017 and FINRISK 2007–DILGOM 2014 cohorts that were not used in model training. In cross-sectional and longitudinal samples, information about clinical characteristic gave better model prediction than only using information about the 53 core metabolic measures (Table 11). However, the full model containing information about clinical characteristics and metabolic measures gave the best model estimate (Table 11).

In the cross-sectional model, glucose, albumin, and triglycerides in LDL had the highest importance scores with systolic BP (Study IV Figure 3). Glucose, albumin, and triglycerides in LDL had approximately positive linear relation with systolic BP in partial dependence plot (Study IV Figure 4).

In the longitudinal model, glycerol, average VLDL size, and acetoacetate had the highest importance scores with future systolic BP (Study IV Figure 3). Glycerol, average VLDL size, and acetoacetate had a stepwise association with future systolic BP each demonstrating additional peak from monotone relationship in partial dependence plot (Study IV Figure 4). However, following the general shape of the graphs, glycerol had positive association and average VLDL size and acetoacetate negative associations with future systolic BP.

Table 11. Root-mean-square error for multivariable gradient boosting model fit

Sample	Step	Clinical characteristics	Metabolic measures	Full model
Cross-sectional	Training	16.95 mmHg	16.36 mmHg	15.50 mmHg
Cross-sectional	Test	16.70 mmHg	18.03 mmHg	16.27 mmHg
Longitudinal	Training	14.84 mmHg	16.46 mmHg	13.22 mmHg
Longitudinal	Test	18.52 mmHg	19.78 mmHg	17.61 mmHg

Clinical characteristics were sex, BMI, current smoking, diabetes, antihypertensive medication, exercise, and lipid medication, and baseline SBP (only in longitudinal model). Metabolic measures were the core 53 circulating biomarkers. Full model was adjusted for both clinical covariates and metabolic measures.

# 6 Discussion

## 6.1 Gut microbiota and BP (I-II)

In Study I, we performed a systematic literature review on hypertension research related to gut microbiota. We reviewed 17 observational human studies and 22 animal studies, using pre-defined criteria for the included interventional designs. In Study II, we investigated the link between hypertension, gut microbiota, and dietary salt in a cross-sectional human cohort using shallow shotgun sequencing while adjusting for relevant confounding factors. The major improvements to previous research were the objectively measured BP, register based medication information, and large cohort size.

Our literature review included several prior animal studies that have reported how fecal microbiota transplantation and various interventions targeting dietary salt, antihypertensive medication, probiotics and SCFAs affect the gut microbiota and BP of the host. These animal studies have invoked hypotheses for the pathophysiological mechanisms explaining the effect of gut microbiota on hypertension and motivated the implementation of large-scale epidemiological human studies. In addition to our Study II, three other large cohort studies have reported associations between gut microbiota and human hypertension.

In the following paragraphs, we first summarize the main findings from the human cohort studies and then review in more detail the evidence for the association between BP with the overall gut microbial composition and distinct microbial abundances. In the TwinsUK study, no associations were observed between 68 microbiota markers and self-declared hypertension after correcting for multiple testing (Jackson et al., 2018). However, the low prevalence of self-reported hypertension (27.6%) in TwinsUK cohort compared to expected European prevalence (43.5%) and the lack of objective blood pressure measurements could explain these non-significant results (Jama et al., 2018). In the CARDIA study, a negative association was observed between objectively measured systolic blood pressure with alpha diversity and *Robinsoniella*-genus (Sun et al., 2019). In our study (FINRISK 2002; Study II), 45 microbial genera and 19 *Lactobacillus* species were

associated with objectively measured BP. In the 24-hour urinary sodium subsample of FINRISK 2002, *L. paracasei* was negatively and *L. salivarius* positively associated with both BP and urinary sodium, a proxy for dietary sodium intake (Study II). In the HELIUS study, gut microbiota explained 4.4% of the objectively measured systolic BP variance and 2.2% of the residual systolic BP adjusted with age, sex, and BMI (Verhaar, Collard, et al., 2020). *Roseburia*, *Clostridium*, *Romboutsia*, and *Ruminococcaceae* were the best negative predictors and *Streptococcus* the best positive predictor of systolic BP (Verhaar, Collard, et al., 2020).

### 6.1.1 Overall gut microbial composition and BP

#### 6.1.1.1 Alpha diversity

Three of the four previous studies report associations between alpha diversity and hypertension. In the CARDIA study, Shannon's diversity index was negatively associated with systolic BP ( $\beta = -1.33$  per 1-SD change, 95% CI -2.60 to -0.05,  $P = 0.04$ ) adjusted for age, sex, race, antihypertensive medication, BMI, education, dietary quality score, physical activity, smoking status, clinical field center, and sequence run (Sun et al., 2019). In the FINRISK 2002 study, Shannon's index was negatively associated with systolic BP adjusted for age and sex, but the significance was lost when adjusting additionally for BMI, smoking, exercise, diuretics, beta blockers, calcium channel blockers, and renin–angiotensin system blockers (Study II). In the HELIUS study, Spearman's correlation between Shannon's diversity index and systolic BP was -0.1 with  $P < 0.01$  (Verhaar, Collard, et al., 2020). Therefore, alpha diversity correlated negatively with BP in the three previous studies and, assuming normal distribution and using 68–95–99.7 rule, the difference between high or low alpha diversity would be 2–5 mmHg at the population level. Based on the results of all these studies, the lack of the significant results in the fully adjusted models may indicate that alpha diversity does not have independent role in the association with BP or that the effect size for the association is small.

#### 6.1.1.2 Beta diversity

Of the four cohort studies, only the FINRISK 2002 study reported results for the association between gut microbial beta diversity and hypertension (Study II). The used PERMANOVA model is sensitive to the order of studied covariates, favoring previously introduced covariates. Therefore, the BP variables were included in the models last. Beta diversity explained 0.05% of the variance of diastolic BP in the age- and sex-adjusted model and 0.02% in the multivariable-adjusted model. Also,

other relevant clinical covariates including age, sex, and BMI also had  $R^2$  of <0.5% with beta diversity. In particular, beta diversity explained 0.06% of the variance of diabetes, whereas diabetes and metformin use has been associated with changes in gut microbiota (Forslund et al., 2015). In summary, beta diversity had only minor  $R^2$  with all clinical covariates and the  $R^2$  for diastolic BP appeared to be one order of magnitude lower than those observed for age, sex, and BMI. Also, only significant association in fully adjusted models was observed with diastolic BP.

#### 6.1.1.3 Multivariable gradient boosting model

Instead of ecological diversity measures, the general gut microbial profile can also be studied using multivariable gradient boosting machine learning models. The HELIUS study was the only study that employed such modeling to estimate the proportion of variance gut microbiota explains about BP (Verhaar, Collard, et al., 2020). The authors defined an *ad hoc* formula for the proportion of variance defined as  $R^2 = 1 - \text{Var}(y - \hat{y}) / \text{Var}(y)$ , where  $y$  is the measured and  $\hat{y}$  the estimated BP. To adjust the analyses for the main clinical covariates, the authors also studied residual BP obtained after fitting a linear regression model for BP with age, sex, and BMI. The characteristics and main results of the HELIUS study are presented in Table 12.

Table 12. Overall population and ethnic subsamples of HELIUS study

Ethnicity	N	Age	Women	BMI	HT	HTX	SBP	Res SBP	DBP	Res DBP
Overall	4672	50 ± 12	52%	27 ± 5	42%	22%	4%	2%	4%	2%
Dutch	1328	51 ± 13	48%	25 ± 4	34%	16%	5%	0.6%	0.4%	n.a.
South Asian Surinamese	575	52 ± 11	52%	27 ± 5	49%	30%	n.a.	0.6%	n.a.	0.1%
African Surinamese	1128	52 ± 11	60%	28 ± 5	53%	31%	n.a.	0.7%	n.a.	0.1%
Ghanaian	462	48 ± 9	55%	28 ± 5	59%	29%	n.a.	n.a.	n.a.	n.a.
Moroccan	605	46 ± 11	46%	28 ± 5	24%	8%	0.8%	0.4%	n.a.	0.6%
Turkish	436	44 ± 11	51%	29 ± 5	29%	15%	n.a.	n.a.	0.5%	0.6%

BMI, body mass index; HT, hypertension; HTX, antihypertensive medication; n.a., model lacked predictive power; Res, residual variance adjusted for age and sex.

While age, sex, and BMI had consistent characteristics between ethnicities in the HELIUS study, notable differences were observed in hypertension (24–59%),

antihypertensive medications (8–31%), diabetes (5–24%), and antidiabetic medication (2–20%). The observed associations for unadjusted systolic and diastolic BP in subgroup analyses were highly variable. However, the  $R^2$  with BP residuals appeared more consistent, varying 0.4–0.7% for residual systolic BP and 0.1–0.6% for residual diastolic BP.

The large number of negative values (marked n.a. in Table 12) observed for  $R^2$  in the HELIUS study indicates that the *ad hoc* formula used may not be a generally suitable definition for the  $R^2$ . The study also adjusted for clinical covariates using residual BP as opposed to including the covariates in the model; furthermore, the study did not include all relevant confounders of elevated BP. Although not ideal, this remains a sensible definition for  $R^2$  in gradient boosting until a more robust definition is formulated. Although estimators such as RMSE or c-statistic allow comparing different models, they do not provide information about the clinical significance of the differences found. Even considering the limitations, the HELIUS study provided a reasonable estimation for the link between overall gut microbiota and BP.

### 6.1.2 Associations for distinct genera and species

Gut microbiota can be studied at different levels of taxonomic ranks (domain > kingdom > phylum > class > order > family > genus > species). In addition to the overall gut microbial composition, associations between distinct taxa with BP can be studied using metagenomics libraries, including DESeq2.

In the CARDIA and HELIUS studies, a negative association was reported between *Robinsoniella*, *Roseburia*, *Clostridium*, *Romboutsia*, and *Ruminococcaceae* and systolic BP (Sun et al., 2019; Verhaar, Collard, et al., 2020). *Robinsoniella* and *Romboutsia* were not part of the common microbial genera in FINRISK 2002 (Study II). *Clostridium*, *Roseburia*, and *Ruminococcus* were also detected in FINRISK 2002, but these genera were not associated with BP. Notably, CARDIA and HELIUS used 16S rRNA sequencing, and therefore reported results for distinct OTUs rather than particular genera (as in shotgun metagenomics), which partially explains why these associations were not observed in FINRISK 2002.

In FINRISK 2002, we observed 45 distinct microbial genera associated with BP indices. A total of 27 of these 45 genera belong to the *Firmicutes*, a phylum that has been previously associated with obesity, diabetes, and chronic kidney disease (Tang et al., 2017). More specifically, BP was associated with a large number of SCFA producing bacteria (Table 3). *Anaerostipes*, *Bacteroides*, *Blautia*, *Coprococcus*, *Dialister*, and *Phascolarctobacterium* were positively and *Prevotella* negatively associated with BP. The genus *Lactobacillus* has been linked with acetate production, and we observed 12 positive and 29 negative associations for 19 distinct

*Lactobacillus* species and BP. In particular, *L. paracasei* was negatively associated with both systolic BP ( $P = 0.02$ ) and urinary sodium excretion ( $P < 0.001$ ), and *L. salivarius* was positively associated with both pulse pressure ( $P < 0.001$ ) and urinary sodium excretion ( $P = 0.004$ ).

In a subsample (N = 200) of the HELIUS study, fecal acetate and propionate were positively associated with systolic BP (Verhaar et al., 2020). In our Study IV, circulating acetate was negatively associated with systolic BP and hypertension, and positively associated with diastolic BP and the systolic BP change in follow-up.

### 6.1.3 Summary for gut microbiota and BP

Multiple epidemiological study designs have demonstrated consistently that different characteristics of gut microbiota are associated with BP. While the ecological diversity may be too generally defined to capture practical information for epidemiological research, the multivariable gradient boosting method was able to give a reasonable estimate for the magnitude of the effect. Additionally, distinct gut microbial taxa have been both positively and negatively associated with BP. Some of the bacteria associated with BP have also been linked to dietary sodium and SCFA production. However, different studies have mostly found associations for disjoint sets of gut microbial taxa, potentially due to the differences in sequencing techniques used. The low number of studies combined with potential ethnic and geographic differences in microbial taxa may also contribute to the disparity.

## 6.2 The circulating metabolites and BP (III-IV)

In Study III, we investigated the association BP had with 545 low-abundance plasma eicosanoids and related oxylipins, family of metabolites that have been previously linked to numerous pathophysiological processes that are central to BP regulation (see section 2.2.3.3). Additionally, an eicosanoid risk formula defined in the discovery cohort (FINRISK 2002) was associated with BP in the replication cohort (FHS). In Study IV, we examined the link between BP and high-abundance serum metabolic measures. Our multivariable gradient boosting modeling revealed that baseline serum lipid, and particularly LDL-derived and VLDL-derived cholesterol measures, and glucose metabolism abnormalities were associated with follow-up BP. The information about baseline metabolic measures improved the prediction of follow-up BP compared to a model that used only common clinical covariates.

### 6.2.1 Human lipidome

In cross-sectional analyses, most PUFAs were negative associated with BP (Study IV). Our results were consistent with previously published large cohort studies, including Women's Health Study and Brisighella Heart Study, and provided new information about cross-sectional and longitudinal multivariable associations between BP and lipids in large, well-phenotyped cohorts (Paynter et al., 2011; Cicero et al., 2014).

Our Study III is the first publication that comprehensively examines the association between eicosanoids and BP in humans using high-throughput LC–MS. Although prior studies have been limited by the number of metabolites studied, they have been able to demonstrate that a small set of eicosanoids are associated with renal function, vascular tone, and hypertension (Laffer et al., 2003; Minuz et al., 2008; Taddei et al., 2006; Ward et al., 2005, 2008).

Our eicosanoid risk score included both intermediate and potentially terminal metabolites. 11-dehydro-2,3-dinor-TXB<sub>2</sub> can be measured from urine indicating that the eicosanoid is a terminal metabolite (DeFilippis et al., 2013). In a small human study, individuals with prior myocardial infarction compared to healthy controls demonstrated differences in TXA<sub>2</sub> production assessed by measuring urine 11-dehydro-2,3-dinor-TXB<sub>2</sub> (DeFilippis et al., 2013). 12-HHTrE is a non-enzymatic degradation product of TXA<sub>2</sub> and PGH<sub>2</sub> (Maddipati et al., 2014). 12-HHTrE has been linked to PGI<sub>2</sub> synthesis, and the primary downstream metabolite of 12-HHTrE, 12-oxoheptadeca-5(Z)-8(E)-10(E)-trienoic acid, is an antagonist of TXA<sub>2</sub> receptors (Csanyi et al., 2007). Therefore, 12-HHTrE related pathways could potentially increase PGI<sub>2</sub> modulated vasodilation (Csanyi et al., 2007). Adrenic acid is a polyunsaturated 22-carbon fatty acid which serves as a substrate for eicosanoid production, and adrenic acid-derived metabolites have been linked to modulate adrenal cortical artery relaxation (Kopf et al., 2010).

Our results demonstrate that eicosanoids are strongly associated with BP: both positive and negative associations were observed, and we were able to replicate the main findings using an independent cohort. However, laborious analyte identification forced us to focus more detailed analyses in small subset of eicosanoids. High correlation between analytes unavoidably introduces arbitrariness in metabolite selection. In the future, improved methodology could allow general analyte identification and utilization of metabolic databases to find novel pathways between eicosanoids and BP even using the current data.

### 6.2.2 Amino acids

In our cross-sectional sample, we observed a positive association for leucine, isoleucine, and alanine and a negative association for glutamine and glycine with BP

(Study IV). Leucine and isoleucine belong to branched-chain amino acids, a group of essential amino acids that may have a role in the cell signaling of impaired insulin action and aggravated oxidative stress (Z.-Y. Zhang et al., 2018). In our longitudinal multivariable model, glycine, leucine, phenylalanine, and histidine were among the top 15 model covariates (Study IV). Repeated measurements of circulating amino acid levels, finer dietary information, or both may be required to accurately assess the role of amino acids in cardiovascular health and BP.

### 6.2.3 Energy metabolism-related measures

Insulin resistance, diabetes, hypertension, and CAD are related comorbidities (Hall et al., 2015). We observed positive cross-sectional associations for glucose, pyruvate, lactate, glycerol, acetoacetate, and beta-hydroxybutyrate with hypertension (Study IV). These metabolites were also among the top 15 covariates in multivariable models. Therefore, our results highlight the importance of energy metabolism abnormalities in the development of hypertension.

### 6.2.4 Fluid balance-related measures

We observed a positive cross-sectional association between albumin and BP. Albumin was also among the top covariates in our cross-sectional multivariable model. A partial dependence plot revealed a positive linear relationship between albumin and systolic BP. In the cross-sectional Oslo Health Study ( $N = 5,171$ ) and the Neuroprotective Model for Healthy Longevity among the Malaysian Elderly study ( $N = 2,322$ ), albumin was positively associated with BP (Eshkoor et al., 2016; Høstmark et al., 2005). However, a normotensive Japanese ( $N = 2,240$ ) observed a negative longitudinal association between albumin and hypertension onset. However, a normotensive Japanese ( $N = 2,240$ ) observed negative longitudinal association between albumin and hypertension onset (Oda, 2014). Therefore, the association between albumin and BP may be multifaceted or non-causal.

### 6.2.5 Inflammation markers

Numerous studies have reported associations between markers of inflammatory activity such as cytokines and acute phase reactants (Harrison et al., 2011; Barrows et al., 2019). Downstream acute phase reactants have been favored in clinical research, because they are comparatively stable relative to propagators of inflammation cascade, such as IL-6 with a circulating half-life  $<2$  h (Danesh et al., 2008). We observed positive cross-sectional association between BP and a novel low-grade inflammation biomarker, glycoprotein acetyl (Study IV). Eicosanoids

have been reported to modulate inflammatory- and anti-inflammatory responses (Dennis & Norris, 2015; Harizi et al., 2008). We observed that eicosanoids and related oxylipins demonstrated strong overall association with BP (Study III).

## 6.3 Limitations of the study

### 6.3.1 Study I

Our literature review was performed according to an established protocol and a university library informatician was consulted with the search terms. However, our results must be interpreted in the context of their limitations. First, we performed single author screening of the included manuscripts. Second, the choice to focus on specific intervention types was artificial. Third, we did not provide numerical summary statistics. These limitations could potentially result in false exclusion of valid publications and mistakes in summary tables.

### 6.3.2 Study II

Our study was able to improve prior study designs using objective BP measurement, register based information about medication use, and shotgun metagenomics. However, some limitations remain. First, fecal sampling is proxy for the gut microbiota. Second, the samples were stored for prolonged time before analysis. Third, shotgun metagenomics has method specific limitations (Thomas & Segata, 2019). Fourth, our 24-hour urine sodium subsample had limited size (11.9%) and urine collection offers only limited information about dietary habits (Rakova et al., 2017). Fifth, hypertensive individuals may have received guidance to limit their dietary sodium intake. Sixth, different analysis methods and the use of different databases can produce heterogenous results (Nearing et al., 2021). Microbes observed in stool do not accurately represent all sites of the gastrointestinal tract and, in particular, the microbes in small intestine and mucosal layer of colon may not be detected in accurate proportions in stool. The methodological limitations reduce reproducibility between different metabolomic studies and could induce both type I and type II errors in results. Limited size of the 24-hour urinary sodium subsample reduces the power of the statistical analysis. Lifestyle guidance provided to hypertensive individuals produces confounding bias to the statistical models that can potentially reduce the observed effect sizes.

### 6.3.3 Study III

Our study has several strengths, including a large, unselected population sample, external replication of our results, and assays of a large number of eicosanoids. However, our study also has its limitations. First, LC–MS is highly sensitive, and we were unable to find all the metabolites observed in discovery cohort in replication cohort. Second, metabolite identification was laborious and therefore performed only for six metabolites. Third, many eicosanoids have short half-lives imposing chemical instability in LC–MS analysis. Fourth, not all identified metabolites were eicosanoids. These limitations reduce the reproducibility of results between different studies and make difficult to utilize human metabolome databases to find potential functional pathways behind the observed associations.

### 6.3.4 Study IV

Our study has several strengths, including large cross-sectional and moderate longitudinal population sample sizes, access to repeated measurements, and consistent biomarker quantification. However, several limitations exist. First, NMR provided only limited window to serum high abundance metabolic measures. Second, our longitudinal sample size may have been insufficient to capture all potential associations. Third, baseline examinations ranged over a period of 15 years imposing differences in freezing times between studies. Fourth, the effect sizes observed were modest. These methodological limitations reduce the potential to capture novel biomarkers that are associated with BP and the limited prospective data reduces the power of performed statistical analyses.

## 7 Summary

The overall motivation for this study was to integrate modern metabolomics and metagenomics into hypertension research using the large and well-phenotyped Finnish cohort studies. The specific aims were to 1) estimate the relation between the gut microbiota and hypertension, 2) study the metabolic profile of hypertension, and to 3) establish if a family of small molecule activators and suppressors of systemic inflammation, eicosanoids, are associated with BP.

The four large human microbiota studies published to date demonstrate that gut microbiota is associated with human hypertension (Jackson et al., 2018; Sun et al., 2019; Verhaar, Collard, et al., 2020). The results of Study II, together with findings from prior studies, also suggest that dietary sodium may affect the gut microbial species, depleting potentially beneficial species. As our study is mainly observational, we can only speculate on the underlying causes of these findings. First, gut microbial end products may influence the gastrointestinal permeability and induce inflammation in the gut wall. Second, some metabolites, including SCFAs, have the potential to be absorbed in circulation where they can interact with host receptors. Third, gut microbiota may also modulate the sympathetic response of the host. However, the true significance of these effects on population level BP and health remains unclear. Third-generation sequencing, using a multiomics approach, and having access to additional longitudinal data could enable future epidemiological studies to better answer this question. Additionally, our growing understanding of microbiota and BP enables us to better plan human interventions that investigate the effect of dietary challenges to gut microbiota, circulating metabolites, and BP.

Eicosanoids and related oxylipins demonstrate a strong association with BP (Study III). As eicosanoid compounds affect numerous physiological processes that are central to BP regulation, they may offer new insights about the pathogenesis of hypertension, as well as serve as potential targets for therapeutic intervention. The largest challenge to fully utilize these data, however, is the non-trivial metabolite identification. However, the growing databases of human metabolites could allow better metabolite identification in the future, which, in turn, may be used to improve risk prediction and find potential therapeutic targets.

We also used high abundance metabolic measures to identify a serum signature associated with BP and BP change in follow-up using conventional statistics and machine learning approaches (Study IV). Our results suggest that serum lipids, and particularly LDL-derived and VLDL-derived cholesterol measures, and glucose metabolism abnormalities are associated with hypertension onset. Use of serum metabolite determination could be used to identify individuals at high risk of developing hypertension in time range of 7–11 years.

Our studies improve the current knowledge of the associations of gut microbiota and circulating metabolites with BP. Metabolomics and metagenomics offer novel approaches to improve hypertension risk prediction and to discover potential targets for therapeutic intervention of elevated BP.

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## Original Publications

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I





Systematic Review

# Targeting Gut Microbiota to Treat Hypertension: A Systematic Review

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**Abstract:** While hypertension remains the leading modifiable risk factor for cardiovascular morbidity and mortality, the pathogenesis of essential hypertension remains only partially understood. Recently, microbial dysbiosis has been associated with multiple chronic diseases closely related to hypertension. In addition, multiple small-scale animal and human studies have provided promising results for the association between gut microbial dysbiosis and hypertension. Animal models and a small human pilot study, have demonstrated that high salt intake, a risk factor for both hypertension and cardiovascular disease, depletes certain *Lactobacillus* species while oral treatment of *Lactobacilli* prevented salt-sensitive hypertension. To date, four large cohort studies have reported modest associations between gut microbiota features and hypertension. In this systematic literature review, we examine the previously reported links between the gut microbiota and hypertension and what is known about the functional mechanisms behind this association.



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**Keywords:** blood pressure; dietary sodium; gut microbiota; hypertension; lactobacillus; salt intake

## 1. Introduction

High blood pressure (hypertension) remains the leading modifiable risk factor for cardiovascular morbidity and mortality [1]. As the prevalence of treatment resistant hypertension is approximately 15%, a need for novel therapeutic modalities for high blood pressure exists [2]. In addition to a shortage of treatment options, the pathogenesis of essential hypertension remains elusive [1,3]. In fact, while increased risk of hypertension has been associated with both adverse lifestyles and over 900 genetic loci, common genetic risk variants explain only <6% of the variance in systolic blood pressure (BP) [4].

Gut microbiota dysbiosis may represent a potentially modifiable risk factor for high blood pressure as it has recently been associated with multiple conditions (e.g., obesity, metabolic syndrome, diabetes, and cardiovascular disease) that are closely associated with hypertension [5]. However, the literature on the potential links between the gut microbiota and blood pressure has not been reviewed to date.

In this systematic literature review, we examine the relation of gut microbiota and hypertension. The specific review questions addressed are: (1) is gut microbiota associated with hypertension, (2) is hypertension associated with functional changes in the gut microbiota, (3) what is the effect size of such interactions, and (4) could gut microbiota modulation be used as a treatment modality for hypertension?

## 2. Materials & Methods

### 2.1. Search Strategy

We performed a systematic literature review for original research articles using Medical Literature Analysis and Retrieval System Online (MEDLINE), Excerpta Medica database

(EMBASE), and Cochrane Library. The literature search consisted the intersection of hypertension research (“blood pressure” or “hypertension” in title, author keywords, or Medical Subject Headings [MeSH] keywords) and microbiota studies (“Gastrointestinal Microbiome” MeSH keyword or words microbiota, microbiota or metagenomics in title or abstract). While the focus of the literature review was limited to gut microbiota, the latter search term was given a general form to reduce the false exclusion of relevant research. The literature search was performed on 4 September 2020 without language and publication date restrictions.

## 2.2. Study Selection, Data Extraction, and Data Analysis

Our systematic literature review followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [6]. The inclusion criteria were published original research articles that reported results between sequenced gut microbiota and essential hypertension. In particular, studies focusing on associations for diseases other than hypertension (e.g., renal disease, liver disease, or sleep apnea) were excluded. Studies on oral microbiota were also excluded, due to the expected differences in the environmental conditions between the oral cavity and gut. We also deemed the studies between the maternal microbiota and offspring hypertension to be outside the scope of our literature review. Finally, we included the articles with the following study interventions: stool transfer, orally administrated probiotics, sodium, antihypertensive medication, and short chain fatty acids (SCFAs), or genomic knockout models.

Duplicate search results were automatically detected using Digital Object Identifiers. Manual screening was performed by single author using first titles, second abstracts, and finally full-text. Included manuscripts were mutually non-exclusively grouped in animal models and human studies. In animal models, the effect of interventions to circulating metabolites, vasculature, gut wall, and gut content are presented in a summary table. In human studies, the associations observed between hypertension, high dietary salt, gut microbiota, and SCFAs are presented in a second summary table. When original research articles reported associations between a large number of different groups or between multiple interventions, the one most suited to answer to previously described attributes was selected. A preliminary literature review did not reveal compatible numerical properties in published gut microbiota-hypertension studies that could be combined to perform a meta-analysis. We used in the review two software tools implemented in R. Revtools [7] was used to conduct the article screening and PRISMAstatement [8] to draw the flow charts of study inclusions and exclusions.

## 3. Results

The literature search identified 669 potential original research articles. We excluded 180 duplicate search results, 264 manuscripts after title screening, and 132 manuscripts in abstract screening. In full-text screening, we excluded four incomplete clinical trials, four review articles, 20 manuscripts missing gut microbiome sequencing and 27 manuscripts due to the intervention type. Finally, we included 38 original research articles in our literature review (Figure 1). We summarize in the following section the 22 animal studies that examined the potential causal relation between gut dysbiosis and hypertension (Table 1). These promising results from animal models have given rise to 13 small studies, four large cross-sectional epidemiological studies and one interventional pilot study on the association between gut dysbiosis and human hypertension (Table 2).

**Table 1.** The effect of rodent interventions to circulating metabolites, vasculature, gut wall, and gut content.

Study	Animals	Sequencing	Intervention	Increased or Upregulated in Serum, Plasma, Vasculature, or Organs	Decreased or Downregulated in Serum, Plasma, Vasculature, or Organs	Increased or Upregulated in Gut	Decreased or Downregulated in Gut
Adnan [9]	WKY rats	16S rRNA (V4)	FMT from SHRSP		Erysipelotrichaceae, Dorea, Anaerostipes, Bacteroidales, Micrococcaceae, Ruminococcus, Deferribacteres, Deferribacteres, Mucispirillum, Deferribacteraceae, Deferribacteres, Lactococcus, Desulfovibrionales, Roseburia, Coprococcus, Lachnospiraceae, Clostridiales, Firmicutes		Bacteroidetes, Bacteroidia, Erysipelotrichi, Erysipelotrichales, Allobaculum, Actinobacteria, Bacteroidaceae, Bacteroides, Actinobacteria, Bifidobacteriales, Bifidobacterium, Enterobacteriales, Gammaproteobacteria, Enterobacteriaceae, Betaproteobacteria, Sutterella, Alcaligenaceae, Bacillales, Bacillaceae, Coprobacillus, Coriobacteriales, Coriobacteriia, Adlercreutzia, Holdemania, Enterococcus
Bier [10]	DSS rats	16S rRNA (V4)	4% NaCL			<i>Erwinia, Christensenellaceae, Corynebacteriaceae, acetate, propionate, isobutyrate</i> Increases in rest: Streptococcaceae, Veillonellaceae, Clostridiaceae (negative association with SBP), Helicobacteriaceae, Lactobacillus	<i>Anaerostipes</i>
Chakraborty [11]	DSS rats	16S rRNA (V3-V4)	Diurnal timing dependent alterations or disrupted circadian rhythm		Mannose, trans-4-hydroxyproline, xylose, N,N,N-trimethyl-5-aminovalerate (GO), indoleacetate, ferulic acid 4-sulfate, gentisate (GO), indoleacetylglucine (GO)	<i>Anaeroplasmataceae; 25 upregulated metabolites</i>	Decreases in rest: Sutterella (positive association with SBP), Erysipelotrichaceae
Cheema [12]	C57BL/6 and GF mice	16S rRNA (V3-V4)	Angiotensin II	4-ethylphenyl sulfate (GI), p-cresol sulfate (GI), p-cresol glucuronide (GI), spermidine		<i>Rikenellaceae</i>	<i>Lachnospiraceae; 71 downregulated metabolites (including N,N,N-trimethyl-5-aminovalerate, spermidine, indoleacetate, trans-4-hydroxyproline, xylose)</i>
Chen [13]	Sprague-Dawley rats	16S rRNA (V3-V4)	4% NaCl (20% fructose in water)	Renin, Ang-II			<i>Desulfovibrionaceae</i>
Ferguson [14]	C57Bl/6 mice	16S rRNA (V4)	8% NaCl			<i>Firmicutes, Prevotella, mesenteric arterial immune cells (CD45+, CD3+, CD4+, and CD8+ T cells)</i>	<i>Bacteroidetes, Leuconostoc, Streptococcaceae, Lachnospiraceae UCG-06, Lachnospiraceae FCS020</i>
Gómez-Guzmán [15]	SHR	qRT-PCR	K8 and LC9	Vascular relaxation induced by acetylcholine	Heart weight, ventricular weight and kidney weight relative to body weight Heart rate and increased coronary flow	Lactobacillus	Bacteroides, Clostridium
Kim [16]	C57B16 mice	16S rRNA (V3-V4)	Butyrate			<i>Akkermansia muciniphila; mRNA for occludin, ZO-1, and claudin 4; CCR2+CD4+IL-17+Th17 cells</i>	
Li [17] (2017)	GF C57BL/6L mice	16S rRNA (V4)	FMT from hypertensive human			<i>Coprobacillus, Prevotella</i>	<i>Anaerotruncus, Coprococcus, Ruminococcus, Clostridium, Roseburia, Blautia, Bifidobacterium</i>
Marques [18]	C57Bl/6 mice	16S rRNA (V3-V4)	Acetate		Cardiac fibrosis and left ventricular hypertrophy	<i>Bacteroides acidifaciens</i>	
Mell [19]	DSS rats	16S rRNA (V1-V3)	FMT from salt-resistant DSS	Acetate and heptanoate			
Robles-Vera [20] (2018)	Wistar rats	16S rRNA (V3-V4)	L-NAME	Aortic ring NADPH oxidase activity, TNF- $\alpha$ , IL-1 $\beta$ , and ROR $\gamma$		<i>Propionibacterium, propionate; Th17/Treg activity in mesenteric lymph nodes</i>	<i>Veillonellaceae</i> Parabacteroides, Bifidobacterium, Olivibacter, Dysgonomonas, Pedobacter, Flavobacterium, and Desulfovomaculum; expression of barrierforming junction transcripts in the colon
Robles-Vera [21] (2020)	SHR	16S rDNA (V4-V5)	Acetate	Vascular relaxation induced by acetylcholine	Left ventricle weight relative to body weight; (microbial) endotoxins	<i>zonula occludens-1, occludin, mucin-2, mucin-3, IL-18, Treg</i>	<i>Lactobacillus, Peptostreptococcaceae, Th17 levels of tight junctional proteins (occludin, tight junction protein 1, cingulin); Bifidobacterium (SHR)</i>
Santisteban [2]	Sprague Dawley rats (SHR)	16S rDNA (V4-V5)	Angiotensin II	FITC-dextran		<i>Fibrotic area and muscularis layer in small intestine; CD3+ T-cells, CD68+ macrophages, Iba1+ macrophages; Streptococcus (SHR)</i>	
Sharma [22]	SHR (Ang II)	16S rDNA (V4-V5)	Chemically modified tetracycline-3			<i>Ruminococcus and Oscillospira; number of goblet cells and villi length</i>	<i>Proteobacteria, Parabacteroides and Blautia; thickness of the muscularis layer and fibrotic area</i>

**Table 1.** Cont.

Study	Animals	Sequencing	Intervention	Increased or Upregulated in Serum, Plasma, Vasculature, or Organs	Decreased or Downregulated in Serum, Plasma, Vasculature, or Organs	Increased or Upregulated in Gut	Decreased or Downregulated in Gut
Toral [23] (2018)	C57BL/6J mice	16S rRNA	LC40			<i>Bifidobacterium</i> , ( <i>Lactobacillus fermentum</i> CECT5716)	<i>Anaerostipes</i> , <i>Hespellia</i> , <i>Prevotella</i>
Toral [24] (2019a)	WKY rats	16S rDNA (V4-V5)	FMT from SHR	lipopolysaccharides		Bacteroidia, Bacteroidales, Bacteroidetes, Odoribacter, Odoribacteraceae, Coprococcus, Turicibacteraceae, Turicibacterales, Turicibacter; TNF- $\alpha$ , tyrosine hydroxylase, and noradrenaline mRNA	<i>Blautia</i> , Enterococcaceae, Enterococcus; zonula occludens-1 and mucin-2 mRNA
Toral [25] (2019b)	WKY rats	16S rDNA (V4-V5)	FMT from SHR	Aortic NADPH oxidase, TNF- $\alpha$ , INF $\gamma$ , IL-17, ROR $\gamma$	Aortic relaxation induced by acetylcholine	CX3CR1, Itga4, and CCR9 in mesenteric lymph nodes Acidovorax, Aeromonadaceae, Bacteroides, Enterococcus, Methylophilaceae, Pseudomonas, Variovorax paradoxus; response to endothelium-dependent vasorelaxation (ACh)	
Waghulde [26]	DSS rats	16S rRNA (V1-V3)	Deletion of <i>Gper1</i>				Clostridiaceae, Fusobacterium, Lactobacillus, Pediococcus, Turicibacter
Wilck [27]	FVB/N mice	16S rDNA (V4-V5)	4% NaCl (1% in water)			<i>Parasutterella</i> ; CD4 $^+$ ROR $\gamma$ t $^+$ T <sub>H</sub> 17	<i>L. murinus</i> , <i>Lactobacillus</i> , <i>Oscillibacter</i> , <i>Pseudoflavonifractor</i> , <i>Clostridium XIVa</i> , <i>Johnsonella</i> , <i>Rothia</i> ; indole-3-lactic acid, indole-3-acetic acid
Wu [28]	SHR	16S rDNA	ATR blocker	LBP		<i>Lactobacillus</i> ; acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid; cingulin, occludin and tight junction protein 1	
Yan [29]	Wistar rats	16S rRNA (V3-V4)	8% NaCl (3% in water)	cortisone	aldosterone	corticosterone and enzymes of corticosterone synthesis (Cyp11a1, Cyp11b1, and Hsd11b1)	<i>B fragilis</i> YCH46; arachidonic acid in HSD; enzyme of cortisone inactivation (Hsd11b2)
Yang [30]	SHR	16S rRNA (V4-V5)	ACE inhibitor		Marker of gut permeability (I-FABP)	<i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Actinobacteria</i> ; number of goblet cells, villi length	<i>Bacteroidetes</i> ; fibrotic area
Zhang [31]	C57BL/6J mice	16S rRNA (V4-V5)	4% NaCl	Ang I, glucose, albumin, total proteins, sodium	LDL-C		<i>Lactobacillus</i>

ACE, angiotensin-converting enzyme; Ang, angiotensin; ATR, Angiotensin II type 1 receptor; BFM, *Bifidobacterium breve* CECT7263; DSS, Dahl salt-sensitive; GF, Germ free; GO, denotes metabolite product associated with microbial origin; GPR, G protein-coupled receptors; FMT, fecal microbiota transplant; HT, hypertension; K8, *Lactobacillus coryniformis* CECT5711; L-NAME, NG-nitro-L-arginine methyl ester (nitrogen oxide synthase blocker); LBP, lipopolysaccharide-binding protein. LC40, *Lactobacillus fermentum* CECT5716; LC9, *Lactobacillus gasseri*; LDL-C, low-density lipoprotein cholesterol; SHRSP, spontaneously hypertensive stroke prone rats; T<sub>H</sub>, helper T cell.

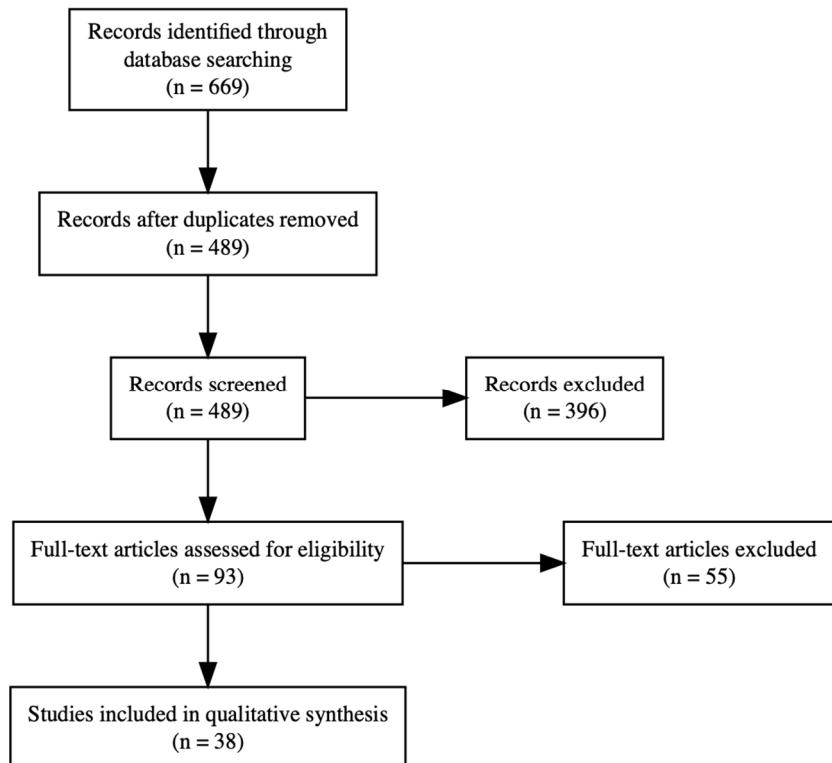
**Table 2.** The associations observed between human hypertension, high dietary salt, gut microbiota, and SCFAs.

Study	Population	Sequencing	Enriched in Hypertension or Positively Associated with BP Indices	Enriched in Normotension or Negatively Associated with BP Indices	SCFA in Hypertension	High Salt Diet
Calderón-Pérez [32]	29 non-treated HT and 32 NT subjects in Reus, Spain	16S rRNA	Bacteroides coproccola, Bacteroides plebeius; Lachnospiraceae	Ruminococcaceae NK4A214, Ruminococcaceae UCG-010, Christensenellaceae R-7, Faecalibacterium prausnitzii, Ruminococcaceae hominis	Decreased serum acetate, isobutyrate, burate and isovalerate; increased stool acetate, propionate, butyrate, valerate	
Dan [33]	67 HT and 62 NT subjects in Beijing, China	16S rRNA (V3-V4)	<i>Acetobacteroides</i> , <i>Alistipes</i> , <i>Bacteroides</i> , <i>Barnesiella</i> , <i>Butyrivimonas</i> , <i>Christensenella</i> , <i>Clostridium sensu stricto</i> , <i>Cosenzaea</i> , <i>Desulfovibrio</i> , <i>Dialister</i> , <i>Eisenbergiella</i> , <i>Faecalitalea</i> , <i>Megasphaera</i> , <i>Microvirgula</i> , <i>Mitsuokella</i> , <i>Parabacteroides</i> , <i>Proteiniborus</i> , <i>Terrisporobacter</i>	<i>Acetobacteroides</i> , <i>Acidaminobacter</i> , <i>Adlercreutzia</i> , <i>Anaerotruncus</i> , <i>Asteroplasma</i> , <i>Bulleidia</i> , <i>Cellulosilyticum</i> , <i>Clostridium III</i> , <i>Clostridium IV</i> , <i>Clostridium XIVa</i> , <i>Coprobacter</i> , <i>Enterococcus</i> , <i>Enterorhabdus</i> , <i>Flavonifractor</i> , <i>Gemmiger</i> , <i>Guggenheimella</i> , <i>Intestinimonas</i> , <i>Lachnospiraceae_incertae_sedis</i> , <i>Lactivibrio</i> , <i>Lactobacillus</i> , <i>Macilibacteroides</i> , <i>Marvinbryantia</i> , <i>Olsenella</i> , <i>Paraprevotella</i> , <i>Parasutterella</i> , <i>Phascolarctobacterium</i> , <i>Prevotella</i> , <i>Romboutsia</i> , <i>Ruminococcus</i> , <i>Sporobacter</i> , <i>Sporobacterium</i> , <i>Sutterella</i> , <i>Vampirovibrio</i> , <i>Veillonella</i> , <i>Victivallis</i>		

Table 2. Cont.

Study	Population	Sequencing	Enriched in Hypertension or Positively Associated with BP Indices	Enriched in Normotension or Negatively Associated with BP Indices	SCFA in Hypertension	High Salt Diet
de la Cuesta-Zuluaga [34]	441 subjects in Colombia	16S rRNA (V4)			Increased fecal acetate, propionate, and butyrate	
Ferguson [14]	39 subjects with normal and 93 with high sodium intake in USA	16S rRNA	<i>Prevotella</i>			Increase in Firmicutes, Proteobacteria, Prevotella, Ruminococcaceae, and Bacteroides
Han [35]	99 non-treated HT, 56 pre-HT and 41 NT subjects in Kailuan, China	Shotgun metagenomic	Streptococcus virus phiAbc2, <i>Salmonella</i> phage vB SemP Emek, <i>Mycobacterium</i> phage Toto	Cronobacter phage CR3, <i>Cnaphalocrocis medinalis</i> granulovirus		
Huart [36]	38 HT, 7 borderline HT, and 9 NT male subjects in Belgium	16S rDNA (V1-V3)				Increased stool acetate, butyrate, and propionate
Kim [16]	22 HT and 18 NT subjects in Florida, USA	Shotgun metagenomic	Parabacteroides johnsonii, Klebsiella, Anaerotruncus, <i>Eubacterium</i> siraeum, Alistipes indistinctus, <i>Prevotella</i> bivia, Ruminococcus torques, Alistipes finegoldii associated	Bacteroides thetaiotaomicron, Paraprevotella clara, Paraprevotella		Decrease in plasma butyrate
Li [17] (2017)	99 HT, 56 pre-HT and 41 NT subjects in Tangshan, China	Shotgun metagenomic	<i>Acidiphilum</i> , <i>Faecalibacterium</i>			
Li [37] (2019)	104 HT, 63 non-treated HT, 26 NT with hyperlipidemia and 42 NT subjects in Xinxiang, China	16S rRNA (V3-V4)	<i>Lactococcus</i> , <i>Alistipes</i> , <i>Subdoligranulum</i> , <i>Megasphaera</i> , <i>Megamonas</i>	Anaerotruncus, Ruminiclostridium, <i>Robinsoniella</i> , Clostridium, Intestinimonas, Butyrivibacillus, Subdoligranulum, Treponema, Holdemania, Roseburia, Butyrivibrio, Oscillibacter, Marvinbryantia, Akkermansia, Oribacterium, Pyramydobacter <i>Clostridium sensu stricto</i> 1, <i>Romboutsia</i> , <i>Erysipelotrichaceae</i> UCG.003, <i>Ruminococcus</i> 2, <i>Intestinibacter</i>		
Liu [38]	94 HT and 94 NT subjects in Xianyang, China	Bacteria primers	<i>Eubacterium rectale</i>	<i>Bifidobacterium</i> , <i>Bacteroides thetaiotaomicron</i>		
Mushtaq [39]	50 HT and 30 NT subjects	16S rRNA (V3/V3-V4), Bacteria primers	<i>Prevotella</i> , <i>Megasphaera</i> , <i>Parasutterella</i> , <i>Escherichia-Shigella</i> , <i>Phascolarctobacterium</i> <i>faecium</i> , Acidaminococcus, Actinomyces, Anaerostipes, Bacteroides, Blautia, Cellulomonas, Clostridioides, <i>Collinsella</i> , Coprococcus, Desulfovibrio, Dialister, Dielma, Dorea, Eisenbergiella, Enorma, Enterobacter, Erysipelatoclostridium, Faecalitalea, Holdemania, Intestinibacter, Lactococcus, Megasphaera, Mitsuokella, Paraprevotella, Phascolarctobacterium, Ruthenibacterium, Sanguibacteroides, Sutterella, Turicibacter	<i>Faecalibacterium prausnitzii</i> , <i>Bacteroides uniformis</i>		
Palmu [5]	3291 HT and 3662 NT subjects with an urinary sodium subsample of 829 in Finland	Shotgun metagenomic		Adlercreutzia, Alloprevotella, Anaerotruncus, Coprobacillus, Faecalibacterium, Fournierella, Hungatella, Parasutterella, Prevotella, Sellimonas, Senegalimassilia, Solobacterium, Tyzzerella		Increase in <i>Lactobacillus salivarius</i> , decrease in <i>Lactobacillus paracasei</i>
Silveira-Nunes [3]	48 HT and 32 NT subjects in Minas Gerais, Brazilia	16S rRNA (V3-V4)	<i>Lactobacillus</i> <i>salivarius</i> , <i>Bacteroides</i> <i>plebeius</i> , <i>Eggerthella</i>	<i>Roseburia facies</i> , <i>Faecalibacterium prausnitzii</i> , <i>Parabacteroides distasonis</i> , <i>Fusobacterium</i> , <i>Coprobacillus</i>		
Sun [40]	183 HT and 346 NT subjects in USA	16S rRNA (V3-V4)	<i>Catabacter</i> , <i>Robinsoniella</i>			
Verhaar [1]	1937 HT and 2735 NT subjects in Amsterdam, Netherlands	16S rRNA (V4)	<i>Streptococcus</i>	<i>Roseburia</i> , <i>Clostridium</i> <i>sensu stricto</i> 1, <i>Roseburia hominis</i> , <i>Romboutsia</i> , <i>Ruminococcaceae</i> , <i>Enterorhabdus</i>	Higher stool acetate and propionate	
Walejko [41]	10 black HT, 12 white HT, 10 black NT, and 20 white NT in USA	Shotgun metagenomic				Decrease in <i>L. salivarius</i> , <i>L. rhamnosus</i> , <i>L. plantarum</i> , <i>L. delbrueckii</i> , <i>L. casei</i> , <i>L. brevis</i>
Wilck [27]	12 healthy males with 6 g sodium chloride intervention in Berlin, Germany	Shotgun metagenomic				
Yan [42]	60 HT and 60 NT subjects in China	Shotgun metagenomic	<i>Klebsiella</i> , <i>Streptococcus</i> , <i>Parabacteroides</i>	<i>Roseburia</i> , <i>Faecalibacterium prausnitzii</i>		

HT hypertensive; NT, normotensive.



**Figure 1.** PRISMA flow chart.

### 3.1. Microbial Data Is Compositional in Nature

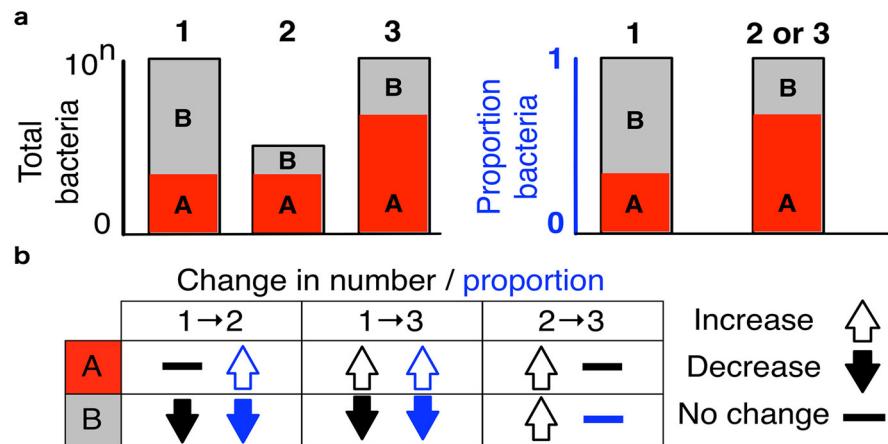
The microbial abundances share a subtle but highly consequential property imposed by limits of high-throughput sequencing. The observed count of one species affects the observed counts of all other species making the data inherently compositional (Figure 2) [43]. The experimental setup of high-throughput sequencing resembles the frequent example used in the teaching of probability calculation where a fixed number of different colored marbles are removed from a bag of unknown number of marbles. When the microbial load (total number of marbles) is not known, the data provides only information about the relative proportions of studied characteristics and using inappropriate statistical methods can lead up to a 100% false discovery rate [44]. One popular method to render microbial counts comparable across samples is the centered log-ratio transformation where bacterial counts are divided by the geometric mean of all observed counts. [43] In addition to appropriate statistical methodology, flow cytometry could be used to estimate the cell counts (while not total microbial load) improving the interpretation of 16S rRNA sequenced data in particular [44,45].

### 3.2. Animal Studies

#### 3.2.1. Changes in Gut Microbiota Are Associated with Hypertension in Rodents

Fecal microbiota transplantation from hypertensive donors to normotensive rats has been demonstrated to increase BP and induce changes in the abundances of multiple gut microbial species [9,19,24,25]. Notably, cross-species fecal microbiota transplantation from hypertensive humans to mice promotes hypertension [17]. While various associations between BP and gut microbiota have been observed (Table 1), *Lactobacillus* abundances appear to be particularly susceptible to sodium in salt-induced hypertension [27,31]. The association between probiotic intake and blood pressure has been studied in experimental animal models. In mice, *L. fermentum* treatment prevents hypertension and reduces endothelial dysfunction [23]. Consistently, long-term administration of *Lactobacillus fermentum* or *L. coryniformis* plus *L. gasseri* have been demonstrated to reduce systolic BP and decrease vascular inflammation in spontaneously hypertensive rats [15]. Increased

nitric oxide production may play a critical role in the beneficial (antihypertensive) effect of *Lactobacilli* [20].



**Figure 2.** Gut metagenome sequencing data are compositional. In this illustration, we observe three samples (1, 2 and, 3) and two microbial species (A and B). The sequence counts of microbial samples do not provide information about the absolute microbial abundances (black) making the observed data inherently compositional (blue). Therefore, real change in the total abundance of one microbial species can impose perceived change in other observed species (bottom left). Conversely, multiple proportionate changes in the real microbial abundances may be hidden from the observed abundances (bottom right). Adapted from Gloor et al. [43]. Licensed under Creative Commons Attribution License (CC BY, 2017).

BP has a well-defined morning diurnal rhythm peak, dropping 10–20% at rest [11]. There is some evidence that gut microbiota shares synchronous time-of-day variation with BP that also correlates with the levels of circulating renal markers [11]. In rats, microbial pathways characterized with biosynthesis were upregulated at active phase of day (night-time) while metabolite degradation pathways were upregulated at rest (daytime) [11]. This finding may have an impact on clinical hypertension care also as reduced nocturnal systolic BP dipping has been associated with an increased risk of cardiovascular events and diurnal variation of BP may affect both medication timing and microbial-targeted strategies [11,46].

### 3.2.2. Increased BP Is Associated with Inflammation and Gut Wall Pathology

The onset of hypertension has been associated with adrenergic nervous system activation that becomes more pronounced with increasing BP [47]. Dietary habits and psychosocial environment (stress) are possible drivers of this sympathetic activity that could alter host-microbiota cross-talk perpetuating peripheral and neural inflammation [22]. In hypertensive rats, green fluorescent protein staining demonstrated robust retrograde labeling from small intestine to the paraventricular nucleus of the hypothalamus compared to normotensive rats [2]. Changes in gut microbiota also increase plasma noradrenaline concentration (a marker of sympathetic activity), altered T<sub>H</sub>17/Treg balance in mesenteric lymph nodes, and levels of pro-inflammatory cytokines (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], interleukin [IL]-1 $\beta$ , IL-6, IL-17a, and interferon- $\gamma$ ) in brain paraventricular nucleus [24,25]. In addition, increased tyrosine hydroxylase (a rate-limiting enzyme in the synthesis of norepinephrine) immunoreactivity was observed in the small intestine of hypertensive compared to normotensive rats [2]. In a model of angiotensin II-induced hypertension, infusion of an anti-inflammatory agent in the cerebral ventricles alleviated the activation of microglia (resident macrophages), decreased the concentration of proinflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) in the paraventricular nucleus, and induced changes in several gut microbial communities [22].

In addition to an augmented paraventricular nucleus-gut connection and increased markers of inflammation, hypertension has been associated with increased intestinal perme-

ability and reduced mRNA levels of intestinal gap junction proteins in rats (Table 1) [2,24]. In addition, spontaneously hypertensive rats (SHRs) have lower mucin transcripts levels, the gel-like protective barrier proteins, compared to Wistar Kyoto rats [21]. Circulating bacterial wall components, such as lipopolysaccharides, can activate vascular toll-like receptors contributing to low-level chronic inflammation exacerbating hypertension [21]. However, treatment with renin-angiotensin system inhibitors has been reported to ameliorate gut wall pathology [28,30]. Candesartan treatment increases the intestinal expression of genes encoding tight junction proteins and serum levels of lipopolysaccharides-binding protein in SHRs [28]. Captopril treatment is associated with an increased number of goblet cells, increased villi length, reduced ileal fibrosis and reduced gut wall permeability serum markers in SHRs [30]. Intracerebroventricular administration of an anti-inflammatory agent also prevents gut wall pathology by reducing fibrosis and thickness of muscularis layer in the gut, and increasing villi length and the number of goblet cells [22]. In summary, hypertension has been associated with gut wall pathology and augmented paraventricular nucleus -gut connectivity in animal models.

### 3.2.3. Changes in Gut Microbiota Are Associated with Circulating Metabolites in Animal Models

SCFAs are microbial fermentation products of undigested carbohydrates that have the ability to enter host circulation (Table 1) [48]. In germ-free animals, a 100-fold reduction in cecal and circulating SCFA levels has been reported compared to conventional animals [48]. In particular, three major SCFAs, acetate, propionate, and butyrate, have been reported to have borderline undetectable concentrations in the plasma of the germ-free mice [49].

Hypertension has been associated with a decreased numbers of acetate- and butyrate-producing bacteria [20]. Angiotensin II type 1 receptor blocker treatment of SHRs has been reported to increase fecal acetate, propionate, and butyrate levels [28]. In an animal model of mineralocorticoid excess, dietary acetate increased the relative abundance of acetate-producing gut bacteria while reducing blood pressure and cardiac hypertrophy compared to untreated mice [18]. Acetate and butyrate treatment has been reported to alleviate the gut-wall pathology and lower BP in hypertensive rats and mice while no changes were observed in normotensive rats [16,21].

The SCFAs have been demonstrated to affect renin release in juxtaglomerular cells and modulate BP through G-protein coupled receptors [48]. A family of SCFA binding G-protein coupled receptors has been recently discovered [26]. A model of genomic excision of G-protein coupled estrogen receptor has given support to host-microbial cross-talk demonstrating that host genomic change modulates gut microbiota which in turn affects host BP [26]. The role of gut microbiota on the circulating metabolites was further studied in model of germ-free and conventional mice [12]. While hypertension induced changes were observed in the fecal and plasma metabolites of conventional mice, no changes were observed in germ-free mice [12]. In conclusion, the host and gut appear to share two-way interaction which is in part governed by SCFAs.

### 3.2.4. High Dietary Salt and Gut-Immune Axis in Animal Models

The deleterious effect of high dietary salt on cardiovascular health has been recently associated with the gut-immune axis [27]. High salt intake has been reported to modulate gut microbiota, particularly depleting *L. murinus* in mice (4% dietary and 1% drinking water NaCl vs. 0.5% dietary NaCl for 14 days) while the growth of various *Lactobacilli* was inhibited by sodium (half maximal growth inhibition 0.6 mol/L in vitro vs. 0.3 mol/L colonic NaCl concentration under high dietary salt) [27]. *L. murinus* administration reduces consistently salt-sensitive hypertension and prevents the increase in IL-17A producing CD4<sup>+</sup> T<sub>H</sub>17 among small intestinal, colonic and splenic lamina propria lymphocytes [27]. The changes in gut microbiota in hypertension have been associated with increased T<sub>H</sub>17/Treg ratio in mesenteric lymph nodes and activation of adaptive immune response [25]. The B7 ligand co-stimulated T cell activation and modulation of T<sub>H</sub>17/IL17 axis have been proposed to share an essential role in the development of endothelial dysfunction, increased

vascular oxidative stress, and hypertension upon fecal microbiota transplantation from SHR to Wistar Kyoto rats [25].

Salt-induced hypertension has been demonstrated to increase the number of immune cells in mesenteric arterial arcade and aorta (CD45<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells) [14]. In addition to the decrease of *Lactobacillus* species, various gut microbial changes have been associated with high dietary salt (4–8% dietary NaCl; Table 1) [10,13,14,31]. A novel mechanism for salt-induced hypertension has been proposed: salt induced changes in gut microbiota upregulate the production of corticosterone which enters circulation causing mineralocorticoid excess (pseudoaldosteronism), leading to hypertension, hypokalemia and inhibition of aldosterone synthesis [29].

### 3.3. Human Studies

#### 3.3.1. Gut Microbiota Is Associated with Human Hypertension

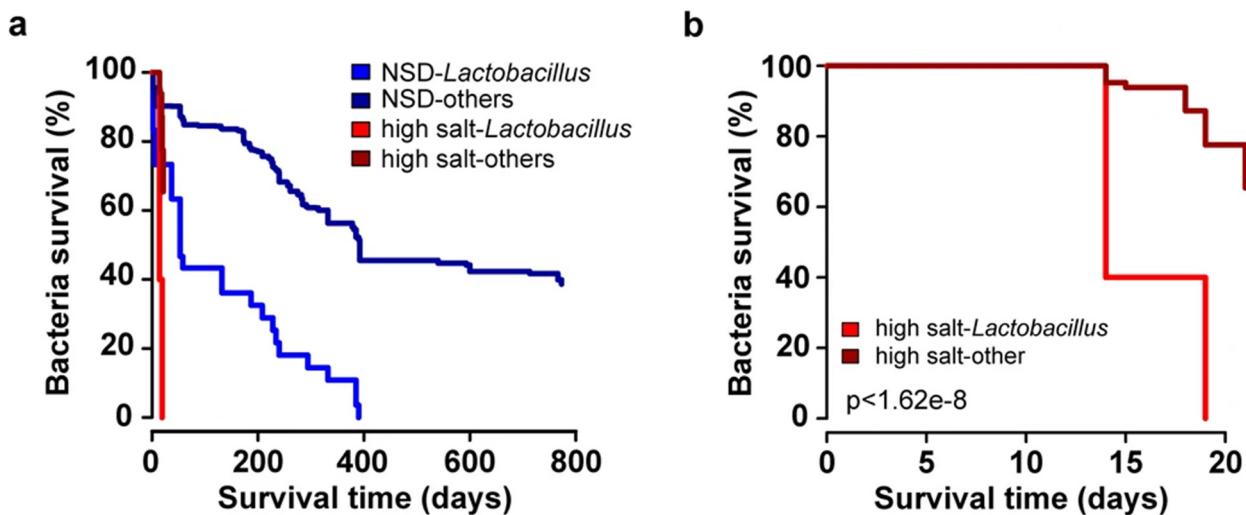
Numerous scientific publications have reported changes in gut microbial abundances and serum metabolites between groups of normotensive, pre-hypertensive and hypertensive individuals (Table 2) [17,35,37–39,42]. In particular, Black hypertensive individuals have been reported to have higher BP, greater prevalence of treatment resistant hypertension, increased pro-inflammatory potential in gut microbiota, and greater oxidative stress markers in plasma as compared to White hypertensive individuals [41]. Even subtypes of hypertension, namely isolated systolic and diastolic hypertension, share distinct gut microbiota profiles [33].

Four large cross-sectional studies have reported associations between gut microbiota and human hypertension. In the TwinsUK (N = 2737, 89% female, age 60 ± 12; 16S rRNA; record identified through other sources) study, no associations were observed between 68 various microbiota markers and self-declared hypertension after correcting for multiple testing [50]. In the Coronary Artery Risk Development in Young Adults (CARDIA; N = 529, 54% female, age 55 ± 3; 16S rRNA) study, a negative association was observed both for microbial alpha diversity and the abundance of *Robinsoniella*-genus with systolic BP [40]. In the FINRISK 2002 study (N = 6953, 55% female, age 49 ± 13; shotgun metagenomics), 45 microbial genera and 19 *Lactobacillus* species were associated with BP indices [5]. In the HEalthy Life In an Urban Setting (HELIUS; N = 4672, 52% female, age 50 ± 12; 16S rRNA) study, gut microbiota explained 4.4% of the overall unadjusted systolic BP variance but the proportion of variance explained was strongly divergent in different ethnic groups (4.8% for Dutch and <0.8% for others) [1]. In previous studies, the observed association between gut microbiota and hypertension was insignificant for British [50], African [1], Ghanaian [1], South Asian [1], and Turkish participants [1]. While significant associations have been observed in American [40], Finnish [5], Moroccan [1], and Dutch [1] participants, only the gut microbiota of the Dutch cohort demonstrated a high level of explained variance with regard to BP [1]. Altogether, it appears that associations between gut microbiota and BP exist, but they vary between different ethnic populations.

#### 3.3.2. High Dietary Salt Is Associated with Gut Microbiota in Human Hypertension

While the deleterious effect of high-salt diet to BP and cardiovascular health is well documented, most studies have focused on the pathophysiology of the high-salt diet in kidneys, vasculature, and the sympathetic nervous system [27]. To study the effect of high-salt diet on gut microbiota, a pilot study (N = 12) was performed in healthy males receiving slow-releasing 6 g sodium chloride supplementation (total salt intake 13.8 ± 2.6 g/day for 14 days) [27]. Sodium supplementation resulted in an increase in nocturnal BP, increase in peripheral blood CD4<sup>+</sup> IL-17A<sup>+</sup>TNF- $\alpha$ <sup>+</sup>T<sub>H</sub>17 cells, and reduction of *Lactobacillus* species (Figure 3) [27]. In the FINRISK 2002 study (N = 6953; cross-sectional study), genus-level *Lactobacillus* was not associated with BP while positive and negative associations of multiple *Lactobacillus* spp. and BP were detected [5]. In particular, *L. paracasei* was negatively associated with BP, whereas *L. salivarius* was positively associated with only pulse pressure [5]. In addition, *L. paracasei* was negatively, and *L. salivarius* was positively

associated with dietary sodium intake ( $N = 829$  for 24-h urinary sodium subsample) [5]. *Lactobacilli* are not a dominant member of the human feces, observed in only 15–42% of individuals [5,27]. *Prevotella* and *Bacteroides* have been positively associated with high-salt diet in human [14]. In the FINRISK 2002 study, *Bacteroides* was positively associated with BP and *Prevotella* had a borderline significant negative association with diastolic BP [5]. In summary, while gut bacteria demonstrate consistent links with high-salt diet and hypertension, genus level abundances may offer too generalized and even contradictory information to capture all the relevant associations.



**Figure 3.** Effects of high-salt challenge in healthy human subjects. The left panel displays Kaplan-Meier survival curves for gut bacteria in individuals under high-salt intervention (red;  $N = 12$ ) and healthy controls (blue;  $N = 121$ ). The bright shades of red and blue indicate the *Lactobacillus* species while the dark shades indicate the set of all other detected gut bacteria. The right panel displays the results for the salt intervention group during the first 20 days of the intervention. In summary, high-salt challenge decreases the abundances of several gut microbial species while the loss of *Lactobacillus* species is particularly rapid. NSD, normal salt diet. Adapted by permission from the Springer Nature: Nature, Salt-responsive gut commensals modulates TH17 axis and disease, Wilck et al. [27].

### 3.3.3. SCFAs Are Altered in Human Hypertension

Hypertensive individuals are reported to have decreased levels of circulating SCFAs, such as acetate, isobutyrate, butyrate and isovalerate, and increased levels of fecal SCFAs, such as acetate, butyrate, propionate, and valerate, compared to normotensive individuals [1,16,32,34,36]. The relationship between the number of SCFA-producing gut bacteria and host SCFA levels appears to be convoluted [32]. In particular, BP was negatively associated with fecal SCFA-producing bacteria, but positively associated with fecal SCFA levels in the HELIUS study [1]. While trimethylamine N-oxide, a product of bacterial metabolism derived from phosphatidylcholine (found in red meat, dairy products, eggs, and fish) has been associated with atherosclerosis and cardiovascular health, it was not related to hypertension in a small human study [32]. All in all, the previous studies demonstrate changes in SCFA production and absorption in human hypertension [1,32].

### 3.3.4. Gut Microbiota May Modulate Host Inflammatory Response

Similar to animal models, human hypertension is associated with increased intestinal wall permeability [16]. Mucosal dendritic cells detect local pathogens and regulate intestinal immune homeostasis [14]. High-salt diet has been associated, in addition to changes in circulating SCFAs, with increased formation of isolevuglandins in CD11c<sup>+</sup> antigen-presenting cells leading to T cell activation and production of IFN- $\gamma$  [14]. While the effect of SCFAs is governed by the activated receptor, SCFAs are linked to important anti-inflammatory effects suppressing the production of TNF- $\alpha$  and IL-6 [3]. In particular,

IL-17A producing CD4<sup>+</sup> T<sub>H</sub>17 cells are reported to transmit immune reaction in hypertension [16,27]. The gut microbiota may therefore modulate host inflammatory response and BP also in humans.

#### 4. Conclusions

##### 4.1. Summary

Several animal studies have suggested that gut microbiota dysbiosis and hypertension could be causally related. These results are in part supported by results from four large-scale observational epidemiological studies and from one interventional pilot study performed in humans.

Although several studies have reported on the potential association between the Firmicutes/Bacteroidetes ratio and blood pressure, [9,11,13,18,21,23,28,39] this approach has several challenges. These challenges include the lack of information on which of the two taxa is the main driver of the ratio and the use of relatively heterogenic and coarse phylum-level information. We have therefore refrained from focusing on these studies in this review.

Although variation in the gut microbiota has been associated with BP, the proportion of BP variance explained by gut microbiota seems to be only modest in most human cohort studies (<1%), when accounting for potential confounding factors. In addition, the observed associations vary greatly in different ethnic populations. However, the key gut microbial characteristics (diversity indices, microbial abundances, and proportions of variance) vary by study either due to technical factors or due to biological variation, which makes comparing and replicating results across studies challenging. Gut microbiota has been proposed to demonstrate reproducible patterns of variation, coined enterotypes, that could offer an useful tool to simplify the complexity of the gut ecosystem and possibly simplify studying the comorbidity of hypertension [51]. Due to the large number of bacterial species in gastrointestinal tract and limitations of available taxonomic resolution, most studies have only studied genus level abundances which may offer overly general information that only partially captures the interactions between the gut and the host. Our recent preprint demonstrates that increased taxonomic resolution improved the predictive performance of machine learning models in liver disease and proposes the use of species or even greater levels of resolution to improve our understanding of the role of gut microbiota to human health [52].

##### 4.2. Future Directions

To assess the role of SCFAs in host, careful consideration is necessary to differentiate the associations reported for SCFA producing bacteria, fecal SCFA levels, and circulating (bioactive) SCFA levels. Finding methods for accurate, absolute scale and possible non-direct measurement of the gut microbiota and its functional pathways could offer improvements in studying the microbiota-BP associations in large-scale epidemiological studies. In addition, human studies could be improved: (1) by using more accurate methods of BP measurement, such as home or ambulatory BP; (2) by performing deep metagenomic sequencing that would study species and strain level abundances; and (3) by implementing large-scale interventional studies that aim to manipulate dietary sodium intake and gut *Lactobacillus* species to establish a causal relationship between the gut microbiota and BP in humans.

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## Abbreviations

BP	blood pressure.
CD	cluster of differentiation
IL	interleukin
mRNA	messenger RNA
SCFA	short chain fatty acid
SHR	spontaneously hypertensive rat
T <sub>H</sub>	helper T cell
Treg	regulatory T cell
TNF- $\alpha$	tumor necrosis factor- $\alpha$

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III



ORIGINAL RESEARCH

# Association Between the Gut Microbiota and Blood Pressure in a Population Cohort of 6953 Individuals

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**BACKGROUND:** Several small-scale animal studies have suggested that gut microbiota and blood pressure (BP) are linked. However, results from human studies remain scarce and conflicting. We wanted to elucidate the multivariable-adjusted association between gut metagenome and BP in a large, representative, well-phenotyped population sample. We performed a focused analysis to examine the previously reported inverse associations between sodium intake and *Lactobacillus* abundance and between *Lactobacillus* abundance and BP.

**METHODS AND RESULTS:** We studied a population sample of 6953 Finns aged 25 to 74 years (mean age,  $49.2 \pm 12.9$  years; 54.9% women). The participants underwent a health examination, which included BP measurement, stool collection, and 24-hour urine sampling ( $N=829$ ). Gut microbiota was analyzed using shallow shotgun metagenome sequencing. In age- and sex-adjusted models, the  $\alpha$  (within-sample) and  $\beta$  (between-sample) diversities of taxonomic composition were strongly related to BP indexes ( $P < 0.001$  for most). In multivariable-adjusted models,  $\beta$  diversity was only associated with diastolic BP ( $P = 0.032$ ). However, we observed significant, mainly positive, associations between BP indexes and 45 microbial genera ( $P < 0.05$ ), of which 27 belong to the phylum *Firmicutes*. Interestingly, we found mostly negative associations between 19 distinct *Lactobacillus* species and BP indexes ( $P < 0.05$ ). Of these, greater abundance of the known probiotic *Lactobacillus paracasei* was associated with lower mean arterial pressure and lower dietary sodium intake ( $P < 0.001$  for both).

**CONCLUSIONS:** Although the associations between overall gut taxonomic composition and BP are weak, individuals with hypertension demonstrate changes in several genera. We demonstrate strong negative associations of certain *Lactobacillus* species with sodium intake and BP, highlighting the need for experimental studies.

**Key Words:** blood pressure ■ gastrointestinal microbiota ■ hypertension ■ *Lactobacillus* ■ salt intake

**D**ysbiosis of the gut microbiota has been recently linked to various chronic diseases, such as obesity, metabolic syndrome, diabetes mellitus,<sup>1</sup> and cardiovascular disease,<sup>2</sup> and changes in lifestyle.<sup>3</sup> Additional evidence, primarily from animal studies, suggests an association between microbiota and hypertension.<sup>4–7</sup> Furthermore, high salt

intake, a risk factor for both hypertension and cardiovascular disease, was shown to deplete certain *Lactobacillus* species in mice while treating the mice with *Lactobacillus* prevented salt-sensitive hypertension.<sup>8</sup> Findings from these studies are consistent with those from human studies, in which consumption of salted snacks has been indicated as a significant

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## CLINICAL PERSPECTIVE

### What Is New?

- We advance the prior scarce and conflicting knowledge on the associations between gastrointestinal microbes and hypertension in a large, representative population sample using standardized blood pressure measurements, adjustment for relevant confounders, and stool metagenomic sequencing.
- Although the associations between overall gut taxonomic composition and blood pressure are weak, individuals with hypertension demonstrate changes in several microbiota genera, with most of these genera belonging to the *Firmicutes* phylum.
- Interestingly, we also demonstrate strong negative associations of certain *Lactobacillus* species with both dietary sodium intake and blood pressure.

### What Are the Clinical Implications?

- The observed associations between the gut microbial composition and hypertension offer novel insights on the potential mechanisms through which diet affects the gut microbiome and blood pressure.

(Coronary Artery Risk Development in Young Adults) study participants,<sup>14</sup> an SD increase in gut microbiota α (within-sample) diversity was related to a modest 1.29 mm Hg lower systolic blood pressure (BP;  $P=0.049$ ). In a commentary, Jama and coauthors<sup>15</sup> proposed several improvements in the experimental design of future studies examining the relation between the microbiota and hypertension, such as the use of standardized BP measurements, adjustment for medication use, and replacing 16S rRNA profiling with metagenome sequencing.

Herein, we aim to advance the current knowledge on the association between the gut metagenome and BP in a well-phenotyped, large random population sample of 6953 individuals while adjusting for relevant confounders, including antihypertensive medication classes. A 24-hour urine sodium sample was available for 829 individuals. We therefore also performed a more focused analysis on the interrelations between sodium intake, gut *Lactobacillus* abundance, and BP to gain additional insight on the potential mechanisms through which the microbiota might affect BP.<sup>8</sup>

## METHODS

### Availability of Data and Materials

The data that support the findings of this study are available from Finnish Institute for Health and Welfare Biobank (<https://thl.fi/en/web/thl-biobank>). The data are not publicly available because they contain information that could compromise research participant privacy/consent. The source code for the analyses is openly available at 10.5281/zenodo.3622730.

### Study Sample

The Finnish Institute for Health and Welfare has performed population surveys every 5 years since 1972 to monitor the development of cardiovascular risk factors in the Finnish population.<sup>16</sup> A random population sample of 13 437 individuals, aged 25 to 74 years, from 6 geographic regions was invited to take part in the FINRISK 2002 study.<sup>17</sup> Of the 8799 individuals who took part in the FINRISK 2002 study (participation rate, 65.5%), we excluded 1568 who did not provide stool samples, 20 because of low total read count ( $N<50\ 000$ ), and 258 because of missing relevant covariates, for a final study sample of 6953 individuals who were included in the analysis. For a subsample of 829 participants, 24-hour urine collection for estimating dietary sodium intake was performed.<sup>18</sup> The study was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa University Hospital District, and all participants gave written informed consent.

### Nonstandard Abbreviations and Acronyms

<b>BP</b>	blood pressure
<b>CARDIA</b>	Coronary Artery Risk Development in Young Adults
<b>ICD-8</b>	<i>International Classification of Diseases, Eighth Revision</i>
<b>ICD-9</b>	<i>International Classification of Diseases, Ninth Revision</i>
<b>ICD-10</b>	<i>International Classification of Diseases, Tenth Revision</i>
<b>KO</b>	Kyoto Encyclopedia of Genes and Genomes Orthology group

correlate of the human gut microbiota.<sup>9</sup> In addition, the gut microbiota has been functionally linked to complications of hypertension, such as arterial thrombosis.<sup>10–12</sup> These prior results therefore highlight the potential of the gut microbiota as a therapeutic target for hypertension.

Despite these promising results from animal studies, human data are scarce and conflicting. In the TwinsUK cohort,<sup>13</sup> self-reported hypertension was not related to 68 various microbiota markers. In another publication based on a subsample of 529 CARDIA

## Health Examination and 24-Hour Urine Collection

After completing a questionnaire on sociodemographic information, lifestyles, medications, and medical history at home, the participants attended a physical examination at a local study site. The participants underwent measurements for height and weight. A nurse measured sitting BP 2 times from the right arm using a mercury manometer and a 14×40-cm cuff after a 5-minute rest. Participants in the 24-hour urinary sodium subsample were instructed to start the collection on a Sunday morning and return the container the following day. A sample of urine was frozen at -20°C and later analyzed using an ion-selective electrode (Optima analyzer; Thermo Electron Oy, Vantaa, Finland).<sup>18</sup> Urine sodium excretion was calculated as the product of urine sodium concentration and daily excreted urinary volume.

## Stool Sampling and Storage

Stool samples were collected at home after the physical examination in 50-mL Falcon tubes and were mailed to Finnish Institute for Health and Welfare using prepaid packages over 1 to 2 days. The samples were then frozen in -20°C and kept unthawed until 2017, when they underwent metagenomic sequencing.

## Stool DNA Extraction and Library Preparation

Microbiota analysis was performed at the University of California, San Diego, using whole genome untargeted shallow shotgun metagenomic sequencing against mapped reference databases, following a previously published protocol.<sup>19</sup> In brief, Illumina-compatible libraries were prepared from isolated DNA and normalized to 5- $\mu$ g input per sample. Samples were pooled using the iTru<sup>20</sup> dual-indexing system and sequenced using Illumina Hi-Seq 4000 for paired-end 150-bp reads. Sequence reads were mapped against taxonomy using SHOGUN v1.0.5 against National Center for Biotechnology Information RefSeq database (version 82; May 8, 2017).<sup>21,22</sup> Functional profiles were calculated from a combination of observed and predicted Kyoto Encyclopedia of Genes and Genomes Orthology group (KO) annotations from the RefSeq genomes following the predicted parameters of the SHOGUN tool.<sup>21</sup>

## Outcome Variables and Covariates

The mean of the 2 measurements was used to determine systolic and diastolic BP. Hypertension was defined as systolic BP  $\geq$ 140 mm Hg, diastolic BP  $\geq$ 90 mm Hg, or use of antihypertensive medication.

Pulse pressure was defined as systolic minus diastolic BP. Mean arterial pressure was defined as follows: [(2×diastolic BP)+systolic BP]/3.

Body mass index was defined as weight (kilograms) divided by the square of the body height (meters). Participants were asked to assess their leisure-time physical activity by a 4-option multiple choice question. The 4 options for leisure-time activity were (1) sedentary, (2) light activity for >4 hours per week, (3) fitness training or other strenuous exercise for >3 hours per week, and (4) competitive sports. Information on medication use was retrieved from the Finnish national Drug Purchase Register,<sup>23</sup> which captures all prescription drug purchases in Finland. Finnish pharmacies fill prescriptions for a maximum of 3 months, and antihypertensive medication use was defined as a drug purchase occurring within the 4 months preceding baseline. Medications with the following Anatomical Therapeutic Chemical classification<sup>24</sup> codes were considered anti-hypertensive medications: diuretics (C03\*),  $\beta$  blockers (C07\*), calcium channel blockers (C08\*), and renin–angiotensin system inhibitors (C09\*). Prevalent diabetes mellitus was defined as self-reported diabetes mellitus, a previous diagnostic code indicating diabetes mellitus in the nationwide Care Register for Health Care (*International Classification of Diseases, Tenth Revision [ICD-10]*, codes E10-E14 or *International Classification of Diseases, Eighth Revision/International Classification of Diseases, Ninth Revision [ICD-8/ICD-9]*, code 250), a previous diabetes mellitus medication purchase (Anatomical Therapeutic Chemical classification code A10\*), or special reimbursement code for diabetes mellitus medications in the Drug Reimbursement Register. Smoking was defined by self-reported current daily smoking.

## Statistical Analysis

We compared characteristics between individuals who were and were not included in the urinary sodium subsample using the 2-sample T test and the  $\chi^2$  test of equality of means. Unless otherwise noted, we adjusted the analyses for age, sex, body mass index, smoking, exercise, diabetes mellitus, diuretic use,  $\beta$ -blocker use, calcium channel blocker use, and renin-angiotensin system inhibitor. We calculated a diversity (Shannon index) using species-level data with the R package *microbiome*.<sup>25</sup> We calculated the dissimilarity matrix ( $\beta$  diversity) and principal coordinates using Bray-Curtis dissimilarity on compositional microbial species-level abundance using R packages *Phyloseq*<sup>26</sup> and *Vegan*.<sup>27</sup> The  $\alpha$  diversity is a measure of within-sample diversity (eg, number of microbial species observed or number of questions needed on average to identify random microbe within sample), and the  $\beta$  diversity is a measure of between-sample

diversity (eg, euclidean distance, where each axis represents single species or ratio of number of species shared and number of species in total). We analyzed the associations between  $\beta$  diversity and BP variables using permutational multivariate ANOVA with 999 permutations. We studied common microbial genera (prevalent in at least 1% of sample population with a relative abundance  $>0.1\%$ ) using DESeq2 with the Benjamini-Hochberg correction.<sup>28,29</sup> We analyzed associations of the genera with (1) BP indexes and (2) 24-hour urinary sodium excretion. We further studied the association between *Lactobacillus* and BP in subgroups by sex and antihypertensive medication. We performed more focused analysis for the detected *Lactobacillus* species, replicating the previous 2 steps. We log(x+1) transformed the KO groups. We used fully adjusted linear regression models to estimate the associations between KO groups and systolic BP. We visualized separately the associations between KO groups and systolic BP using the FuncTree package.<sup>30</sup> For the module, pathway, and biological process layers, we used node sizes that corresponded to the average inverse  $P$  value of all KO groups that could be assigned to that node. The source code for the analyses is openly available at 10.5281/zenodo.3622730.<sup>31</sup> We used R<sup>32</sup> version 3.6.0 for all statistical analyses.

## RESULTS

The characteristics of the main study sample and the 24-hour urinary sodium subsample are reported in Table. A small, but statistically significant, difference between the 2 samples was observed for age ( $P<0.001$ ), diastolic BP ( $P=0.001$ ), pulse pressure ( $P<0.001$ ), heavy exercise ( $P=0.011$ ),  $\beta$ -blocker use ( $P=0.046$ ), and renin-angiotensin system blocker use ( $P=0.030$ ). We observed 134 *Lactobacillus* species (Table S1) and 91 common microbial genera (4.7% of all available genera; Table S2).

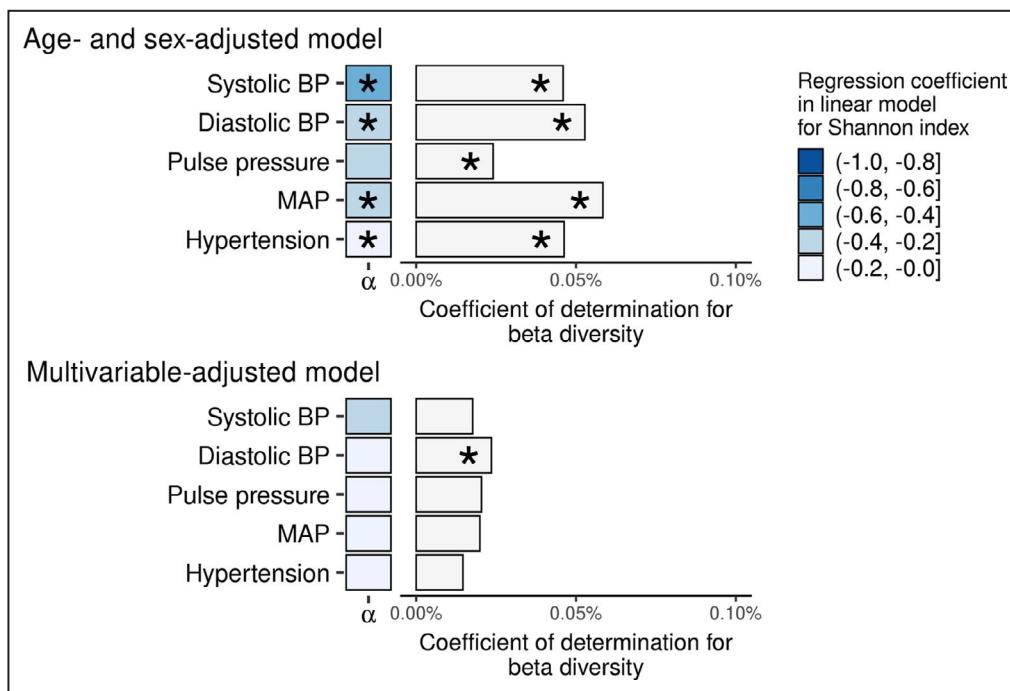
In models adjusted for age and sex (Figure 1, Table S3), an SD increase in microbiota  $\alpha$  diversity was inversely associated with systolic BP (effect size,  $-0.54$  mm Hg; 95% CI,  $-0.96$  to  $-0.12$  mm Hg;  $P=0.012$ ), diastolic BP (effect size,  $-0.31$  mm Hg; 95% CI,  $-0.56$  to  $-0.06$  mm Hg;  $P=0.016$ ), mean arterial pressure (effect size,  $-0.39$  mm Hg; 95% CI,  $-0.66$  to  $-0.12$  mm Hg;  $P=0.005$ ), and hypertension (odds ratio, 0.91; 95% CI, 0.86–0.96;  $P<0.001$ ). However,  $\alpha$  diversity was not related to any BP variable in the multivariable-adjusted models (Figure 1, Table S3).

In the age- and sex-adjusted models, all BP indexes were significantly associated with  $\beta$  diversity ( $P\leq0.038$  for all; Figure 1, Table S4). The coefficients of determination between  $\beta$  diversity and

**Table. Characteristics of the Study Sample**

Characteristics	All	Urinary Sodium Subsample	P Value
No.	6953	829	
Age, mean (SD), y	49.2 (12.88)	47.2 (10.94)	<0.001
Women, N (%)	3819 (54.9)	460 (55.5)	0.720
BMI, mean (SD), kg/m <sup>2</sup>	27.0 (4.66)	26.7 (4.54)	0.117
Systolic BP, mean (SD), mm Hg	135.6 (20.20)	134.6 (18.65)	0.113
Diastolic BP, mean (SD), mm Hg	79.1 (11.23)	80.3 (11.12)	0.001
Pulse pressure, mean (SD), mm Hg	56.5 (16.39)	54.4 (14.16)	<0.001
Arterial pressure, mean (SD), mm Hg	97.9 (12.67)	98.4 (12.40)	0.263
Hypertension, N (%)	3291 (47.3)	378 (45.6)	0.283
Current smoker, N (%)	1637 (23.5)	210 (25.3)	0.189
Diabetes mellitus, N (%)	390 (5.6)	36 (4.3)	0.081
Exercise, N (%)			
Light	1466 (21.1)	167 (20.1)	0.545
Moderate	3922 (56.4)	445 (53.7)	0.109
Heavy	1565 (22.5)	217 (26.2)	0.011
Antihypertensive medication, N (%)	1253 (18.0)	122 (14.7)	
Diuretics	232 (3.3)	20 (2.4)	0.145
$\beta$ Blockers	714 (10.3)	68 (8.2)	0.046
Calcium channel blockers	293 (4.2)	32 (3.9)	0.670
RAS blockers	569 (8.2)	51 (6.2)	0.030

Continuous variables are presented as mean (SD), and categorical values are presented as count (percentage). Characteristics between individuals who were and were not included in the urinary sodium subsample were compared using the 2-sample T test and the  $\chi^2$  test of equality of means. BP indicates blood pressure; BMI, body mass index; and RAS, renin-angiotensin system.



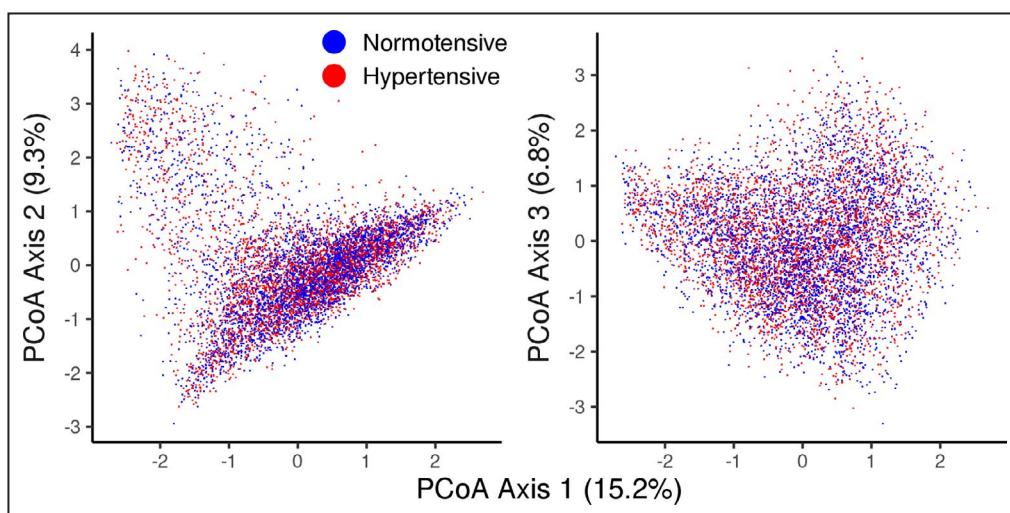
**Figure 1.** Associations between blood pressure (BP) variables and microbial diversity.

The blue heat maps on the left express the change in BP variables per 1-SD increase in  $\alpha$  diversity (Shannon index); log odds are reported for hypertension. The bar plots on the right represent the proportion of variability ( $R^2$ ) in  $\beta$  diversity (Bray-Curtis distance) explained by BP indexes. Multivariable-adjusted model is adjusted for age, sex, body mass index, smoking, exercise, diuretics,  $\beta$  blockers, calcium channel blockers, and renin-angiotensin system blockers. Significant results are marked with asterisk ( $P < 0.05$ ).  $\alpha$  indicates effect size for  $\alpha$  diversity; and MAP, mean arterial pressure.

BP variables varied between 0.02% and 0.06%. In multivariable-adjusted models, only diastolic BP ( $R^2=0.02\%$ ;  $P=0.032$ ) was significantly related to  $\beta$  diversity (Figure 1, Table S4). The first 3 principal coordinate axes explained 31.3% of the variation in bacterial abundances, but visual inspection did not

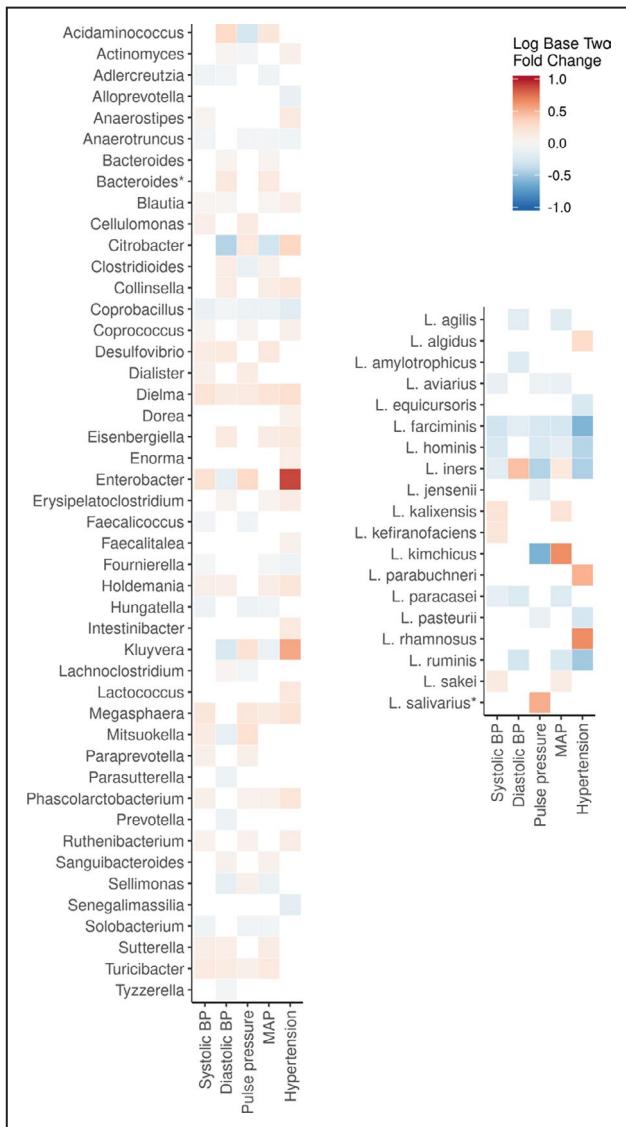
reveal clustering of hypertensive and normotensive individuals (Figure 2).

We then studied the associations between genus-level abundances and BP indexes. We observed 122 significant associations between 45 distinct microbial genera and BP indexes with false discovery



**Figure 2.** Principal coordinate analysis (PCoA) for species-level bacterial abundances (Bray-Curtis distance).

rate-corrected  $P<0.05$  (Figure 3, Table S5). These associations are shown in subgroups by sex and antihypertensive medication use in Figures S1 and S2. The results differed by subgroup for several species. Of all covariates, inclusion of body mass index in the age- and sex-adjusted model resulted in most of the

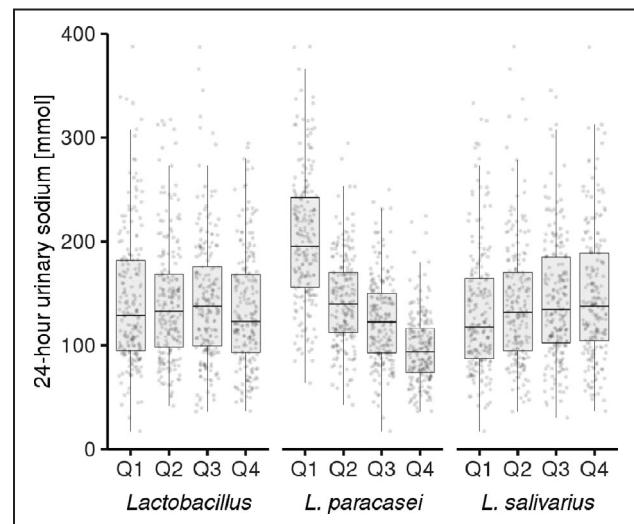


**Figure 3. Associations for common microbial genera and *Lactobacillus* species with blood pressure (BP) indexes.**

We observed 45 distinct microbial genera and 19 *Lactobacillus* species that were significantly associated with BP indexes using DESeq2 ( $P<0.05$  for all). The heat map expresses the fold change associated with BP indexes in base 2 logarithm ratios of microbial abundances. For hypertension, the range signifies a change of microbial abundance from 0.5 (blue) to 2 (red) times the bacterial abundance in normotensive participants. For continuous variables, the fold change is expressed per 1-SD change in BP variable. The models are adjusted for age, sex, body mass index, smoking, exercise, diuretics,  $\beta$  blockers, calcium channel blockers, and renin-angiotensin system blockers. Association with bacterial plasmid is denoted using asterisk. MAP indicates mean arterial pressure.

reduction (from 39 to 23; 59%) in the number of significant genera-hypertension associations. The association between the *Lactobacillus* genus and BP indexes was nonsignificant, but species-level analyses revealed 41 significant associations for 19 (14.0%) distinct *Lactobacillus* species with false discovery rate-corrected  $P<0.05$ . Of these associations, 12 were positive and 29 were negative (Figure 3, Table S6). We observed 481 KO groups associated with systolic BP (false discovery rate-corrected  $P<0.05$ ; Table S7). Internal node calculation revealed that several of the most prominent pathways were related to lipid metabolism, gluconeogenesis, and xenobiotic metabolism (Figure S3).

Finally, we performed a more focused analysis on the association between genus- and species-level *Lactobacillus* abundances and sodium intake in the subsample of participants with 24-hour urinary sodium excretion (mean,  $142.3\pm62.9$  mmol) data available. *Lactobacillus* prevalence was 15.5% at the detection limit of 0.1% relative abundance in this subsample. *Lactobacillus* genus was not associated with 24-hour urinary sodium excretion (false discovery rate-corrected  $P=0.984$ ; Figure 4). At the species level (Figure 4), *Lactobacillus paracasei* demonstrated



**Figure 4. *Lactobacillus* abundance in groups by 24-hour urinary sodium excretion.**

We observed significant association between 24-hour urinary sodium excretion and two *Lactobacillus* species with false discovery rate-corrected  $P<0.05$  while the genus-level association remained insignificant. For *Lactobacillus paracasei* ( $\log_2$  fold change,  $-0.018\pm0.002$ ;  $P<0.001$ ), the 24-hour urinary sodium excretion levels were as follows: quartile (Q) 1,  $205.4\pm68.5$  mmol; Q2,  $143.0\pm43.8$  mmol; Q3,  $123.9\pm41.1$  mmol; and Q4,  $96.6\pm33.7$  mmol. For *Lactobacillus salivarius* ( $\log_2$  fold change,  $0.007\pm0.002$ ;  $P=0.004$ ), the 24-hour urinary sodium excretion levels were as follows: Q1,  $132.7\pm60.3$  mmol; Q2,  $142.3\pm63.3$  mmol; Q3,  $144.0\pm58.3$  mmol; and Q4,  $150.1\pm68.6$  mmol. We visualize the associations using quartiles of DESeq2-fitted abundances against 24-hour urinary sodium.

a strong, negative association with ( $\log_2$  fold change,  $-0.018 \pm 0.002$ ;  $P < 0.001$ ) urinary sodium excretion. *Lactobacillus salivarius* was positively ( $\log_2$  fold change,  $0.007 \pm 0.002$ ;  $P = 0.004$ ) associated with urinary sodium excretion.

## DISCUSSION

We investigated the relation between the gut metagenome and objectively measured BP in a large, representative population cohort while adjusting for relevant confounding factors. In age- and sex-adjusted models, we observed strong associations between overall gut taxonomic composition and BP. However, these associations were weaker in multivariable-adjusted models. We observed significant associations between 45 distinct microbial genera and BP indexes, of which 27 belong to the phylum *Firmicutes*. Interestingly, certain *Lactobacillus* species demonstrated strong associations with both BP indexes and 24-hour urinary sodium excretion. Our functional analysis suggests that microbiome-driven processes associated with lipid metabolism, gluconeogenesis, and xenobiotic metabolism may be associated with BP. However, experimental designs with deep microbiome sequencing are needed for more detailed information on the causal/functional mechanisms and, ultimately, the clinical significance of our findings.

Several prior animal studies have suggested an association between intestinal dysbiosis and hypertension.<sup>4</sup> A lower gut microbiota  $\alpha$  diversity was reported among hypertensive cases in a small case-control study with a study sample of 11 rats and 17 human patients.<sup>5</sup> In another experimental study by Adnan et al,<sup>6</sup> 6 normotensive rats were gavaged with microbiota from hypertensive rats, which led to increases in the *Firmicutes/Bacteroides* ratio and systolic BP. Finally, Durgan et al<sup>33</sup> suggested that a causal relationship between gut microbial dysbiosis and obstructive sleep apnea-induced hypertension could exist in groups of 6 to 9 rats allocated to high-fat and normal chow diet. These early animal studies have suggested that gut dysbiosis and hypertension could be causally related, offering a basis for large-scale epidemiological studies in humans.

Two previous cohort studies have assessed the association between 16S rRNA-sequenced gut microbiota and hypertension in humans. Jackson et al<sup>13</sup> studied the link between gut microbiota markers and self-reported common diseases in a sample of 2737 TwinsUK study participants. However, no associations were observed between 68 various microbiota markers and self-declared hypertension after correcting for multiple testing. As previously noted,<sup>15</sup> the prevalence of self-reported hypertension (27.6%)

in TwinsUK was lower than expected and, therefore, the lack of objective BP measurements could explain these nonsignificant results. In another study by Sun et al,<sup>14</sup> the authors examined the association between gut microbiota and objectively measured BP in 529 CARDIA study participants. This study reported a negative association between gut microbial  $\alpha$  diversity and systolic BP. The authors also observed a single significant genus-level association between systolic BP and *Robinsoniella*, which was not included in common microbial genera (prevalence,  $\geq 1\%$ ; abundance,  $\geq 0.1\%$ ) used in this study. Our study builds on these earlier efforts through the use of shotgun metagenomic sequencing and a large, representative study sample with detailed drug purchase data, standardized BP measurements, and adequate statistical power. Although the multivariable-adjusted associations between overall gut taxonomic composition and BP observed in our study were small, we demonstrate mainly positive associations between 45 microbial genera and BP indexes. A total of 27 of these 45 genera belong to the phylum *Firmicutes*, highlighting the previously observed potentially harmful links of increased *Firmicutes* with hypertension, obesity, diabetes mellitus, and chronic kidney disease.<sup>5,34</sup>

In a previous publication, Wilck et al<sup>8</sup> studied the effects of high dietary salt intake on gut-immune axis through induction of interleukin-17A-producing T-helper 17 cells, which can also contribute to hypertension. The authors reported that *Lactobacillus murinus* was depleted by high dietary salt in mouse models, whereas *L. murinus* treatment prevented salt-sensitive hypertension by modulating T-helper 17 cells. In the same study, markedly increased salt intake also led to reduced *Lactobacillus* species abundance, increased T-helper 17 cell levels, and increased BP in a small sample of 12 men and women. In line with the results by Wilck et al,<sup>8</sup> we observed mainly negative, but also positive, associations between *Lactobacillus* species and BP indexes. Although the *Lactobacillus* genus was not associated with 24-hour urinary sodium excretion, we observed a strong association between an increased *L. paracasei* abundance and decreased urinary sodium excretion ( $P < 0.001$ ; Figure 4). A positive association between *L. salivarius* and sodium excretion was also observed ( $P = 0.004$ ). The directions of the effects for both *Lactobacillus* species were consistent in models for BP and dietary sodium. However, the *Lactobacillus*-related results were somewhat different in subgroups by sex and antihypertensive medication use. In particular, the association between *L. paracasei* and BP was stronger in women. The association between *L. paracasei* and BP was observed in users and nonusers of antihypertensive medications.

The underlying mechanisms of these associations require further study, but the probiotic supplementation with *L paracasei* has been previously shown to reduce interleukin-17 levels in acutely ill patients and *Lactobacillus casei* group on the whole to induce potential weight loss.<sup>35,36</sup>

Although our study has several advantages, our results must be interpreted in the context of their limitations. First, although fecal sampling is a noninvasive and feasible method for assessing microbiota, it is only a proxy for the gut microbiota. In particular, the stable microbial niche in mucosal layer of the gastrointestinal tract could account for major physiological effects with minor contribution to stool sampling.<sup>37</sup> Second, the relatively long storage time of the samples (15 years at -20°C) could lead to some deterioration of the samples. However, the taxonomic composition of our samples was similar to what has been previously observed in larger cohort studies.<sup>19</sup> Third, metagenomics is a novel field with reported limitations (eg, those related to sequencing and labeling DNA in stool samples).<sup>38</sup> Fourth, despite 24-hour urine collection being a more accurate method for estimating sodium intake than spot urine samples, it remains a cross-sectional snapshot of dietary habits.<sup>39</sup> Fifth, the hypertensive participants of our study may have received instructions to reduce their sodium intake, leading to weaker or reverse causation.

## CONCLUSIONS

This study is by far the largest to examine the association between human gut microbiota and objectively measured BP. Although the associations between overall gut taxonomic composition and BP are weak, individuals with hypertension demonstrate changes in several microbiota genera, with most of these genera belonging to the *Firmicutes* phylum. Interestingly, we also demonstrate strong negative associations of certain *Lactobacillus* species with both dietary sodium intake and BP (Figures 3 and 4). This finding provides additional population-level evidence to those from experimental studies demonstrating that distinct *Lactobacillus* species are depleted by high dietary salt, whereas treatment with these same species might prevent salt-sensitive hypertension.<sup>8</sup> Our research needs to be expanded (1) by estimating the effect of the reported associations for public health, (2) by determining the functional role of gut microbiota by combining taxonomic profiling with simultaneous determination of the gut and plasma metabolome, and (3) by conducting additional studies that directly manipulate *Lactobacillus* species levels in the gut to establish the causal relation between these species and hypertension. The

observed associations between the gut microbial composition and hypertension offer novel insights on the potential mechanisms through which diet affects the gut microbiome and BP. In addition, our results raise hypotheses on how the microbiome could be manipulated to improve hypertension control.

## ARTICLE INFORMATION

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Author contributions: Drs Salomaa, Knight, Lahti, and Niiranen designed the work; Drs Havulinna, Jain, Salido, Sanders, Brennan, Humphrey, Vartiainen, Laatikainen, Jousilahti, and Salomaa acquired the data; Drs Palmu, Saloenssaari, Sanders, Lahti, and Niiranen analyzed the data; and Drs Cheng, Inouye, Jain, Jousilahti, Salomaa, Knight, Lahti, and Niiranen supervised the work. All authors wrote the article and gave final approval of the version to be published.

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### Disclosures

Dr Salomaa has received honoraria from Novo Nordisk and Sanofi for consultations. He also has ongoing research collaboration with Bayer Ltd (all unrelated to the present study). The remaining authors have no disclosures to report.

### Supplementary Materials

Tables S1–S7

Figures S1–S3

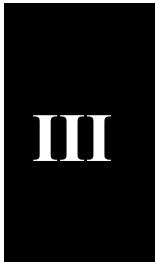
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ORIGINAL RESEARCH

# Eicosanoid Inflammatory Mediators Are Robustly Associated With Blood Pressure in the General Population

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**BACKGROUND:** Epidemiological and animal studies have associated systemic inflammation with blood pressure (BP). However, the mechanistic factors linking inflammation and BP remain unknown. Fatty acid–derived eicosanoids serve as mediators of inflammation and have been suggested to regulate renal vascular tone, peripheral resistance, renin-angiotensin system, and endothelial function. We hypothesize that specific proinflammatory and anti-inflammatory eicosanoids are linked with BP.

**METHODS AND RESULTS:** We studied a population sample of 8099 FINRISK 2002 participants randomly drawn from the Finnish population register (53% women; mean age, 48±13 years) and, for external validation, a sample of 2859 FHS (Framingham Heart Study) Offspring study participants (55% women; mean age, 66±9 years). Using nontargeted liquid chromatography–mass spectrometry, we profiled 545 distinct high-quality eicosanoids and related oxylipin mediators in plasma. Adjusting for conventional hypertension risk factors, we observed 187 (34%) metabolites that were significantly associated with systolic BP ( $P <$ Bonferroni-corrected threshold of 0.05/545). We used forward selection linear regression modeling in FINRISK to define a general formula for individual eicosanoid risk score. Individuals of the top risk score quartile in FINRISK had a 9.0 (95% CI, 8.0–10.1) mm Hg higher systolic BP compared with individuals in the lowest quartile in fully adjusted models. Observed metabolite associations were consistent across FINRISK and FHS.

**CONCLUSIONS:** Plasma eicosanoids demonstrate strong associations with BP in the general population. As eicosanoid compounds affect numerous physiological processes that are central to BP regulation, they may offer new insights about the pathogenesis of hypertension, as well as serve as potential targets for therapeutic intervention.

**Key Words:** blood pressure ■ eicosanoids ■ hypertension ■ liquid chromatography–mass spectrometry ■ metabolite

**A**vast majority of patients with hypertension (>95%) are classified as having primary (essential) hypertension, a heterogeneous condition of hypertension that has no identifiable cause (by definition). Essential hypertension is most likely the consequence of an interaction between genetic factors and environmental factors (eg, obesity, insulin resistance, sedentary lifestyle, stress, and sodium intake).<sup>1</sup>

Intriguingly, all of the aforementioned factors are also related to chronic low-grade inflammation, underscoring the need to further investigate inflammation as a potential mainstay pathologic mechanism underlying hypertension.<sup>2</sup>

The upstream initiation of inflammatory activity in humans is governed mainly by substrates and products of polyunsaturated fatty acids.<sup>3</sup> Termed

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## CLINICAL PERSPECTIVE

### What Is New?

- Fatty acid-derived eicosanoids serve as mediators of inflammation and have been suggested to regulate renal vascular tone, peripheral resistance, renin-angiotensin system, and endothelial function.
- We assayed a comprehensive panel of >500 distinct high-quality eicosanoids and related oxylipin mediators in community-based samples of >10 000 individuals using liquid chromatography–mass spectrometry and relate these eicosanoids and eicosanoid profiles to blood pressure traits.

### What Are the Clinical Implications?

- We observed that 187 (34%) eicosanoids and related oxylipin mediators were significantly associated with systolic blood pressure.
- Individuals in the top quartile of a 6-metabolite risk score had a 9.0 mm Hg higher systolic blood pressure and 2-fold greater odds of hypertension compared with individuals in the bottom quartile.
- In conclusion, as eicosanoid species affect numerous physiological processes that are central to blood pressure regulation, they may offer new insights about the pathogenesis of hypertension, as well as serve as potential new targets for therapeutic intervention.

20-hydroxyeicosatetraenoic acid, and genetic polymorphisms that regulate the levels of these eicosanoids are altered in small samples of individuals and animals with hypertension.<sup>10–12</sup>

Until recently, sensitive methods for detecting and quantifying eicosanoids in large sample sizes were lacking. However, mass spectrometry (MS) based analytics now allow for the rapid large-scale quantification of several hundred upstream eicosanoids in human plasma.<sup>13,14</sup> Our goal was to gain a more detailed understanding of how upstream inflammatory mediators are related to an individual's prevalence for hypertension. We quantified a comprehensive panel of >500 distinct high-quality upstream eicosanoids and related oxylipin mediators in FINRISK 2002 (n=8099) and FHS (Framingham Heart Study Offspring) (n=2859) cohort participants using liquid chromatography–MS (LC-MS) and related these eicosanoids and eicosanoid profiles to BP traits.

## METHODS

### Availability of Data and Materials

The data that support the findings of this study are available from Finnish Institute for Health and Welfare Biobank (<https://thl.fi/en/web/thl-biobank>). The data are not publicly available because they contain information that could compromise research participant privacy/consent. The source code for the analyses is openly available at 10.5281/zenodo.3604123.

### Cohorts

The FINRISK 2002 study used a random population sample of individuals, aged 25 to 74 years, from 6 geographical areas of Finland. The sampling was stratified by sex, region, and 10-year age group for a population sample of 13 500 individuals; the overall participation rate was 65.2% (n=8798). The sampling has been previously described in detail.<sup>15</sup> Plasma LC-MS was performed successfully on n=8292 participants. After excluding 193 participants with missing covariate data, n=8099 individuals were included in the analyses as the discovery cohort for the present investigation.

The first-generation (ie, the "original") cohort of the FHS included a random sample of two thirds of the adult population of Framingham, MA, who were enrolled in a longitudinal community-based cohort study in 1948. The FHS Offspring includes 5124 participants, children of the first-generation cohort and their spouses, who have been reexamined every 4 to 8 years since the first examination in 1971. The characteristics and study protocol of FHS Offspring cohort have been published.<sup>16</sup> For this study, we considered n=3002 individuals who participated in the 8

### Nonstandard Abbreviations and Acronyms

<b>12-HHTrE</b>	12-hydroxyheptadecatrienoic acid
<b>FHS</b>	Framingham Heart Study
<b>LC-MS</b>	liquid chromatography–mass spectrometry
<b>MS</b>	mass spectrometry
<b>TXA<sub>2</sub></b>	thromboxane A2
<b>TXB<sub>2</sub></b>	thromboxane B2

eicosanoids, the small-molecule derivatives of arachidonic acid and other polyunsaturated fatty acids serve as both activators and suppressors of systemic inflammatory activity.<sup>3</sup> Data derived mainly from animal studies suggest that eicosanoid compounds affect renal vascular tone, urine sodium excretion, peripheral resistance, kidney disease, renin-angiotensin-aldosterone system, and endothelial function, factors that are central to blood pressure (BP) regulation itself.<sup>4–9</sup> Published data have also demonstrated that a few, select eicosanoids, such as

examination cycle of FHS Offspring in 2005 to 2008 and had assays for eicosanoids with LC-MS. After excluding 143 participants with missing covariates, we included n=2859 participants as the replication cohort.

## Ethical Approval

The FINRISK 2002 study was approved by Coordinating Ethics Committee of the Helsinki University Hospital District. FHS Offspring was approved by Boston University Medical Center's Institutional Review Board. All participants in both studies provided written informed consent. Participants' consent to publication of information was not required because the participants remain unidentifiable.

## Clinical Evaluation and Definitions

Participants of both cohorts provided a medical history, including information on medication use, and underwent a physical examination and laboratory assessment of cardiovascular risk factors at baseline. The methods of these examinations have been described previously in detail.<sup>15,16</sup> At all examinations, a healthcare professional performed 2 (FHS Offspring) or 3 (FINRISK) sequential BP measurements using a mercury column sphygmomanometer on seated participants, according to a standardized protocol. We defined the BP at a given examination as the mean of all sequentially measured BP values. We defined hypertension as BP  $\geq 140/90$  mm Hg or use of antihypertensive medication. Antihypertensive medication use was based on self-report in both studies. We defined pulse pressure as systolic minus diastolic BP and mean arterial pressure as [(2×diastolic BP)+systolic BP]/3. We defined body mass index (BMI) as weight (kg) divided by height (m) squared and current smoking as self-reported daily use of tobacco products. In FINRISK 2002, prevalent diabetes mellitus was defined as self-reported diabetes mellitus, a previous diagnostic code indicating diabetes mellitus in the nationwide Care Register for Health Care (*International Classification of Diseases, Tenth Revision [ICD-10]*,<sup>17</sup> codes E10–E14; *International Classification of Diseases, Ninth Revision [ICD-9]*, code 250; or *International Classification of Diseases, Eighth Revision [ICD-8]*, code 250), a previous diabetes mellitus medication purchase (Anatomical Therapeutic Chemical code A10\*) in the nationwide Prescribed Drug Purchase register, or a diabetes mellitus medication code in the nationwide Reimbursed Medication Register. In FHS Offspring, prevalent diabetes mellitus was defined as a fasting plasma glucose  $\geq 7.0$  mmol/L or self-reported use of glucose-lowering medications. Using data from the Hospital Discharge and Drug Reimbursement Registers, we defined asthma in FINRISK using diagnostic codes indicating

asthma (*ICD-10* codes J45-J46 or *ICD-8/9* code 493), a previous asthma medication purchase (Anatomical Therapeutic Chemical codes R03BA, R03BC, R03DC, and R03AK), or having a special reimbursement for asthma medications. We estimated glomerular filtration rate using the Chronic Kidney Disease Epidemiology Collaboration formula.<sup>18</sup> We defined NSAID use by a prescription purchase of medication under Anatomical Therapeutic Chemical group M01A (excluding subgroup M01AX and self-care drug purchases).

## Plasma Sampling

In FINRISK, blood samples were drawn after a minimum of 4 hours of fasting, the samples were kept at room temperature for 20 minutes before centrifugation, and the samples were stored at  $-70^{\circ}\text{C}$ . In FHS, fasting samples were drawn, centrifuged for 22 minutes at  $4^{\circ}\text{C}$ , and separated plasma was stored at  $-80^{\circ}\text{C}$  within 90 minutes of collection.

## Eicosanoid Profiling

Using a directed nontargeted LC-MS approach in conjunction with computational chemical networking of spectral fragmentation patterns, we identified 545 eicosanoids and related oxylipins in the FINRISK. The methods of plasma eicosanoid profiling using LC-MS have been previously described in detail.<sup>13,14</sup> Metabolite data were adjusted for technical variation in off-plate pooled plasma samples and in spike-in internal standards. Missing values were replaced with minimum value for each eicosanoid abundance. The 6 eicosanoids and related oxylipin mediators included in the risk score were matched between FINRISK and FHS by comparing their LC-MS profiles. These metabolites were also identified, if possible, through comparisons with reference standards and online databases.

## Genotyping

The methods of single-nucleotide polymorphism (SNP) genotyping and quality control have been previously described in detail.<sup>19</sup> In short, the participants of FINRISK were genotyped on Illumina CoreExome genotyping array. A reference panel of 1000 genomes was further used to impute genotypes.

## Statistical Analysis

We used R version 3.6.1<sup>20</sup> for all analyses. The source code for the analyses is openly available at 10.5281/zenodo.3604123.<sup>21</sup> Unless otherwise noted, we adjusted all analyses for age, sex, BMI, current smoking, diabetes mellitus, antihypertensive medication, and MS batch. We normalized eicosanoid abundances using median absolute deviation; we calculated the median of the absolute difference from the median,

and used this value to scale all analyte values for a given assay plate. We used linear and logistic regression models to examine the associations between each eicosanoid molecule and BP traits (systolic BP, diastolic BP, pulse pressure, mean arterial pressure, and hypertension). We adjusted for multiple comparisons using Bonferroni correction to minimize the probability of type I error.<sup>22</sup> Relations between eicosanoids significantly associated with systolic blood arterial pressure were assessed using Spearman correlation and ordered using hierarchical cluster analysis with complete linkage method. We assessed the multivariable association between eicosanoids significantly related to systolic BP using stepwise linear regression modeling with forward selection and a Bonferroni-corrected inclusion threshold of  $P=0.05/545$ . For the 6 eicosanoids that remained in the models, we calculated eicosanoid risk scores according to the formula  $\beta_1X_1+\beta_2X_2+\dots+\beta_nX_n$ , with  $X_n$  denoting the standardized value for the nth eicosanoid abundance, and  $\beta_n$  denoting the regression coefficient from the regression model for systolic BP containing the statistically significant eicosanoids.<sup>23</sup> We assessed the odds of hypertension and increase in systolic BP by 1-SD increases and by quartiles of the risk scores using unadjusted and multivariable adjusted logistic and linear regression models. We replicated these analyses in FHS Offspring using the eicosanoid abundances in FHS and the regression coefficients from FINRISK.

We also assessed the association between the eicosanoid risk score and systolic BP in subgroups by aspirin use, asthma status, age (younger than versus older than the median age of 49 years), BMI (<30 versus  $\geq 30$  kg/m<sup>2</sup>), glomerular filtration rate (<90 versus  $\geq 90$  mL/min), and NSAID use. We determined the association of the eicosanoid risk score with age, BMI, and estimated glomerular filtration rate using the Pearson correlation. We compared eicosanoid risk score levels between subgroups by aspirin use, asthma status, and NSAID use using the 2-sample *t* test. To analyze the causative role of the eicosanoid risk score, we performed genome-wide association study (GWAS) and 2-sample mendelian randomization (MR) in the study sample. To account for ordered patterns in genetic data, we calculated multidimensional scaling based on raw Hamming distances using PLINK<sup>24</sup> version 1.9. We performed the GWAS for the continuous eicosanoid risk scores and the autosomes using SNPTEST<sup>25</sup> version 2.5.2, adjusted for age, sex, batch, and first 10 multidimensional scaling axes. We included in the mendelian randomization the SNPs that had Hardy-Weinberg equilibrium  $>1E-6$ ,  $P<5E-8$ , and minor allele frequencies  $>0.01$  using TwoSampleMR.<sup>26</sup> For the outcome of the mendelian randomization, we used the GWAS results for automated systolic BP measurements in UK Biobank.<sup>27,28</sup>

We estimated the causative roles using 5 distinct methods: inverse variance weighted,<sup>29</sup> weighted median,<sup>30</sup> weighted mode,<sup>30</sup> simple mode,<sup>31</sup> and MR-Egger.<sup>32</sup>

## RESULTS

The characteristics of FINRISK (n=8099; mean age, 48.0±13.1 years; 53.1% women) and FHS (n=2859; mean age, 66.3±8.9 years; 54.7% women) cohorts are shown in the Table.

### Association Between Eicosanoids and BP Traits

Of the eicosanoids and related oxylipin mediators, 187 (34.3%) were significantly associated with systolic BP, 124 (22.8%) with diastolic BP, 177 (32.5%) with mean arterial pressure, 161 (29.5%) with pulse pressure, and 155 (28.4%) with hypertension in FINRISK (Figure 1, Table S1). We selected systolic BP as our main outcome variable because of its strong association with cardiovascular diseases. We observed 175 (93.6%) positive and 12 (6.4%) negative associations for systolic BP (Figure 1, Table S1). The heat maps of pairwise correlations for the 187 metabolites related to systolic BP are shown in Figure 2 and Figure S1. This analysis revealed strong overall correlations, but only minor clustering of the eicosanoids.

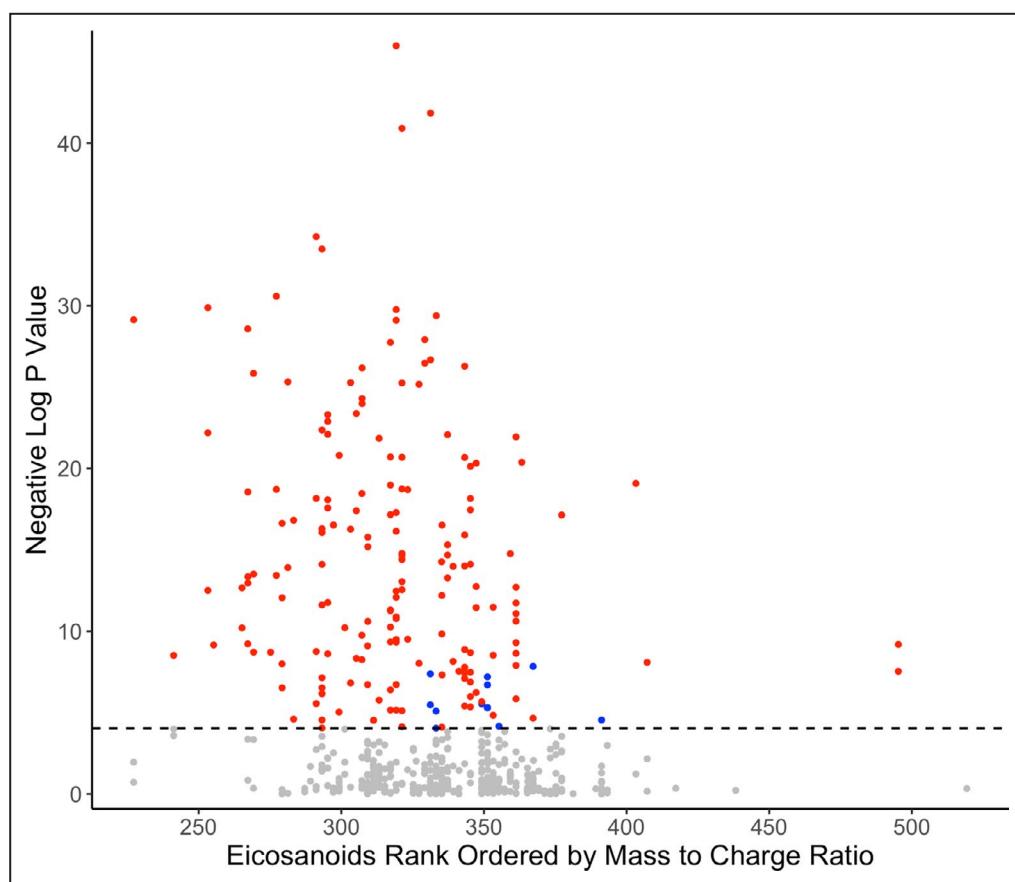
### Independent Determinants of BP and Hypertension

We used forward selection linear regression modeling with a Bonferroni-corrected inclusion threshold to define a set of metabolites that was independently

**Table. Characteristics of the Discovery (FINRISK) and Replication (FHS) Samples**

Characteristics	FINRISK 2002	FHS
No. of subjects	8099	2859
Age, mean (SD), y	48.0 (13.1)	66.3 (8.9)
Women, N (%)	4300 (53.1)	1564 (54.7)
BMI, mean (SD), kg/m <sup>2</sup>	26.9 (4.7)	28.3 (5.4)
Systolic blood pressure, mean (SD), mm Hg	135.1 (20.0)	128.5 (17.2)
Diastolic blood pressure, mean (SD), mm Hg	79.0 (11.3)	73.4 (10.1)
Pulse pressure, mean (SD), mm Hg	56.1 (16.1)	55.1 (16.0)
Mean arterial pressure, mean (SD), mm Hg	97.7 (12.7)	91.8 (10.5)
Hypertension, N (%)	3567 (44.0)	1673 (58.5)
Antihypertensive medication, N (%)	1177 (14.5)	1389 (48.6)
Current smoker, N (%)	2097 (25.9)	256 (9.0)
Diabetes mellitus, N (%)	446 (5.5)	393 (13.7)

Continuous variables are presented as mean (SD). Categorical variables reported as absolute and relative frequencies. BMI indicates body mass index; and FHS, Framingham Heart Study.



**Figure 1.** Manhattan plot for associations between metabolites and systolic blood pressure in FINRISK 2002.

A significant association was observed for 187 of the 545 eicosanoids. Positive correlations are denoted in red, negative in blue, and insignificant in gray. Eicosanoids are ordered by the value of mass/charge ratio. Dashed line represents the Bonferroni-corrected ( $P=0.05/545$ ) level of significance. Analyses are adjusted for age, sex, body mass index, current smoking, diabetes mellitus, antihypertensive medication, and batch.

associated with systolic BP. In FINRISK, these 6 metabolites were 11-dehydro-2,3-dinor thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 12-hydroxyheptadecatrienoic acid (12-HHTrE), 265.1809/3.57 (putative eicosanoid), 295.2279/4.89 (putative eicosanoid), 319.2280/5.67 (unknown), and adrenic acid (Table S2). Of these 6 metabolites, 2 could not be detected in FHS plasma samples (11-dehydro-2,3-dinor-TXB<sub>2</sub> and 295.2279/4.89). Comparing single-metabolite associations, adjusted for relevant covariates, demonstrated that effect sizes between the metabolites were highly consistent across the 2 cohorts (Figure 3, Table S3).

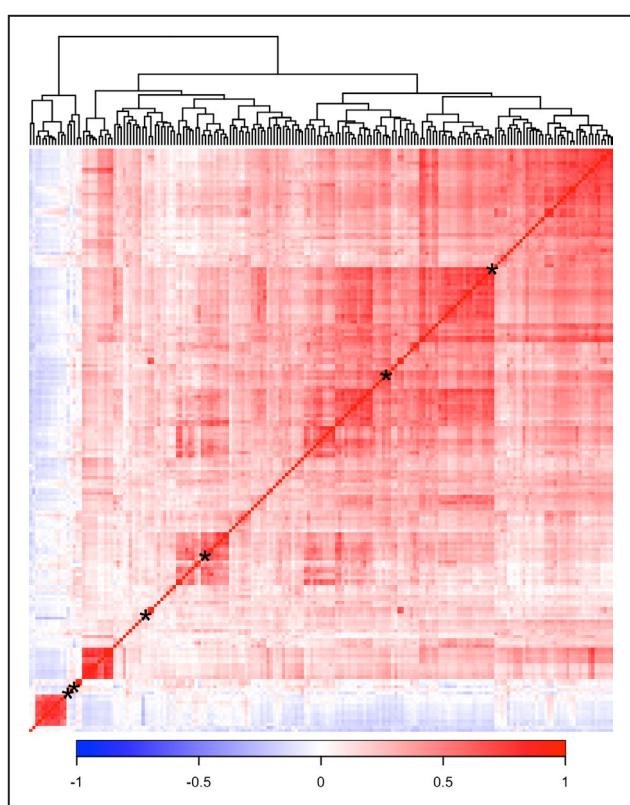
### Eicosanoid Risk Score

We defined an eicosanoid risk score using the effect sizes in FINRISK for the 6 previously mentioned metabolites (Table S2). The abundances of the 2 nondetected metabolites in FHS were treated as zero values. Individuals in the top risk quartile had 9.0 (95% CI, 8.0–10.1) mm Hg higher systolic BP in FINRISK and 6.8 (95% CI, 5.1–8.5) mm Hg higher

systolic BP in FHS compared with individuals in the lowest quartile (Figures 4, 5 and Tables S4, S5, S6). The odds for hypertension were 2.3 (95% CI, 2.0–2.6) and 2.0 (95% CI, 1.6–2.5), respectively, for the top quartile compared with the lowest quartile (Figures 4 and 5). These associations were consistent across FINRISK and FHS. We observed no differences in these associations in subgroups by aspirin use, asthma status, age, BMI, kidney function, and NSAID use (Figure S2). The correlations of the eicosanoid risk score with age, BMI, and estimated glomerular filtration rate were 0.11 ( $P<0.001$ ), 0.07 ( $P<0.001$ ), and -0.02 ( $P=0.04$ ), respectively. We observed no significant differences in the eicosanoid risk score levels in subgroups by aspirin use, asthma status, and NSAID use ( $P>0.5$ ).

### Two-Sample Mendelian Randomization

We observed 222 SNPs in 2 chromosomes significantly associated with the eicosanoid risk score (Figure S3, Table S7). To account for linkage-disequilibrium, in



**Figure 2.** Correlation matrix for the 187 plasma metabolites related to systolic blood pressure in FINRISK 2002.

Relations between eicosanoids were calculated using Spearman correlation and ordered using hierarchical cluster analysis with complete linkage method. Only eicosanoids related to systolic blood pressure were included in the correlation matrix. Asterisk denotes position of the 6 metabolites in our eicosanoid risk score (Figure 3).

each 10-kb window ( $r^2 < 0.001$ ) only the SNP with lowest  $P$  value was retained (Table S8). We used the ( $n=436,419$ ) GWAS results for systolic BP in UK Biobank as the outcome variables.<sup>27,28</sup> The 2-sample mendelian randomization for the 3 SNPs and automated systolic BP measurement was nonsignificant (false discovery rate corrected  $P > 0.26$ ; Table S9).

## DISCUSSION

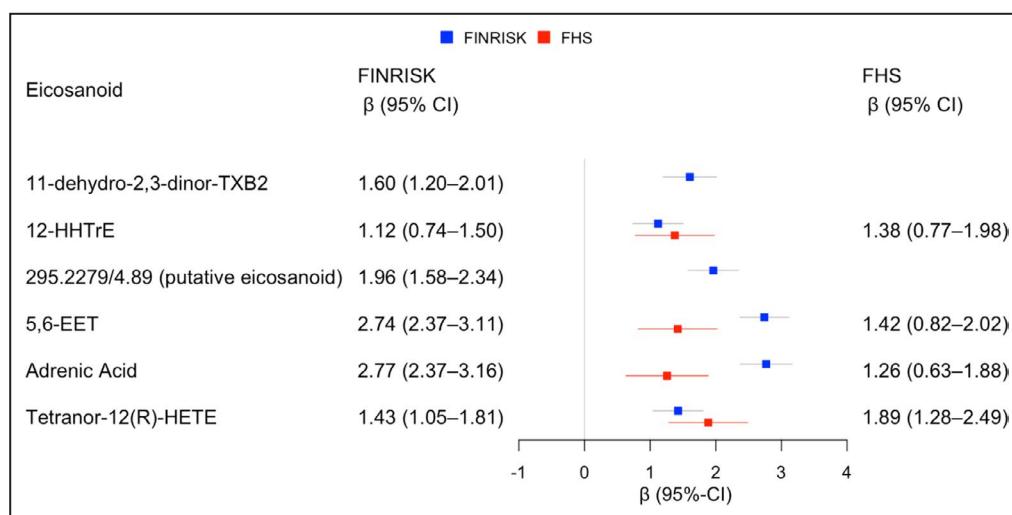
Using a directed nontargeted LC-MS approach in well-phenotyped, large community-based cohorts, we identified 187 eicosanoids and related oxylipins that were associated with systolic BP. FINRISK 2002 participants in the top quartile of the eicosanoid risk score had 9.0 mm Hg greater systolic BP and a >2-fold odds of hypertension, compared with individuals in the lowest quartile. These findings were replicated in the FHS Offspring participants.

The upstream initiation of inflammatory activity in humans is governed mainly by substrates and products of polyunsaturated  $\omega$ -3 and  $\omega$ -6 20-carbon fatty

acids.<sup>7,33</sup> The small-molecule derivatives of arachidonic acid and other polyunsaturated fatty acids, termed eicosanoids, serve as both activators and suppressors of systemic inflammatory activity.<sup>34,35</sup> Most research on systemic inflammation in humans has focused on downstream markers of inflammatory activity, such as cytokines and short-term phase reactants. Recent work by us and others has shown that long-term elevation in these downstream markers is associated with a variety of cardiovascular disease risk traits and outcomes.<sup>36,37</sup> In particular, several cross-sectional and prospective studies in humans have found an association of plasma concentrations of downstream low-grade inflammation markers, such as interleukin-6, intercellular adhesion molecule-1, CRP (C-reactive protein), and tumor necrosis factor- $\alpha$ , with arterial stiffness and hypertension.<sup>38–44</sup> Although downstream markers of inflammation are associated with hypertension and a variety of cardiovascular disease outcomes, evidence for a clinically important, causal role of these biomarkers has been mixed.<sup>45</sup> In addition, despite inflammation being pivotal in the development of atherosclerosis and certain medications with anti-inflammatory properties clearly reduce cardiovascular disease risk, the extent to which any given inflammatory pathway warrants attention as a direct putative target for therapy is unknown.<sup>46</sup> Such results have now led experts to suggest that, where inflammation is concerned, causal factors may be upstream.<sup>45</sup>

This study is the first to comprehensively examine the association between eicosanoids and BP in humans. Prior studies with study samples consisting of tens of hypertensive subjects with a panel of a few, mainly cytochrome P450 pathway eicosanoids have demonstrated that eicosanoids, in general, affect regulation of renal function, vascular tone, and the development of hypertension.<sup>12,47–50</sup> Our results from a large, population-based sample demonstrate that a large number of eicosanoid species are related to BP in both a positive and a negative way. In addition, we demonstrate that a distinct eicosanoid score is related to a >2-fold odds of hypertension. The subgroup analyses demonstrate that the association between our eicosanoid risk score and systolic BP was highly consistent, even in states that affect eicosanoid metabolism and excretion. Although the number of individuals in some subgroups was low, we observed no differences in the relation between the risk score and BP in subgroups by asthma status, age, aspirin use, BMI, and kidney function. Furthermore, the eicosanoid risk score demonstrated only weak correlations with these phenotypes, implying an independent role for eicosanoids in hypertension risk.

Eicosanoids are metabolized via 3 general pathways that involve cytochrome P450 monooxygenases, cyclooxygenases, and lipoxygenases. Several

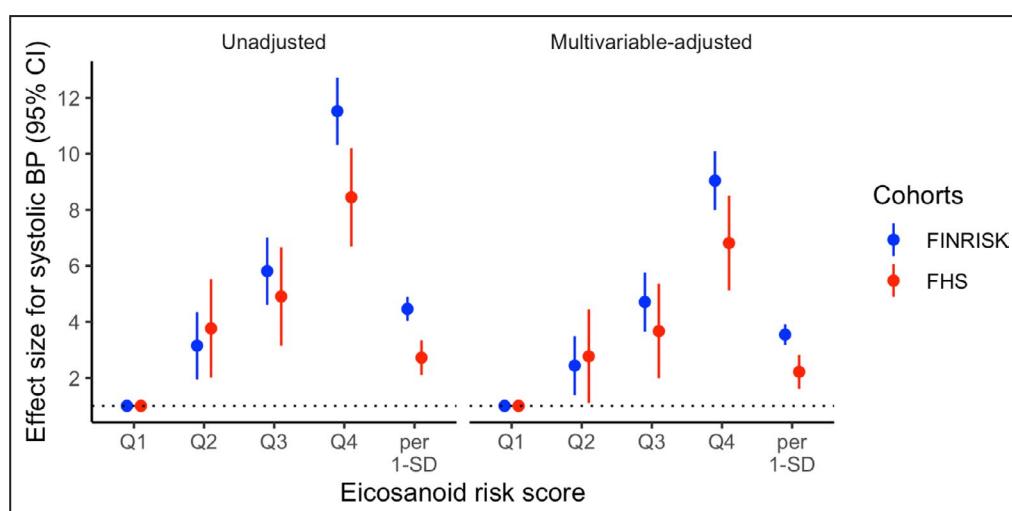


**Figure 3.** The associations between a subset of 6 metabolites and systolic blood pressure (BP) in FINRISK and replication of results in FHS (Framingham Heart Study).

The  $\beta$  coefficients are for the association between 1-SD increase in metabolite concentration and the absolute change of systolic BP (mm Hg) in the 2 study cohorts. All models were adjusted for age, sex, body mass index, current smoking, diabetes mellitus, antihypertensive medication, and batch. Of the 6 eicosanoids observed in FINRISK, 2 were not observed in FHS plasma samples. EET indicates epoxyeicosatrienoic acid; HETE, hexadecatrienoic acid; HHTrE, hydroxyheptadecatrenoic acid; and TXB<sub>2</sub>, thromboxane B<sub>2</sub>.

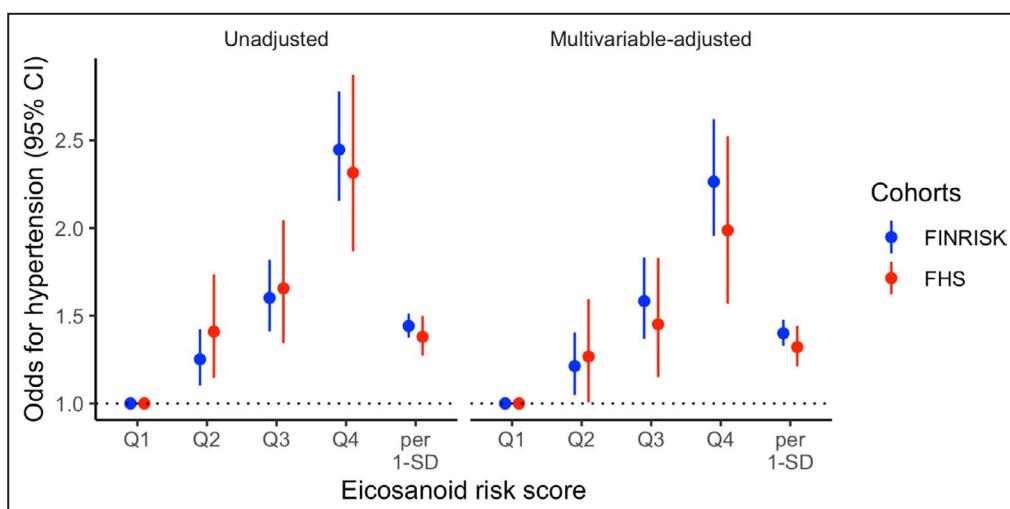
of the identified metabolites that remained in the 6-eicosanoid risk score are members of these pathways. In addition, the key metabolites included in the eicosanoid risk score include both intermediate (eg, adrenic acid and 12-HHTrE) and terminal (eg, 11-dehydro-2,3-dinor-TXB<sub>2</sub>), potentially reflecting key eicosanoid pathways and species related to

BP regulation. The cytochrome P450 pathway metabolizes arachidonic acid to several eicosanoids, including 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids.<sup>8</sup> These metabolites are critical in BP regulation and provide cardioprotective and renoprotective effects in chronic kidney disease.<sup>8</sup> Cyclooxygenase pathway produced prostanoids are



**Figure 4.** The association between the risk score and systolic blood pressure (BP) in FINRISK and FHS (Framingham Heart Study).

We calculated eicosanoid risk score for each participant according to the formula  $\beta_1X_1+\beta_2X_2+\dots+\beta_nX_n$ , with  $X_n$  denoting the standardized value for the nth eicosanoid abundance, and  $\beta_n$  denoting the regression coefficient from the regression model containing the indicated eicosanoids. Analyses are adjusted for age, sex, body mass index, current smoking, diabetes mellitus, antihypertensive medication, and batch. Q indicates quartile.



**Figure 5. The association between the risk score and hypertension in FINRISK and FHS (Framingham Heart Study).**

We calculated eicosanoid risk score for each participant according to the formula  $\beta_1X_1 + \beta_2X_2 + \dots + \beta_nX_n$  with  $X_n$  denoting the standard value for the nth eicosanoid abundance, and  $\beta_n$  denoting the regression coefficient from the regression model in FINRISK containing the indicated eicosanoids. Multivariable analyses are adjusted for age, sex, body mass index, current smoking, diabetes mellitus, antihypertensive medication, and batch. Q indicates quartile.

involved in BP homeostasis and, in particular, short-lived thromboxane A<sub>2</sub> (TXA<sub>2</sub>; half-life, 30 seconds) has important role in various cardiovascular diseases through action on platelet aggregation, vasoconstriction, and proliferation.<sup>51,52</sup> TXA<sub>2</sub> is metabolized to inactive TXB<sub>2</sub>, which is degraded through 2 major pathways (dehydrogenation and  $\beta$ -oxidation) and their combination, which results in the formation of 11-dehydro-2,3-dinor-TXB<sub>2</sub>.<sup>53,54</sup> Currently, factors affecting the relative production of 11-dehydro-2,3-dinor-TXB<sub>2</sub> and analyte prognostic utility are not known.<sup>52</sup> However, 11-dehydro-2,3-dinor-TXB<sub>2</sub> may have a role in atherothrombosis.<sup>54</sup> Another metabolite included in our 6-eicosanoid risk score, 12-HHTre, is a nonenzymatic degradation product of TXA<sub>2</sub> and prostaglandin H<sub>2</sub> (an important precursor for eicosanoids).<sup>55</sup> 12-HHTre is a natural ligand for leukotriene B(4) receptor 2 and is linked to synthesis of prostacyclin (prostaglandin I<sub>2</sub>), a potent vasodilator, and the main metabolite of 12-HHTre has antagonist effect to TXA<sub>2</sub> receptor.<sup>56,57</sup> In addition to eicosanoid pathway products, adrenic acid was also included in our risk score. Adrenic acid is a polyunsaturated 22-carbon fatty acid and mainly a substrate for eicosanoid production, and it has been associated with the regulation of adrenal blood flow.<sup>58</sup> Given the findings from our study and from previous experimental trials, these results provide a strong biological basis for how eicosanoids could affect human BP regulation through several different mechanisms. In particular, elevated 11-dehydro-2,3-dinor-TXB<sub>2</sub> and 12-HHTre levels may be result of elevated TXA<sub>2</sub> activity but their

independent role and possible prognostic utility need to be evaluated in future research.

Our study has several strengths, such as unselected population sample, external replication of our results, and assays of a large number of eicosanoids. However, our results must be interpreted in the context of potential limitations. First, LC-MS is a highly sensitive method for assessing circulating metabolites. Particularly, of the 6 metabolites that remained in the final forward-selection regression model in FINRISK, only 4 were observed in FHS samples. This could in part be explained by the between-cohort age and smoking disparities. Second, the unambiguous metabolite classification and identification is still a challenge in high-throughput LC-MS. However, we have previously demonstrated that these signals are highly consistent with known and putative eicosanoids and related oxylipins in human plasma.<sup>14</sup> Third, many eicosanoids have short half-lives of <1 hour and the variance of the measured metabolites is expected to be highly affected by sample processing methods. However, our plasma samples were stored at -70 to 80°C on-site following a strict protocol, and we studied standardized, rather than absolute, metabolite concentrations. Finally, our study demonstrates a strong proof-of-concept association between eicosanoids and BP. Although much is known of eicosanoid physiology,<sup>4–12</sup> additional mechanistic and experimental studies are needed to assess the precise identities and functions of many of the observed metabolites. The eicosanoids included in the risk score are known to be produced in neuronal

tissues, platelets, leukocytes, and smooth muscle cells,<sup>59</sup> which could be used as starting points for in vitro experiments.

## CONCLUSIONS

Plasma eicosanoids demonstrate strong associations with BP in the general population, and differences between eicosanoid profiles are observed between normotensive versus hypertensive participants. Intriguingly, although most of the associations were positive (harmful species), we observed protective molecules as well. In our mendelian randomization analysis, however, we were unable to demonstrate a causal association between eicosanoids and BP. However, this analysis could be limited by the small GWAS sample size. Additional preclinical analyses are therefore needed to examine the causality between eicosanoids and BP and to clarify whether eicosanoids could serve as potential targets for therapeutic intervention.

## ARTICLE INFORMATION

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### Supplementary Materials

Tables S1–S9

Figures S1–S3

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Comprehensive biomarker profiling of hypertension  
in 36 985 Finnish individuals.  
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**IV**



# Original Article

OPEN

## Comprehensive biomarker profiling of hypertension in 36 985 Finnish individuals

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**Objective:** Previous studies on the association between metabolic biomarkers and hypertension have been limited by small sample sizes, low number of studied biomarkers, and cross-sectional study design. In the largest study to date, we assess the cross-sectional and longitudinal associations between high-abundance serum biomarkers and blood pressure (BP).

**Methods:** We studied cross-sectional ( $N = 36\,985$ ; age  $50.5 \pm 14.2$ ; 53.1% women) and longitudinal ( $N = 4197$ ; age  $49.4 \pm 11.8$ , 55.3% women) population samples of Finnish individuals. We included 53 serum biomarkers and other detailed lipoprotein subclass measures in our analyses. We studied the associations between serum biomarkers and BP using both conventional statistical methods and a machine learning algorithm (gradient boosting) while adjusting for clinical risk factors.

**Results:** Fifty-one of 53 serum biomarkers were cross-sectionally related to BP (adjusted  $P < 0.05$  for all). Conventional linear regression modeling demonstrated that LDL cholesterol, remnant cholesterol, apolipoprotein B, and acetate were positively, and HDL particle size was negatively, associated with SBP change over time (adjusted  $P < 0.05$  for all). Adding serum biomarkers (cross-sectional root-mean-square error: 16.27 mmHg; longitudinal: 17.61 mmHg) in the model with clinical measures (cross-sectional: 16.70 mmHg; longitudinal 18.52 mmHg) improved the machine learning model fit. Glucose, albumin, triglycerides in LDL, glycerol, VLDL particle size, and acetoacetate had the highest importance scores in models related to current or future BP.

**Conclusion:** Our results suggest that serum lipids, and particularly LDL-derived and VLDL-derived cholesterol measures, and glucose metabolism abnormalities are associated with hypertension onset. Use of serum metabolite determination could improve identification of individuals at high risk of developing hypertension.

**Keywords:** amino acids, blood pressure, hypertension, inflammation, lipids, nuclear magnetic resonance spectroscopy

**Abbreviations:** BCAA, branched amino acids; BP, blood pressure; NMR, nuclear magnetic resonance spectroscopy; PUFA, polyunsaturated fatty acids; SCFA, short-chain fatty acid; SFA, saturated fatty acids

## INTRODUCTION

Although many hypertension risk factors have been identified, the exact mechanisms behind the age-related increase in blood pressure (BP) remain elusive. Present-day metabolomics offers a promising method to identify and study metabolic biomarkers associated with current hypertension and future hypertension onset. However, only a few prior studies have reported associations between a large number of serum metabolic measures and hypertension in large population cohorts with a longitudinal design [1]. Therefore, additional research on the cross-sectional and longitudinal associations between metabolic biomarkers and hypertension in large population samples are warranted. This information could potentially be used to elucidate the metabolic underpinnings of elevated BP [2].

Recent development in high-throughput nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiling offers a method for studying advanced lipid measures, fatty acids, amino acids, and inflammatory proteins in large population samples [2,3]. In the present study, we aim to advance the current knowledge on the associations between circulating metabolic biomarkers and BP in well phenotyped, representative cross-sectional and longitudinal population samples of 36 985 and 4197 participants, respectively.

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## METHODS

### Cross-sectional study sample

Our study sample consists of six Finnish population samples, FINRISK 1997, FINRISK 2002, FINRISK 2007, FINRISK 2012, Health 2000, and FinHealth 2017 (Fig. S1, <http://links.lww.com/HJH/B807>). All studies are coordinated by the Finnish Institute for Health and Welfare. The studies were approved by the Coordinating Ethics Committee of the Helsinki University Hospital District and all participants gave written informed consent.

The FINRISK studies have been performed every 5 years since 1972 to monitor the development of cardiovascular risk factors in Finnish population aged 25–74 years [4]. The FINRISK 1997–2012 study samples consist of participants randomly drawn from the national population register from up to six geographical areas in Finland. The proportion of individuals who participated in the health examinations varied between 56 and 72% [4]. Health 2000 is a multidisciplinary epidemiological survey of individuals aged at least 30 years living in mainland Finland [5]. The participants, living in 80 municipalities around Finland, were randomly drawn from the population register in the year 2000 to participate in a health examination. The participation rate for health examination was 83%. In FinHealth 2017 study [6], participants aged at least 18 years were drawn from the national population register for municipalities in mainland Finland to participate in a health examination. The participation rate of the health examination was 58%.

Data were available for a total of 38404 FINRISK, Health 2000, and FinHealth 2017 participants. We excluded 1419 participants because of missing covariates for a final study sample of 36 985 individuals who were included in the analysis (Fig. S1, <http://links.lww.com/HJH/B807>).

### Longitudinal study sample

FINRISK 2007 participants who lived in Helsinki, Vantaa, Turku and Loimaa area were invited to a follow-up examination in 2014 (participation rate for health examination in 2014 54%) [7,8]. All living participants of Health 2000 were invited to participate in a follow-up examination in 2011 (participation rate for health examination 59%) [9]. Data were available for 968 FINRISK 2007 and 3229 Health 2000 participants with repeated BP measurements for a final longitudinal study sample of 4197 participants (Fig. S1, <http://links.lww.com/HJH/B807>).

### Study flow

In the FINRISK and FinHealth 2017 studies, after filling in a questionnaire on sociodemographic information, lifestyles, medications, and medical history at home, the participants attended a physical examination at a local study site. The participants underwent measurements for height and weight and blood samples were drawn mainly after a minimum of 4 h of fasting [4]. A study nurse measured sitting BP three times from the right arm using a mercury sphygmomanometer with an appropriately sized cuff.

In the Health 2000–2011 study, participants were interviewed by centrally trained interviewers on sociodemographic information, lifestyle, medications, and medical

history 1–6 weeks before attending physical examination at local study sites. The participants underwent measurements for height and weight. Overnight fasting blood samples were drawn. A study nurse measured sitting BP two times from the right arm using a mercury sphygmomanometer and a 15 × 43 cm sized cuff; a larger cuff was used when needed.

### Serum samples and storage

In all studies, samples were delivered in dry ice to the Finnish National Institute for Health and Welfare and stored at –70 °C [5,10]. Metabolic analyses were performed using 1H-NMR spectroscopy on highly automated platform (Nightingale Health Plc, Helsinki, Finland; biomarker quantification version 2016) [3]. In short, 350 µm serum aliquots were quantified in molar concentration units independently from other samples in the same well plate or same cohort [11]. Measured NMR concentrations have been reported to be consistent with the available clinical chemistry assays (cholesterol measures, apolipoproteins, total triglycerides, glucose, creatinine, and albumin) performed soon after sample collection [10]. The utilized NMR metabolomics technology has received regulatory approval (CE) and 37 biomarkers in the panel have been certified to diagnostic use [11]. We included in our core analyses 53 circulating biomarkers and also studied 97 lipoprotein measures related to 14 lipoprotein subclasses.

### Outcome variables and covariates

The mean of the last two BP measurements was used to determine SBP and DBP. Hypertension was defined as SBP at least 140 mmHg, DBP at least 90 mmHg, or self-reported use of antihypertensive medication. BMI was defined as weight divided by the square of the body height. Use of lipid medication, diabetes, smoking, and leisure-time physical activity were self-reported. Leisure-time activity was divided into four categories: sedentary, light activity for over 4 h per week, fitness training or other strenuous exercise for over 3 h per week, and competitive sports. Smoking was defined as daily use of tobacco products.

### Statistical methods

All metabolic biomarkers (including percentages) were centered to zero and standardized to unit variance (Table S1, <http://links.lww.com/HJH/B807> and Table S2, <http://links.lww.com/HJH/B807>) to simplify pair-wise comparisons and graphical representation of the results. Unless otherwise noted, we adjusted all analyses for age, sex, BMI, smoking, diabetes mellitus, leisure-time physical activity, antihypertensive medication (unless the dependent variable was hypertension), lipid medication, and cohort. We studied the associations for baseline BP and SBP change in follow-up with baseline metabolic measures using linear and logistic regression models. We adjusted analyses for multiple testing using Benjamini–Hochberg correction [12]. The SBP, and not DBP was studied in longitudinal analysis because of its linear relation with age. We also performed the previous analyses stratified by median age (50.5 years) and sex. To assess the validity of sample pooling, we also performed an inverse variance-weighted fixed-effect meta-

analysis for per cohort results in cross-sectional (six cohorts) and longitudinal (two cohorts) study samples.

In addition to conventional (univariable) statistical approaches, we assessed the multivariable associations of the 53 circulating biomarkers with baseline and follow-up SBP using a XGBoost gradient boosting machine learning algorithm. First, we used three sets of model covariates to assess model fit: only clinical characteristics, only metabolic measures, and the combination of clinical characteristics and metabolic measures [13]. In the longitudinal models, baseline SBP was included in among the covariates. We trained our cross-sectional model using a leave-one-group-out cross-validation in FINRISK 1997–2002 and Health 2011 and used FinHealth 2017 for testing. We used root-mean-square error to measure training fit in cross-validation. In the longitudinal analyses, we used the Health 2000–2011 cohort for training with five-fold cross-validation and the FINRISK 2007–2014 cohort for testing. We performed Bayesian optimization with the R package ‘mlrMBO’ to tune the hyperparameters using 42 (six times the number of hyperparameters) preliminary rounds followed by 100 optimization rounds [14]. We constructed partial dependency plots with the R package ‘pdp’ to study and visualize the (marginal) associations between model covariates and SBP [15]. We estimated the fit of final gradient boosting models using the root-mean-square error.

We used R version 3.6.1 for all statistical analyses and the source code for the analyses is openly available at doi:10.5281/zenodo.3625488 [16,17].

## RESULTS

The characteristics of the cross-sectional ( $N=36\,985$ , mean age  $50.5 \pm 14.2$  years, 53.1% women) and longitudinal ( $N=4197$ , mean age at baseline  $49.4 \pm 11.8$  years, 55.3% women) study samples are reported in Table 1 and Table S3, <http://links.lww.com/HJH/B807>, respectively. The sample selection flow is presented in Fig. S1, <http://links.lww.com/HJH/B807>.

In the conventional cross-sectional analyses performed using linear and logistic regression models, only two amino

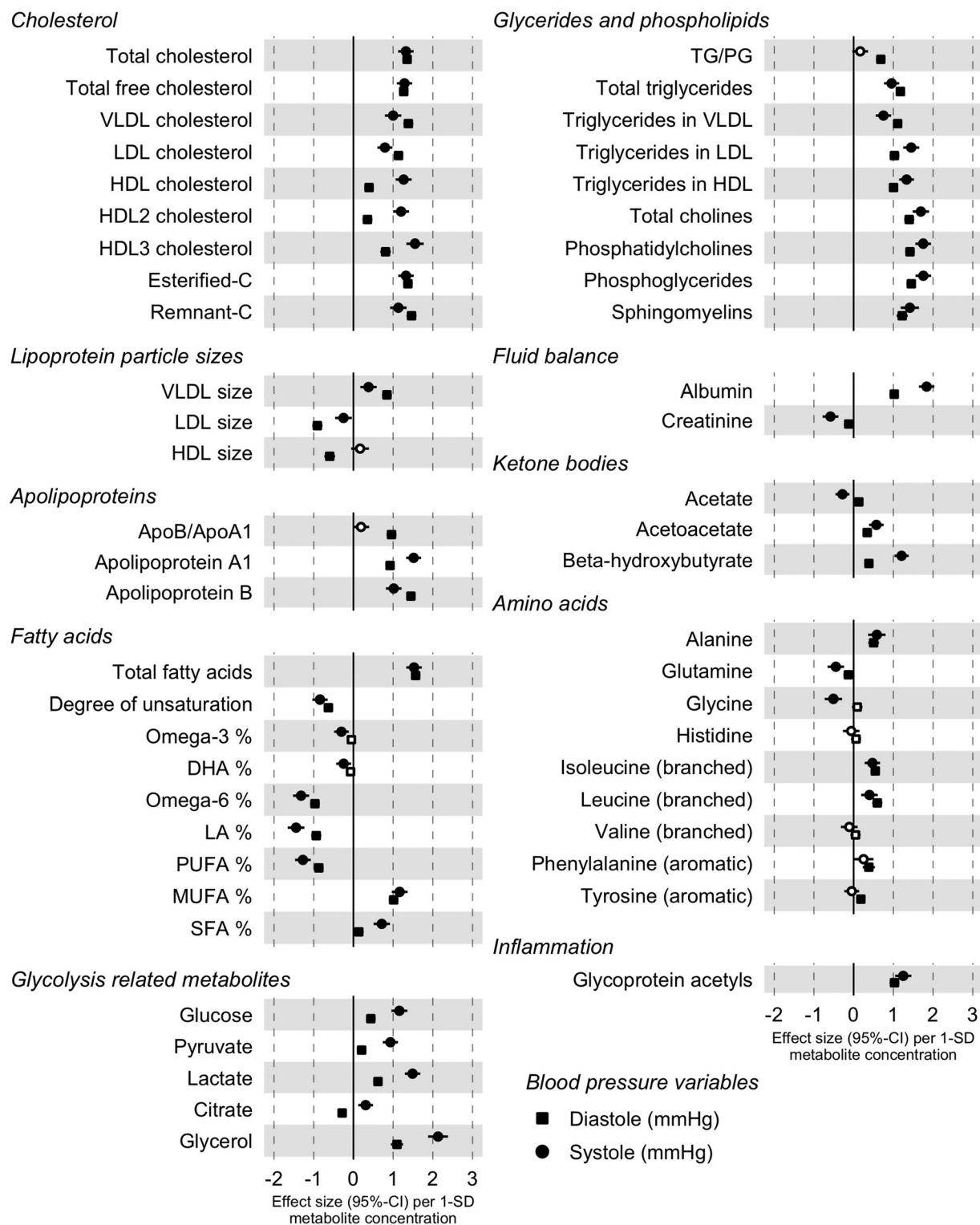
acids (histidine and valine) of all 53 circulating biomarkers included in our analysis were not associated with BP (Fig. 1, Table S4, <http://links.lww.com/HJH/B807>). Six metabolic measures, docosahexaenoic acid, citrate, creatinine, histidine, valine, and tyrosine, were not associated with hypertension (Fig. S2, <http://links.lww.com/HJH/B807>, Table S4, <http://links.lww.com/HJH/B807>). Stratified analysis by sex (Fig. S3, <http://links.lww.com/HJH/B807>, Table S5, <http://links.lww.com/HJH/B807>) and median age (Fig. S4, <http://links.lww.com/HJH/B807>, Table S6, <http://links.lww.com/HJH/B807>) were highly consistent; men compared with women and younger participants compared to older participants had in some cases slightly larger effect sizes. However, acetate was negatively associated with hypertension in women only and acetoacetate positively associated with hypertension in men only. In older than median age participants, total cholesterol, low-density lipoprotein (LDL) cholesterol, esterified cholesterol, and high-density lipoprotein (HDL) cholesterol were not associated with hypertension. Large and extremely large HDL fractions were negatively and medium and small HDL fractions positively associated with hypertension (Fig. S5, <http://links.lww.com/HJH/B807>)

In the longitudinal analyses with conventional statistical approaches ( $N=4197$ ), we examined the associations between baseline metabolites and change in SBP between baseline and follow-up of 7–11 years (Fig. 2, Table S7, <http://links.lww.com/HJH/B807>). We observed that LDL cholesterol [ $\beta=0.74$  mmHg per 1SD normalized concentration; 95% confidence interval (CI) 0.28–1.20 mmHg;  $P=0.01$ ], remnant cholesterol [ $\beta=0.62$  mmHg; 95% CI 0.14–1.10 mmHg;  $P=0.03$ ], apolipoprotein B [ $\beta=0.63$  mmHg; 95% CI 0.14–1.11 mmHg;  $P=0.03$ ], and acetate [ $\beta=0.83$  mmHg; 95% CI 0.25–1.41 mmHg;  $P=0.02$ ] were associated with a BP increase and average HDL particle size [ $\beta=-0.89$ ; 95% CI –1.46 to –0.32 mmHg;  $P=0.01$ ] with a BP decrease during follow-up. Large and extremely large HDL fractions were negatively and other lipoprotein fractions (VLDL, IDL, and LDL) mostly positively associated with SBP change in follow-up (Fig. S6, <http://links.lww.com/HJH/B807>).

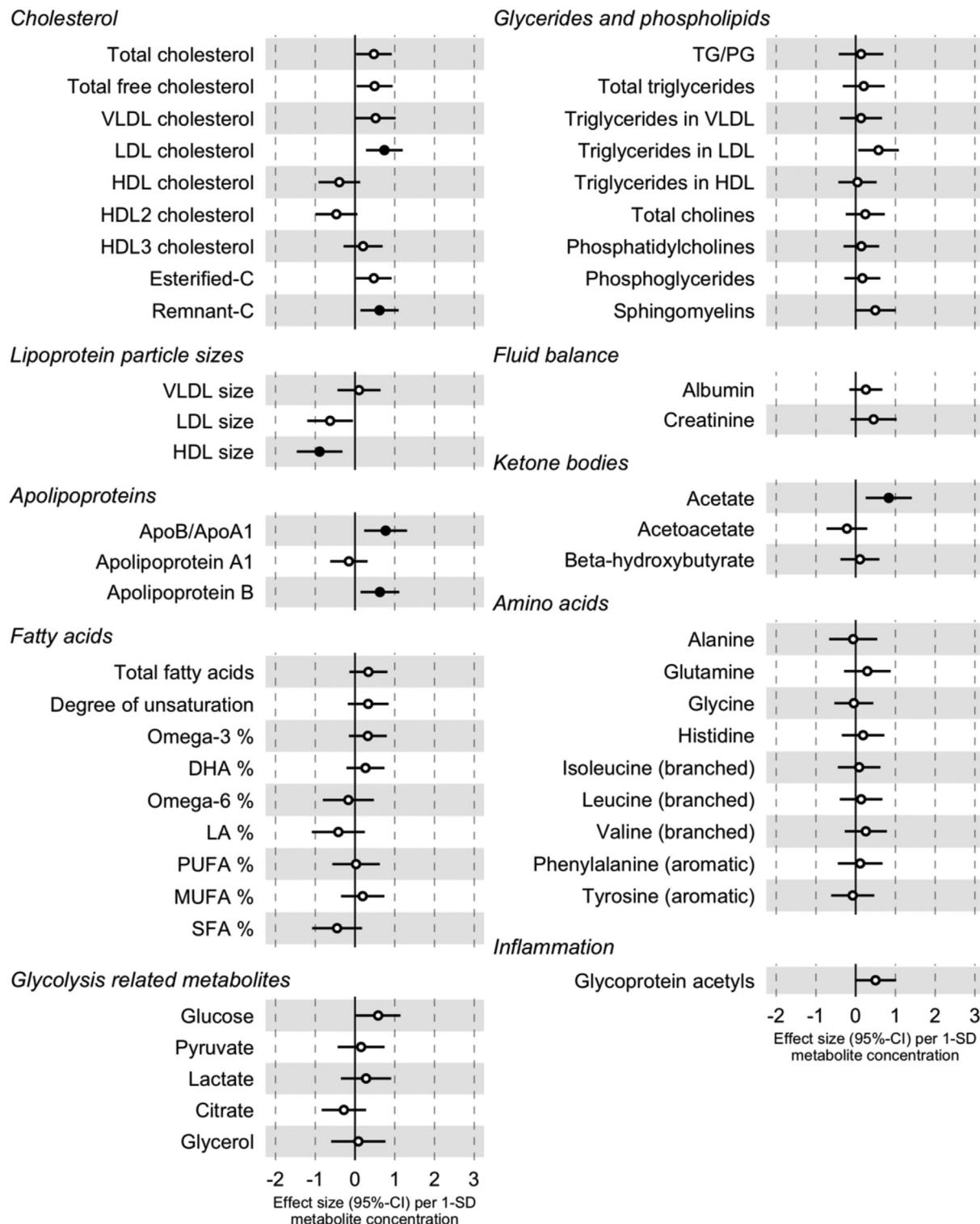
**TABLE 1. Characteristics of the cross-sectional study sample**

Characteristics	Total	FINRISK 1997	Health 2000	FINRISK 2002	FINRISK 2007	FINRISK 2012	FINRISK 2017
<i>N</i>	36 985	7106	6039	7565	5825	5420	5030
Age (years) (SD)	50.5 (14.2)	48.2 (13.1)	52.4 (14.7)	47.9 (13.1)	50.9 (13.9)	51.2 (14.0)	54.1 (16.2)
Female [ <i>N</i> (%)]	19652 (53.1)	3585 (50.5)	3293 (54.5)	4180 (55.3)	3071 (52.7)	2821 (52.0)	2702 (53.7)
BMI (kg/m <sup>2</sup> ) (SD)	27.0 (4.7)	26.6 (4.5)	26.9 (4.6)	26.8 (4.6)	27.1 (4.8)	27.1 (4.9)	27.5 (5.0)
SBP (mmHg) (SD)	134.7 (19.9)	135.6 (19.8)	134.6 (21.0)	134.5 (19.9)	135.8 (20.4)	133.9 (18.8)	133.8 (19.3)
DBP (mmHg) (SD)	80.4 (11.3)	82.3 (11.3)	81.8 (11.2)	78.7 (11.3)	78.9 (11.4)	81.2 (11.0)	79.3 (11.1)
Hypertension [ <i>N</i> (%)]	17424 (47.1)	3328 (46.8)	2838 (47.0)	3242 (42.9)	2855 (49.0)	2668 (49.2)	2493 (49.6)
Current smoker [ <i>N</i> (%)]	8003 (21.6)	1702 (24.0)	1312 (21.7)	1951 (25.8)	1196 (20.5)	1038 (19.2)	804 (16.0)
Diabetes mellitus [ <i>N</i> (%)]	1748 (4.7)	209 (2.9)	325 (5.4)	211 (2.8)	238 (4.1)	341 (6.3)	424 (8.4)
Exercise [ <i>N</i> (%)]							
Light	8502 (23.0)	1604 (22.6)	1652 (27.4)	1697 (22.4)	1188 (20.4)	1145 (21.1)	1216 (24.2)
Moderate	19550 (52.9)	4044 (56.9)	3329 (55.1)	4088 (54.0)	3115 (53.5)	2640 (48.7)	2334 (46.4)
Heavy	8392 (22.7)	1376 (19.4)	980 (16.2)	1694 (22.4)	1426 (24.5)	1529 (28.2)	1387 (27.6)
Competitive	541 (1.5)	82 (1.2)	78 (1.3)	86 (1.1)	96 (1.6)	106 (2.0)	93 (1.8)
Antihypertensive medication [ <i>N</i> (%)]	6799 (18.4)	927 (13.0)	1068 (17.7)	1073 (14.2)	1200 (20.6)	1215 (22.4)	1316 (26.2)
Lipid medication [ <i>N</i> (%)]	3632 (9.8)	237 (3.3)	374 (6.2)	534 (7.1)	827 (14.2)	834 (15.4)	826 (16.4)

Continuous variables are presented as mean (standard deviation) and categorical values as count (percentage). BP, blood pressure.



**FIGURE 1** Cross-sectional associations between the metabolites and blood pressure ( $N=36\,985$ ). Filled circle signifies FDR-corrected  $P$  less than 0.05. Associations are adjusted for age, sex, BMI, current smoking, diabetes, antihypertensive medication, exercise, lipid medication, and cohort. Apo, apolipoprotein; C, cholesterol; DHA, docosahexaenoic acid; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; PG, phosphoglycerides; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglycerides; VLDL, very-low density lipoprotein.



**FIGURE 2** Longitudinal associations between baseline metabolite levels and SBP change ( $N=4197$ ). Filled circle signifies FDR-corrected  $P$  less than 0.05. Associations are adjusted for baseline SBP, age, sex, BMI, current smoking, diabetes, antihypertensive medication, exercise, lipid medication, and cohort. Apo, apolipoprotein; C, cholesterol; DHA, docosahexaenoic acid; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; PG, phosphoglycerides; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglycerides; VLDL, very low-density lipoprotein.

**TABLE 2. Measurement of gradient boosting model fit in cross-sectional and longitudinal samples**

Sample	Step	Full model	Root-mean-square error	
			Clinical characteristics	Metabolic measures
Cross-sectional	Training	15.50 mmHg	16.95 mmHg	16.36 mmHg
Cross-sectional	Test	16.27 mmHg	16.70 mmHg	18.03 mmHg
Longitudinal	Training	13.22 mmHg	14.84 mmHg	16.46 mmHg
Longitudinal	Test	17.61 mmHg	18.52 mmHg	19.78 mmHg

Full model included both clinical characteristics and 53 circulating metabolic biomarkers. In models adjusted with clinical characteristics, we used following covariates age, sex, BMI, current smoking, diabetes, antihypertensive medication, exercise, and lipid medication, and baseline SBP (only in longitudinal model).

The observed cross-sectional associations were consistent across study cohorts (Fig. S7, <http://links.lww.com/HJH/B807>). The results from inverse variance-weighted fixed-effect meta-analysis were also consistent with the pooled cross-sectional (Fig. S8, <http://links.lww.com/HJH/B807>) and longitudinal (Fig. S9, <http://links.lww.com/HJH/B807>) analyses.

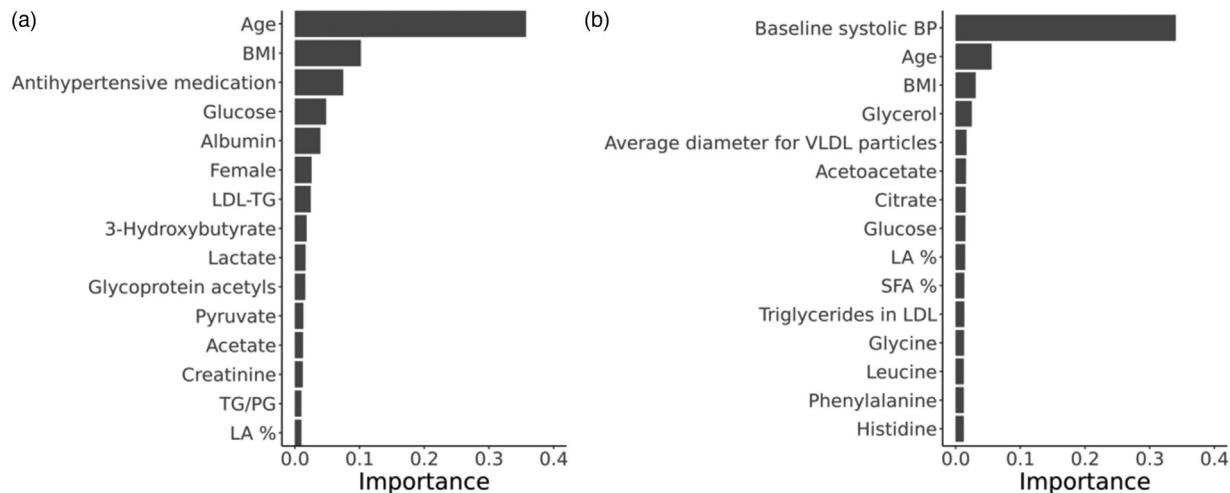
We then examined the overall relation of the metabolic biomarkers with current and future SBP using multivariable gradient boosting machine learning algorithm. First, we compared the predictive accuracy between models that included only clinical characteristics, only metabolic measures, and both the clinical variables and metabolic measures. The hyperparameters used in models are presented in Table S8, <http://links.lww.com/HJH/B807>. Adding metabolic measures (cross-sectional root-mean-square error: 16.27 mmHg; longitudinal: 17.61 mmHg) in the model with clinical measures (cross-sectional: 16.70 mmHg; longitudinal 18.52 mmHg) improved the machine learning model fit (Table 2).

Then, we assessed the independent associations of the metabolic measures with current and future SBP using the same machine learning approach. Expectedly, the clinical characteristics, such as age, BMI, antihypertensive medication (cross-sectional model), and baseline SBP

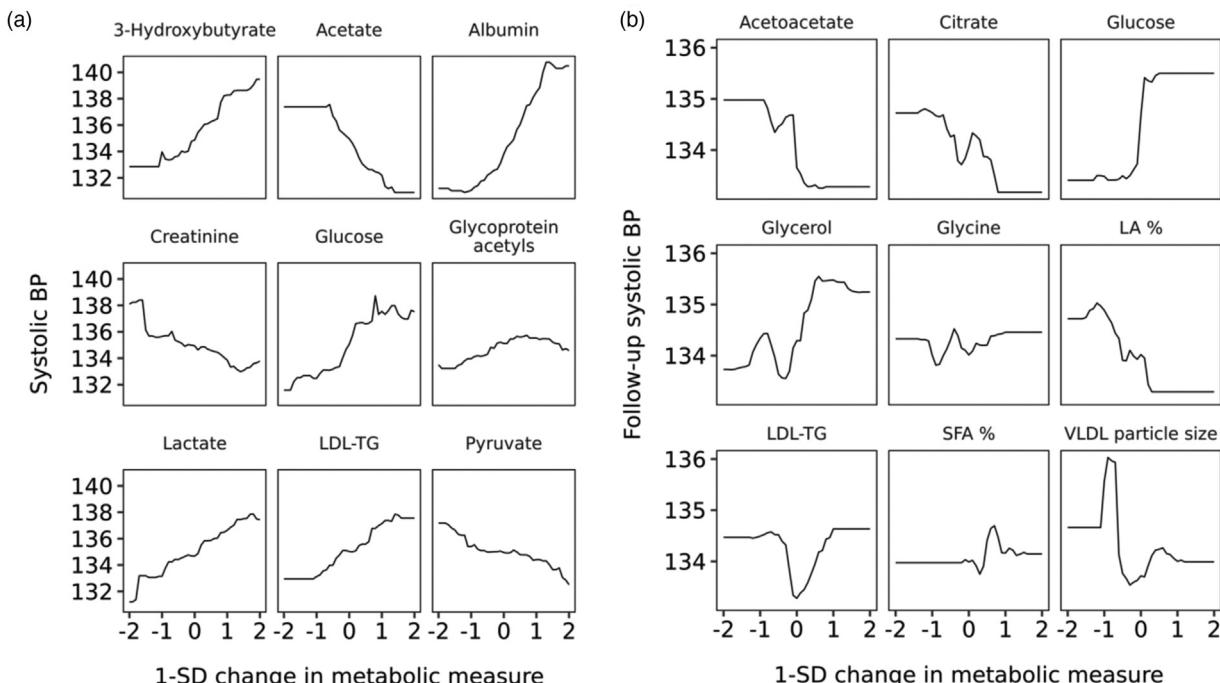
(longitudinal model) contributed the most to the predictive ability of the models (Fig. 3). Of the metabolic measures, glucose, albumin, and triglycerides in LDL were the most important metabolic measures in the cross-sectional model. In the longitudinal model, the three most important metabolic predictors of future SBP were glycerol, average VLDL size, and acetoacetate, which were then followed by other metabolic measures with similar levels of importance. The multivariable-adjusted association between the most important metabolic measures and current or future BP is shown in Fig. 4.

## DISCUSSION

We investigated the cross-sectional and longitudinal associations between serum high-abundance metabolic measures and BP in representative population samples of up to 36 985 and 4197 individuals. In addition to conventional statistical methods where a single metabolic measure was assessed in each model, we used a machine learning approach to identify a metabolic signature related to blood pressure and longitudinal blood pressure change. Our results demonstrate that metabolic measures provide incremental predictive value over conventional clinical variables for current and future SBP. In cross-sectional models,



**FIGURE 3** The top 15 clinical and metabolic measures of current (a) and future (b) blood pressure assessed using a multivariable machine learning approach (gradient boosting). The importance (gain) of a feature represents the total contribution each feature has in the trees of the gradient boosting model. In addition to the 53 metabolic measures, the model was adjusted for age, sex, BMI, current smoking, diabetes, antihypertensive medication, exercise, and lipid medication, and baseline SBP (only in longitudinal model). The sum of the importance of all the features equals one. LA, linoleic acid; LDL, low-density lipoprotein; PG, phosphoglycerides; TG, triglycerides; SFA, saturated fatty acids.



**FIGURE 4** The multivariable-adjusted relation between the top nine metabolites and current (a) and future (b) blood pressure. The top nine biomarkers in the gradient boosting models are represented for cross-sectional (a) and longitudinal (b) samples. Partial dependency plots approximate the relationship between single metabolic measure and outcome variable when the effect of all other model covariates is averaged out. LA, linoleic acid; LDL, low-density lipoprotein; TG, triglycerides; SFA, saturated fatty acids.

glucose, albumin, and triglycerides in LDL were the strongest metabolic correlates of SBP. In longitudinal models, glycerol, average VLDL size, and acetoacetate were the strongest predictors of future SBP. We did not observe any major sex-specific or age-specific differences between the studied 53 metabolic measures and BP.

In our study, triglycerides in LDL were strongly associated with current BP whereas average VLDL was a robust predictor of future BP in the machine learning models. The longitudinal associations between circulating lipoprotein fractions and hypertension have been studied by certain population studies. In the Brisighella Heart Study ( $N=1864$ ), baseline LDL cholesterol level was positively related to rate of new-onset of hypertension [18]. In the Women's Health Study ( $N=17527$ ), average VLDL particle size, as in our study, was positively associated with the development of hypertension. In that study, apolipoprotein B and total triglycerides were also predictive of future hypertension whereas average LDL particle size was negatively associated with the development of hypertension [19]. The association between HDL fractions and hypertension, however, appears to be more convoluted. In the Women's Health Study, medium-HDL and small-HDL particle concentrations were positively associated with future hypertension. In contrast, average HDL particle size, large-HDL particle concentration, and HDL cholesterol were negatively associated with the development of hypertension [19]. In a cross-sectional study of 14 215 normotensive men, baseline HDL cholesterol categorized in five groups demonstrated a U-shaped association with the development of hypertension [20]. HDL particles isolated from

patients with cardiovascular disease have been reported to lack the ability to induce endothelial nitric oxide production and stimulate endothelial repair [21,22]. All in all, results from our and previous studies suggest that serum lipids, and particularly LDL-derived and VLDL-derived cholesterol levels are associated with the development of hypertension.

In the multivariable machine learning model, glycolysis-related metabolic markers glucose and glycerol were associated with BP. In healthy individuals, postprandial insulin secretion related to increasing glucose levels promotes four changes in circulating metabolites reflecting switch from catabolism to anabolism: increased glycolysis (lactate increases) and decreased lipolysis (glycerol decreases), and ketogenesis (beta-hydroxybutyrate and acetoacetate decrease) [23]. Two prior studies have reported on the associations of glycolysis-related metabolic measures, including ketone bodies, with hypertension in humans. In the Bogalusa Heart Study ( $N=1249$ ), fasting glucose was positively associated with SBP [24]. In an American prospective study ( $N=5554$ ), lactate, an indicator of oxidative capacity, was positively associated with development of hypertension in women but not in men [25]. As insulin resistance and diabetes are both related to arterial stiffening, these findings are somewhat expected and highlight the importance of glucose metabolism abnormalities in the development of hypertension [26].

In the cross-sectional univariate and multivariable analyses, we observed a positive association between albumin and blood pressure. Albumin is a key contributor to the vascular colloid-osmotic pressure and important transporter of

hormones, drugs, amino acids, and free fatty acids [27]. In the Oslo Health Study ( $N=5171$ ) and in the Neuroprotective Model for Healthy Longevity among the Malaysian Elderly study ( $N=2322$ ), positive cross-sectional associations were observed between albumin and BP [27,28]. However, a retrospective study of normotensive Japanese ( $N=2240$ ) reported a negative association between blood albumin and risk of hypertension [29]. The authors of this study concluded that the positive cross-sectional associations could be explained by increased vascular volume whereas the negative longitudinal associations could be a result of the anti-inflammatory and antioxidant properties of albumin [29]. As albumin was not a significant correlate of future BP, our cross-sectional findings mainly suggest that the association between albumin and BP may not be causal.

Although nearly all measured fatty acids and amino acids were strongly related to BP in the cross-sectional analyses, none of them demonstrated any strong associations in the longitudinal analyses. Despite several prior studies reporting strong cross-sectional associations of fatty acids and amino acids with BP [30–35], the nature and strength of these associations warrants further study.

### Strengths and limitations

Our study has several strengths, such as a large cross-sectional population sample, access to repeated measurements, and consistent biomarker quantification by the same NMR spectrometry method in all included cohorts. However, our results must be interpreted in the context of potential limitations. First, although feasible to perform in large scale, NMR provided only a limited number of metabolic measures, of which the majority were lipid measures. Second, our longitudinal sample size, although relatively large, may have been insufficient to capture some associations. Third, the dates of baseline examinations ranged over 15 years in our study cohorts resulting differences in freezing times. Fourth, many of the statistically significant associations that were observed had relatively small effect sizes.

In conclusion, we assessed the metabolic profile of hypertension in a representative population sample of up to 36 985 individuals with repeated measurements available for 4197 participants. Our study is the largest study to date to investigate the relation between circulating metabolic measures and hypertension. We identified a metabolic serum signature associated with blood pressure and longitudinal blood pressure change using conventional statistics and machine learning approaches. Our results suggest that serum lipids, and particularly LDL- and VLDL-derived cholesterol levels, and glucose metabolism abnormalities are associated with hypertension onset. Use of serum NMR metabolite determination could improve the identification of individuals at high risk of developing hypertension.

### ACKNOWLEDGEMENTS

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Availability of data and materials: the data that support the findings of this study are available from Finnish Institute

for Health and Welfare Biobank (<https://thl.fi/en/web/thl-biobank>). The source code for the analyses is openly available at doi:10.5281/zenodo.3625488.

Previous presentations: the summary of the study was presented in poster session of the conference of American College of Cardiology (ACC.21) 15–17 May 2021.

### Conflicts of interest

V.S. has received honoraria from Novo Nordisk and Sanofi for consultations and travel support from Novo Nordisk. He also has ongoing research collaboration with Bayer Ltd. (all unrelated to the present study). E.T. and P.W. are shareholders and/or employees of Nightingale Health, Plc, a company offering nuclear magnetic resonance-based metabolic profiling. This work was supported by the Emil Aaltonen Foundation (T.N.), the Paavo Nurmi Foundation (J.P., T.N.), the Finnish Medical Foundation (T.N.), the Finnish Foundation for Cardiovascular Research (V.S.), the Academy of Finland (grant no. 321351 to T.N.; 295741, 307127 to L.L.; 321356 to A.H.; 338818 to M.R.), and the Sigrid Juselius Foundation (M.A.K.). Although Nightingale Health Plc. funded and performed the serum biomarker measurements, the funders play no further role in the design of the study, the collection, analysis, and interpretation of the data; and the decision to approve publication of the finished manuscript. All authors had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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