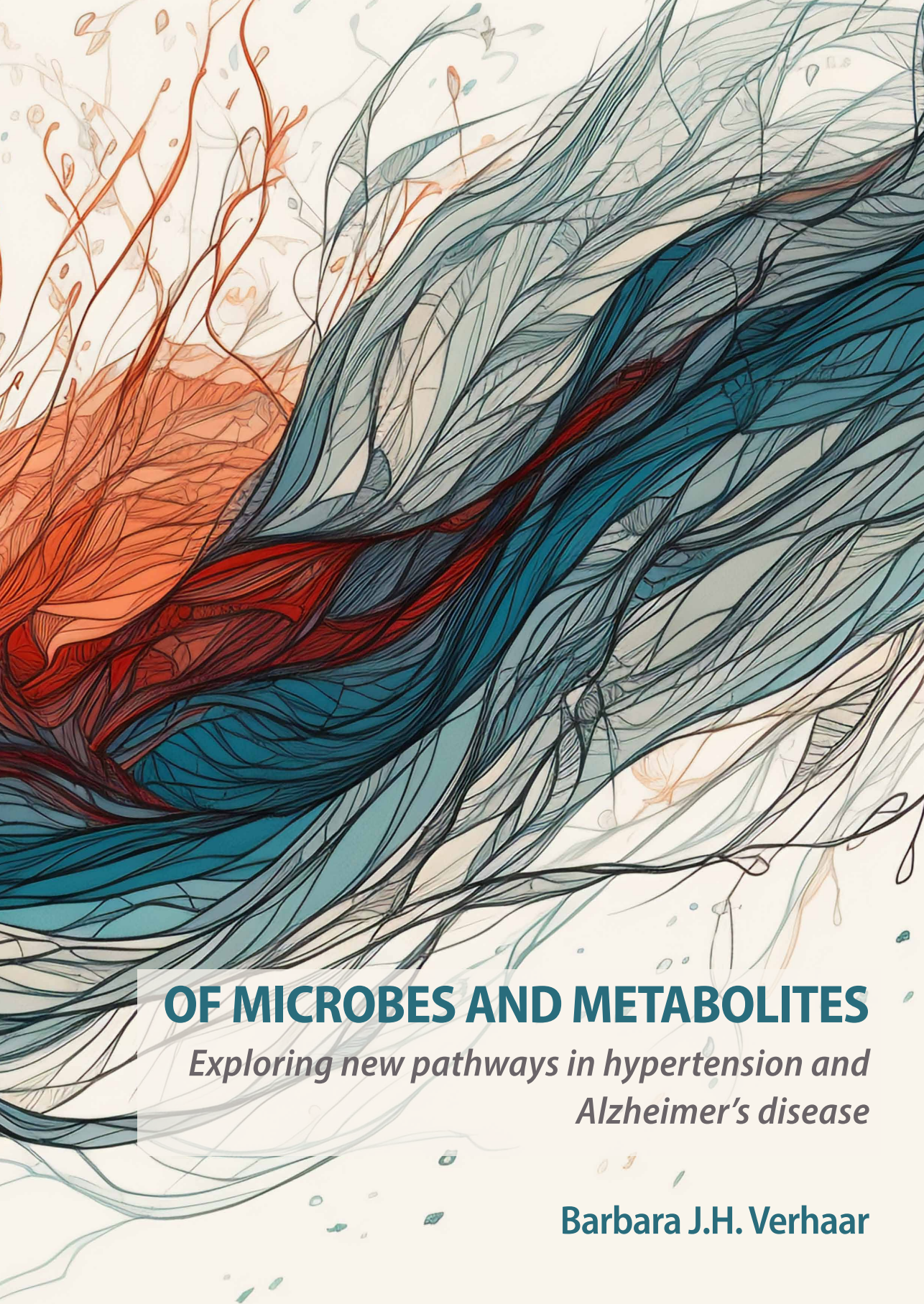

OF MICROBES AND METABOLITES

Exploring new pathways in hypertension and Alzheimer's disease

Barbara J.H. Verhaar

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Exploring new pathways in hypertension and Alzheimer's disease

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Introduction

General introduction

Two public health challenges

By 2030, approximately 20% of the population will be aged 65 years or older.[1] As a consequence, the prevalence of numerous diseases will increase dramatically. Prominent among these diseases is hypertension, a cardiovascular disease that currently affects over 1 billion adults worldwide.[2] Hypertension already accounts for 9.4 million deaths yearly due to complications such as stroke, renal failure and myocardial infarction.[2] Another disease with increasing incidence is Alzheimer's disease (AD), the most common type of dementia. An estimated 47 million people are affected by dementia, with 10 million new cases each year.[3] Due to the changing population age structure, this number is expected to grow to 132 million in 2050.[4]

In this thesis, I set out to investigate the effects of the gut microbiota and its metabolites on these two public health care challenges: hypertension and Alzheimer's disease. Both diseases have a complex etiology that is not completely understood and share lifestyle interventions to reduce their risk, although there is no certain way to prevent either. This introduction will provide some background on the gut microbiota and the pathophysiology of hypertension and Alzheimer's disease. It also includes a general explanation of three techniques used in this thesis: 16S rRNA sequencing of fecal samples, liquid chromatography - tandem mass spectrometry (LC-MS/MS) for identification of plasma metabolites and machine learning analyses.

Gut microbiome: a rapidly evolving field

The human body harbors around 38 trillion microbial cells, which is the same order of magnitude as the total number of human cells in our body.[5] The majority of these microbes reside in our gastrointestinal tract, forming what is known as the gut microbiome. Composed primarily of bacteria and viruses, with smaller contributions from archaea, fungi, and protists, the gut microbiome encompasses the collective genetic material of these microorganisms.[6] Historically, the field depended on bacterial culturing techniques which were unable to culture a large proportion of microorganisms. However, advancements in DNA sequencing techniques over the past two decades have revolutionized our understanding of the gut microbiota's role in health and disease.[7,8] Rather than by culturing, gut

bacterial composition is now determined with either 16S rRNA sequencing or shotgun metagenomic sequencing of DNA in fecal samples (see box "**Two techniques to identify bacteria**"). As a result, thousands of completely new microbial species were discovered, and the composition of the gut microbiota could be described in much more detail, since whole genome information became available for a large number of microbes.[9]

Gut microbiota composition is predominantly shaped by environmental factors.[10] While diet has by far the most impact on microbiota composition, age is another important determinant. The largest changes in the gut microbiota composition take place in early childhood, from the first exposure to the mother's microbiome during birth, to the exposure to (breast)milk and the introduction of the solid food.[11,12] In adulthood, the microbiome changes slowly with advancing age due to a multitude of factors, including comorbidity, polypharmacy and changing lifestyle.[13] Among medications, antibiotics have a particularly profound effect by wholesale killing of bacterial groups.[14] Medication such as metformin, statins, proton pump inhibitors and antihypertensive medication have also been associated with microbiota alterations.[15] However, it is difficult to disentangle the impact of medication use from that of the underlying disease.

The gut microbiome has many functions that benefit the human host. Gut microbes assist in the digestion of our food, by breaking down otherwise indigestible dietary fibers.[16] One of the main products of this fermentation process, short chain fatty acids (SCFA), are used by the cells in the colon (colonocytes) as a main energy source. In addition, the microbiome helps to regulate our immune system and to protect against other harmful bacteria. Microbiota also play a key role in the recycling of bile acids, and the production of a number of vitamins such as vitamin B and vitamin K.[17–19] In the gut, these metabolites are absorbed into the systemic circulation. Other metabolites that are produced by gut bacteria include trimethylamine-N-oxide (TMAO) and phenylacetylglutamine (PAG).[20,21] Both these metabolites have been associated with higher cardiovascular risk.[21,22] Another key mechanism for the effects of the gut microbiota on human health is the enteric nervous system. This large system of nerves is sometimes referred to as "our second brain".[23] There is bidirectional communication between the gut and brain through the vagal nerve, which might explain complaints of stomach aches in stressful situations.[24]

Changes in the structure of the gut microbiome have been observed in a variety of health conditions, including inflammatory bowel disease and diabetes.[25,26] Often, the microbiome alterations are first observed in humans with these conditions, or animal models show that disease outcomes might improve with

Two techniques to identify bacteria

To determine which gut bacteria can be found in the intestinal tract, we sequence the genetic material present in fecal samples. There are two commonly used sequencing techniques: 16S ribosomal RNA gene (rRNA) sequencing and shotgun metagenomic sequencing.

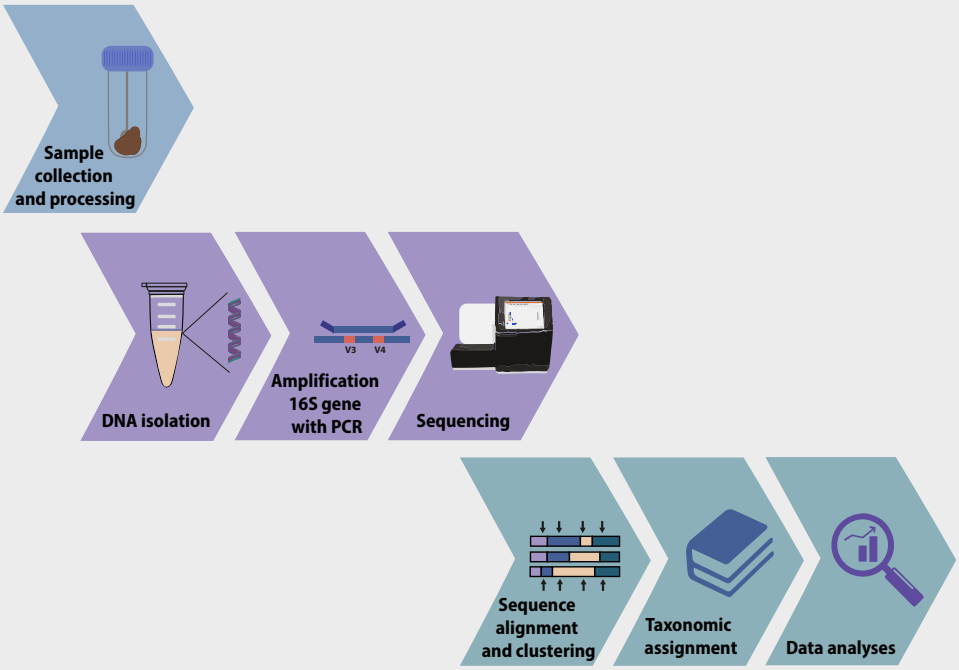
16S rRNA sequencing has accelerated microbiota research since its first use in 1996. This technique uses the DNA that codes for the 16S subunit of the rRNA. The 16S rRNA gene is specific to bacteria and has both highly variable and well-conserved regions. That means that it is easy to find the gene using the region that is well-conserved (i.e., similar between bacteria), while we can also use this gene to distinguish bacteria using regions with high variability between species. These are the steps in the protocol of 16S sequencing:

- DNA isolation: DNA of the host and of micro-organisms in the gut is isolated from fecal samples.
- Amplification of the 16S rRNA gene using polymerase chain reaction (PCR): primers (small pieces of DNA) are designed to match the ends of a region of the 16S gene. Next, the DNA is repeatedly copied using the DNA polymerase enzyme.
- Sequencing: the amplified DNA is sequenced using high-throughput sequencing technologies. The quality of the sequence data is checked and the primer sequences are removed from the data.
- Sequence alignment and clustering: the resulting sequences are compared to a database with reference sequences to identify which microbes we are likely looking at. The next step is to pool similar sequences together into Amplicon Sequence Variants (ASVs) and to count the occurrences of these ASVs per sample.
- Taxonomic assignment: the ASV sequences are assigned taxonomic classifications (i.e., names of bacterial taxa) using a reference database.

In **shotgun metagenomic sequencing**, all microbial DNA in a fecal sample is used for sequencing, not only the 16S rRNA gene. Before sequencing, the DNA is fragmented in small pieces. Using bioinformatic tools, the sequences can then be assembled into longer bits, that can be compared to a database to determine the structure of the microbiome. **Shotgun metagenomic sequencing** is a relatively ex-

pensive technique, that however is becoming increasingly cheaper over time. The advantage of shotgun sequencing compared to 16S sequencing is the improved resolution up to strain level, and the possibility to further analyze the bacterial genomes. From these genomes, we can infer details on their functional profile, enabling pathway analysis.

Figure 1: 16S amplicon sequencing



Schematic overview of the process of 16S amplicon sequencing from sample collection and processing to the data analyses.

microbiome interventions. The next step towards determining causality is to use one of the available strategies to change the gut microbiota composition in humans, in order to improve health outcomes.[27] One such strategy is to supplement certain bacteria with probiotics containing one or more bacterial strains.[28] Alternatively, fecal microbiota transplantation (FMT) can be performed, a procedure in which feces from a healthy donor is transferred into the gastrointestinal tract of a patient with the condition of interest.[29] FMT has been shown to be very effective in *Clostridium difficile* infections, with success rates up to 90%.[30] Other interventions include prebiotics: indigestible dietary supplements such as dietary fibers that selectively stimulate the growth of beneficial bacteria.[31]

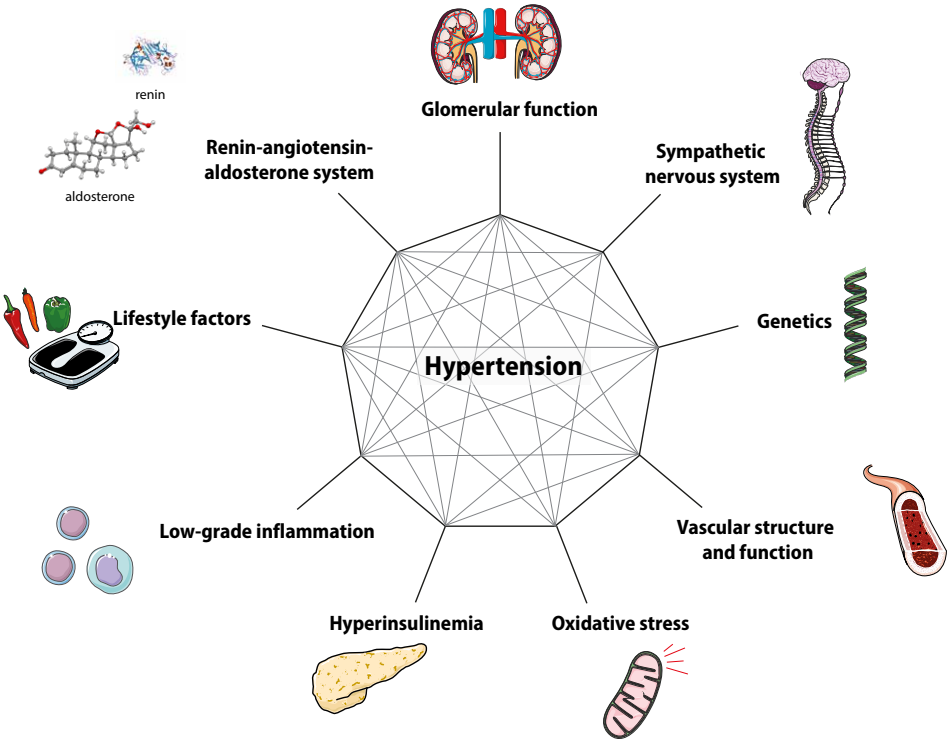
Hypertension: a mosaic of causal factors

High blood pressure, or hypertension, is a health condition that has been recognized for centuries, yet there is still much we do not completely understand about its pathophysiology.[32,33] In its early stages, hypertension rarely causes symptoms, which can lead to many cases going undiagnosed.[34] Even for those who are diagnosed, limited access to treatment and difficulty in managing blood pressure over time can be a significant challenge, especially in low- and middle-income countries with weaker healthcare systems.[2] Detecting hypertension early and providing proper treatment can have substantial health and economic benefits. Failure to do so can result in costly interventions to treat complications, such as cardiac bypass surgery, carotid artery surgery, and dialysis and subsequent need for kidney transplantation, putting a strain on healthcare budgets.[35]

Blood pressure is regulated by an interplay between the sympathetic nervous system, the renin-angiotensin-aldosterone system (RAAS), and the kidneys.[36] These systems work together to maintain a balance between fluid volume and blood vessel tone, which in turn helps to regulate blood pressure. In hypertension, however, this balance is disrupted, leading to a sustained higher setpoint of blood pressure. This may be caused by a variety of factors, including increased peripheral vascular resistance, increased cardiac output, dysfunction of RAAS, or kidney failure.

There are many other factors at play in hypertension, including lifestyle, genetics and vascular inflammation.[37] Lifestyle factors such as a lack of physical activity, high body mass index (BMI), alcohol use, and a Western type diet are associated with higher blood pressure.[38] Common genetic variants explain 3-4% of the variance in systolic and diastolic BP.[39] Many of these genetic variants affect genes that are expressed in vascular tissue such as vascular smooth muscle and

Figure 2: Mosaic theory of hypertension



Schematic overview of factors contributing to the pathophysiology of hypertension.

endothelial cells, which form the inner lining of the vasculature.[40] In a healthy state, endothelial cells produce nitric oxide, an agent that dilates the vessels by preventing contraction of vascular smooth muscle cells.[41] The lower peripheral resistance results in lower blood pressure. In hypertension, there often is endothelial dysfunction, with lower nitric oxide production and more contraction.[42] In addition, the endothelium shows more inflammatory features. Vascular inflammation can amplify the process of atherosclerosis and lead to vascular stiffness.[43,44] Stiff vessels are less compliant, meaning they can absorb less of the pressure wave that travels through the arteries, which leads to a higher blood pressure. The complex interplay of these contributing factors in the etiology of hypertension is sometimes referred to as the mosaic theory of hypertension (**Figure 2**).

However, it remains largely unknown why blood pressure levels, and the response to hypertension treatment, differ between individuals. This is a frustrating reality for patients, who have questions about the causes of hypertension and their response to lifestyle changes or antihypertensive medication. There are three windows of opportunity in hypertension that could provide new leads: the gut microbiota, plasma metabolome, and a focus on diversity.

Three windows of opportunity in hypertension

Animal studies in germ-free rats in the 1960s and 1970s were the first to suggest a connection between gut microbiota composition and blood pressure.[45] Subsequent research, much later, demonstrated that the gut microbiota of hypertensive rats differs from that of control rats. Importantly, it was observed that administering antibiotics to these hypertensive rats had blood pressure-lowering effects.[46] The gut microbiota could establish its effects on blood pressure through the sympathetic nervous system and the vagal nerve, or by producing or modifying metabolites that are absorbed into the systemic circulation.[47,48] These metabolites might affect endothelial function, interfere with RAAS or aggravate vascular inflammation and stiffness. Examples of gut metabolites that have been associated with cardiovascular health are short chain fatty acids (SCFA), lipopolysaccharide (LPS) and trimethylamine-N-oxide (TMAO).

The plasma metabolome, which is a collection of small molecules that can be found in our circulation, might also provide new insights in the causes of hypertension. In several chapters in this thesis, a method called liquid chromatography – tandem mass spectrometry (LC-MS/MS; see box "**Measuring metabolite profiles**") was used to measure plasma metabolites. This collection of metabolites is essentially a fingerprint of metabolic processes in the body. For instance, the plasma metabolome includes dietary products (e.g., caffeine, or fructose) and medication (beta blockers). Sex might have the largest impact on this fingerprint through a range of sex hormone metabolites, and as a result, sex can be easily inferred from plasma metabolite profiles.[49] The gut microbiome also produces or modifies a range of metabolites that are absorbed into our circulation, such as SCFA, TMAO or PAG.[50] Other products that can be found in plasma are intermediates and products of vascular metabolism. Since vascular inflammation is one of the factors contributing to hypertension, these might be metabolites that could teach us more about hypertension pathophysiology.

Another approach that could help us explain underlying mechanisms of hypertension is to focus on diversity. More attention for diversity in medicine in general and hypertension specifically is needed to close the gap in healthcare disparities.[51,52] Three facets of the many faces of diversity are discussed in this thesis, namely diversity in age, sex, and ethnicity. It is well established that hypertension at old age is physiologically different from hypertension at young age. While in young patients, genetic factors and lifestyle have more prominent effects, in older patients, vascular stiffness and loss of kidney function are of greater importance. In addition, clear sex differences exist in life course trajectories of blood pres-

sure.[53] Men have higher blood pressure than women for most of their lives, yet in older age, this difference is reversed and hypertension is more common among women. In general, women have a higher risk of secondary complications with similar blood pressure levels. Lastly, the pathophysiology of hypertension might differ depending on ancestry and its resulting genetic differences. For instance, African-Americans have been shown to have a higher salt-sensitivity.[54] For several chapters in this thesis, we used data of the HEalthy Life in an Urban Setting (HELIUS) study, a multi-ethnic population-based cohort study in Amsterdam with over 25,000 participants.[55] Within this cohort, there is data on self-identified ethnicity as opposed to ancestry. Ethnicity refers to a shared cultural identity among a group of people who share common traditions, beliefs, customs, and sometimes language or religion. Since the HELIUS study is a large cohort that is diverse in age, sex, and ethnicity, this was a suitable starting point to explore the associations between the gut microbiota, plasma metabolome and blood pressure.

Alzheimer's disease: a dangerous pair of proteins

Alzheimer's disease (AD) is named after Alois Alzheimer, a German psychiatrist and neuroanatomist, who first described "a peculiar severe disease process of the cerebral cortex" at a German psychiatry conference in 1906.[56] He described the case of Auguste D., a 51-year-old woman, with rapidly progressive memory complaints, sleep disorders, behavioral problems and confusion. Following Auguste D.'s death, Alzheimer conducted a postmortem examination of her brain, discovering unusual histologic abnormalities. These abnormalities, now recognized as two characteristics of AD, involve the accumulation of abnormally folded amyloid beta in amyloid plaques, and accumulation of tau proteins in neurofibrillary tangles. This process is toxic for neurons and results in loss of neuronal tissue, also known as neurodegeneration.[57] In addition, Alzheimer's disease is associated with chronic neuroinflammation, either as a consequence of the disease process or as an underlying causal factor.[58]

The brain pathology is reflected by a set of biomarkers that we can measure, for example in cerebral spinal fluid (CSF), with positron emission tomography (PET) scans, and with magnetic resonance imaging (MRI) brain scans. Low amyloid levels and increased pathological tau levels in CSF collected by lumbar puncture are indications of Alzheimer's pathology.[59] The accumulation of these proteins can also be visualized using PET-scans. In addition, on MRI the neurodegeneration caused by AD can be recognized as global cortical atrophy (GCA), medial temporal atrophy (MTA) and parietal cortical atrophy (PCA).[60,61] These

Measuring metabolite profiles

LC-MS/MS is a technique that can be used for the identification and quantification of metabolites. As the name shows, it is a combination of two techniques: liquid chromatography (LC) and tandem mass spectrometry (MS/MS). LC is used for separation of metabolites, while MS is used for mass analysis. The process of LC-MS/MS could be set up as follows:

1. **Sample preparation**: The plasma sample is first extracted and purified to remove unwanted proteins and other molecules that may interfere with the analysis.
2. **Chromatographic separation**: The extracted metabolites are separated using liquid chromatography based on their properties, such as polarity and size.
3. **Mass spectrometry (MS1):** The metabolites are ionized and then introduced into the mass spectrometer, which separates the ions based on their mass-to-charge ratio (m/z). The mass spectrometer generates a mass spectrum, which provides information on the molecular weight and structure of the metabolites.
4. **Mass spectrometry (MS2)**: In tandem mass spectrometry (MS/MS), the ions generated in step 3 are further fragmented into smaller pieces. This process allows for the identification of the metabolites based on their specific fragmentation patterns (fingerprints).
5. **Data analysis:** The mass spectra and fragmentation patterns are analyzed using specialized software to identify and quantify the metabolites in the sample by comparing the data to libraries of known metabolites.

In the absence of spiked-in standards with known concentrations, the metabolomics data generated through this process is semi-quantitative. This implies that absolute concentrations of metabolites are not determined. The method provides relative concentrations of the metabolites, allowing for comparison of concentrations between samples.

are scored by radiologists on a 4- or 5-point scale, from normal to abnormal. Vascular damage is more pronounced in vascular dementia, yet also observed in Alzheimer's disease. This vascular damage shows on an MRI as white matter hyperintensities, small (lacunar) infarcts or cerebral microbleeds.[62,63]

Patients with AD usually present with cognitive signs and symptoms, including difficulty with memory, language, and spatial awareness, as well as changes in mood and behavior. A diagnosis of dementia can be made when these complaints lead to functional impairment in daily activities. When cognitive impairment (mostly memory) occurs rather isolated, while daily functioning is largely intact, this is referred to as mild cognitive impairment (MCI).[64,65] Patients with cognitive complaints that do not show any abnormalities on neuropsychological testing are referred to as having subjective cognitive decline (SCD). In a minority of individuals with SCD, underlying Alzheimer's pathology may be causing their memory complaints. In research settings, we can diagnose Alzheimer's disease independent of the cognitive stage.[59] When biomarkers in CSF are indicative of AD pathology, we call this Alzheimer's disease, with stage of SCD, MCI or dementia.

Two potential biomarkers of AD are less established: changes in nutritional status and gut microbiota composition. Weight loss is one of the signs of a changing nutritional status that is observed very early in the disease process in patients with AD. Attention for nutritional status in these patients is important, since malnutrition has been associated with higher mortality and progression of cognitive decline.[66–69] In addition, mouse models suggest that the gut microbiota could affect the progression of disease, by showing that fecal microbiota transplantation from healthy to diseased mice reduced the accumulation of amyloid beta.[70] In this thesis, we explore the relation between AD biomarkers and nutritional status and the gut microbiota in a clinical cohort, the NUDAD project. For the chapter in which we investigate the associations between gut microbiota composition and AD biomarkers, we used machine learning analyses. These machine learning models were applied for multiple projects with microbiome and metabolome data through this thesis (see box "**A very short introduction to machine learning**" for some background).

A very short introduction to machine learning

In this thesis, there are several projects for which we used machine learning analyses. Machine learning is a type of artificial intelligence (AI) that allows computer systems to automatically learn and improve from experience. Machine learning algorithms can be trained on large datasets of input and output data, and they can use this training to make predictions or decisions about new, unseen data. These are four questions that could help in evaluating machine learning models:

- What is the **algorithm** used? The algorithm that is used in this thesis is the XGBoost algorithm,[71] which can either be used in a classification prediction (Alzheimer's disease: yes or no) or a regression prediction (blood pressure as continuous outcome). This algorithm builds decision trees to predict the outcome.

- What is the **design of the model**? We used a nested cross-validation design. That means that within the train set of data, there was another smaller loop of test and train data, in which the hyperparameters of the model were optimized before deciding what the optimal model set-up was. Hyperparameters are variables that define the model set-up, such as the learning rate, the number of trees and the depth of the trees. The resulting model with optimized hyperparameters was tested once on the test set of the outer test-train loop. Thus, data points used to train a model are never used in testing that same model.

- What is the **main model metric**? In other words, on what parameter do we base our evaluation of the model. For instance, this could be explained variance for regression models, in which we try to predict a continuous outcome such as blood pressure. The explained variance is the proportion of variance that we can explain using the machine learning model.

- Is there **leakage** of information from the test to the train data set? In some machine learning designs, a selection of the most promising features (in our case microbes, or metabolites) is made prior to using the machine learning model and splitting the data in a test and train set. The main model metric could be much higher as a result: we can explain more variance because we first selected the features that looked promising based on the complete dataset.

Outline of this thesis

The overall aim of this thesis is to explore the associations between gut microbiota, circulating metabolites and two diseases with a rising incidence: hypertension and Alzheimer's disease.

Part I of this thesis focuses on hypertension. **Chapter 2** describes the associations between gut microbiota composition and blood pressure in a multi-ethnic population cohort. **Chapter 3** reports an overview of the existing literature on gut microbiota composition in hypertension and atherosclerosis: what are the missing links between gut microbiota and cardiovascular health? Next, we will zoom in on one of these mechanisms, the effect of butyrate on blood pressure. The results of a randomized placebo-controlled trial with butyrate in patients with grade I hypertension will be discussed in **Chapter 4**. Since it is likely that there are more yet unknown mechanisms that connect the gut microbiota and blood pressure, we looked for novel associations between plasma metabolites and blood pressure in **Chapter 5**. This project started with a machine learning analysis in a population-based cohort and resulted in a series of *in vitro* experiments to assess the effects of one of the metabolites on endothelial cells. **Chapter 6** revisits the machine learning analyses from the previous chapter through the lens of sex differences: how are plasma metabolites differently associated with blood pressure in women and men?

Part II of this thesis focuses on nutritional status and gut microbiota composition in Alzheimer's disease. **Chapter 7** describes the associations between MRI characteristics and nutritional status in a memory clinic population, including patients with subjective cognitive decline, MCI and early-stage AD dementia. In **Chapter 8**, we explore the associations between gut microbiota composition and the presence of AD biomarkers in the same cohort.

References

1. Population structure and ageing. Eurostat, 2023. Accessed December 17, 2023. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Population_structure_and_ageing.
2. A global brief on hypertension: silent killer, global public health crisis: World Health Day 2013. World Health Organization, 2013. Accessed December 17, 2023. https://www.who.int/publications-detail-redirect/a-global-brief-on-hypertension-silent-killer-global-public-health-crisis-world-health-day-2013.
3. Global action plan on the public health response to dementia 2017–2025. World Health Organization, 2017. Accessed December 17, 2023. https://www.who.int/publications-detail-redirect/9789241513487.
4. Nichols, E. *et al.* Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. *Lancet Public Health* 7, e105–e125 (2022).
5. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* 14, e1002533 (2016).
6. Knight, R. *et al.* The Microbiome and Human Biology. *Annu Rev Genomics Hum Genet* 18, 65–86 (2017).
7. Janda, J. M. & Abbott, S. L. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 45, 2761–2764 (2007).
8. Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J. & Segata, N. Shotgun metagenomics, from sampling to analysis. *Nature Biotechnol* 35, 833–844 (2017).
9. Pasolli, E. *et al.* Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. *Cell* 176, 649-662.e20 (2019).
10. Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–215 (2018).
11. Stewart, C. J. *et al.* Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 562, 583–588 (2018).
12. Laursen, M. F. *et al.* Infant Gut Microbiota Development Is Driven by Transition to Family Foods Independent of Maternal Obesity. *mSphere* 1, e00069-15 (2016).
13. Ghosh, T. S., Shanahan, F. & O'Toole, P. W. The gut microbiome as a modulator of healthy ageing. *Nat Rev Gastroenterol Hepatol* 19, 565–584 (2022).
14. Ianiro, G., Tilg, H. & Gasbarrini, A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut* 65, 1906–1915 (2016).
15. Jackson, M. A. *et al.* Gut microbiota associations with common diseases and prescription medications in a population-based cohort. *Nat Commun* 9, 2655 (2018).
16. Cummings, J. H., Pomare, E. W., Branch, H. W. J., Naylor, C. P. E. & MacFarlane, G. T. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* (1987) doi:10.1136/gut.28.10.1221.
17. Said, H. M. & Kumar, C. Intestinal absorption of vitamins. *Curr Opin in Gastroenterol* 15, 172 (1999).
18. Das, P., Babaei, P. & Nielsen, J. Metagenomic analysis of microbe-mediated vitamin metabolism in the human gut microbiome. *BMC Genomics* 20, 208 (2019).

19. Funabashi, M. *et al.* A metabolic pathway for bile acid dehydroxylation by the gut microbiome. *Nature* 582, 566–570 (2020).
20. Tang, W. H. W. *et al.* Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res* 116, 448–455 (2014).
21. Nemet, I. *et al.* A Cardiovascular Disease-Linked Gut Microbial Metabolite Acts via Adrenergic Receptors. *Cell* 180, 862-877.e22 (2020).
22. Zhu, W. *et al.* Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell* 165, 111–124 (2016).
23. Gershon, M. D. The enteric nervous system: A second brain. *Hospital Practice* (1999) doi:10.3810/hp.1999.07.153.
24. Bonaz, B., Bazin, T. & Pellissier, S. The Vagus Nerve at the Interface of the Microbiota-Gut-Brain Axis. *Front Neuroscience* 12, (2018).
25. Chassaing, B. & Darfeuille–Michaud, A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 140, 1720-1728. e3 (2011).
26. Musso, G., Gambino, R. & Cassader, M. Obesity, Diabetes, and Gut Microbiota: The hygiene hypothesis expanded? *Diabetes Care* 33, 2277–2284 (2010).
27. Cryan, J. F. & Mazmanian, S. K. Microbiota–brain axis: Context and causality. *Science* 376, 938–939 (2022).
28. Quigley, E. M. M. Prebiotics and Probiotics in Digestive Health. *Clin Gastroenterol Hepatol* 17, 333–344 (2019).
29. Leshem, A., Horesh, N. & Elinav, E. Fecal microbial transplantation and its potential application in cardiometabolic syndrome. *Front Immunol* 10, 1341 (2019).
30. Mullish, B. H. *et al.* The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium difficile infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* 67, 1920–1941 (2018).
31. Holscher, H. D. Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes* 8, 172–184 (2017).
32. Esunge, P. M. From blood pressure to hypertension: the history of research. *J R Soc Med* 84, 621 (1991).
33. Kotchen, T. A. Historical Trends and Milestones in Hypertension Research. *Hypertension* 58, 522–538 (2011).
34. Wall, H. K., Hannan, J. A. & Wright, J. S. Patients With Undiagnosed Hypertension: Hiding in Plain Sight. *JAMA* 312, 1973–1974 (2014).
35. Constant, A. F., Geladari, E. V. & Geladari, C. V. "The Economic Burden of Hypertension" in Hypertension and Cardiovascular Disease (ed. Andreadis, E. A.) 351–359 (*Springer International Publishing*, 2016). doi:10.1007/978-3-319-39599-9_21.
36. Burnier, M. & Wuerzner, G. Pathophysiology of hypertension. *Pathophysiology and pharmacotherapy of cardiovascular disease* 655–683 (2015).
37. Harrison, D. G., Coffman, T. M. & Wilcox, C. S. Pathophysiology of Hypertension – the Mosaic Theory and Beyond. *Circ Res* 128, 847–863 (2021).
38. Geleijnse, J. M., Grobbee, D. E. & Kok, F. J. Impact of dietary and lifestyle factors on the prevalence of hypertension in Western populations. *J Hum Hypertens* 19, S1–S4 (2005).

39. Giri, A. *et al.* Trans-ethnic association study of blood pressure determinants in over 750,000 individuals. *Nat Genet* 51, 51–62 (2019).
40. Warren, H. R. *et al.* Genome-wide association analysis identifies novel blood pressure loci and offers biological insights into cardiovascular risk. *Nat Genet* 49, 403–415 (2017).
41. Gamboa, A. *et al.* Contribution of Endothelial Nitric Oxide to Blood Pressure in Humans. *Hypertension* 49, 170–177 (2007).
42. Brandes, R. P. Endothelial Dysfunction and Hypertension. *Hypertension* 64, 924–928 (2014).
43. Sehgel, N. L. *et al.* Increased vascular smooth muscle cell stiffness: a novel mechanism for aortic stiffness in hypertension. *Am J Physiol Heart Circ Physiol* 305, H1281–H1287 (2013).
44. Sun, Z. Aging, Arterial Stiffness, and Hypertension. *Hypertension* 65, 252–256 (2015).
45. Baez, S. & Gordon, H. A. Tone and reactivity of vascular smooth muscle in germfree rat mesentery. The Journal of Experimental Medicine 134, 846 (1971).
46. Yang, T. *et al.* Gut Dysbiosis is Linked to Hypertension. *Hypertension* 65, 1331–1340 (2015).
47. Toral, M. *et al.* Critical Role of the Interaction Gut Microbiota – Sympathetic Nervous System in the Regulation of Blood Pressure. *Front Physiol* 10, 231 (2019).
48. O'Donnell, J. A., Zheng, T., Meric, G. & Marques, F. Z. The gut microbiome and hypertension. *Nat Rev Nephrol* 19, 153–167 (2023).
49. Krumsiek, J. *et al.* Gender-specific pathway differences in the human serum metabolome. *Metabolomics* 11, 1815 (2015).
50. Dekkers, K. F. *et al.* An online atlas of human plasma metabolite signatures of gut microbiome composition. *Nat Commun* 13, 5370 (2022).
51. Abrahamowicz, A. A., Ebinger, J., Whelton, S. P., Commodore-Mensah, Y. & Yang, E. Racial and Ethnic Disparities in Hypertension: Barriers and Opportunities to Improve Blood Pressure Control. *Curr Cardiol Rep* 25, 17–27 (2023).
52. Sharma, A. & Palaniappan, L. Improving diversity in medical research. *Nat Rev Dis Primers* 7, 1–2 (2021).
53. Ji, H. *et al.* Sex Differences in Blood Pressure Trajectories over the Life Course. *JAMA Cardiology* 5, 255–262 (2020).
54. Maraboto, C. & Ferdinand, K. C. Update on hypertension in African-Americans. *Progress in Cardiovascular Diseases* 63, 33–39 (2020).
55. Snijder, M. B. *et al.* Cohort profile: The Healthy Life in an Urban Setting (HELIUS) study in Amsterdam, the Netherlands. *BMJ Open* 7, 1–11 (2017).
56. Alzheimer, A. Über eigenartige Krankheitsfälle des späteren Alters. *Zeitschrift für die gesamte Neurologie und Psychiatrie* 4, 356 (1911).
57. Scheltens, P. *et al.* Alzheimer's disease. *The Lancet* 397, 1577–1590 (2021).
58. Heneka, M. T. *et al.* Neuroinflammation in Alzheimer's disease. *Lancet Neurol* 14, 388–405 (2015).
59. Jack, C. R. *et al.* NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. *Alzheimers Dement* 14, 535–562 (2018).
60. Scheltens, P. *et al.* Atrophy of medial temporal lobes on MRI in" probable" Alzheimer's disease and normal ageing: diagnostic value and neuropsychological correlates. *J Neurol Neurosurg Psychiatry* 55, 967–972 (1992).

61. Pasquier, F. *et al.* Inter-and intraobserver reproducibility of cerebral atrophy assessment on MRI scans with hemispheric infarcts. *Eur Neurol* 36, 268–272 (1996).
62. Fazekas, F., Chawluk, J. B., Alavi, A., Hurtig, H. I. & Zimmerman, R. A. MR signal abnormalities at 1.5 T in Alzheimer's dementia and normal aging. *Am J Roentgenol* 149, 351–356 (1987).
63. Wardlaw, J. M. *et al.* Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. *The Lancet Neurology* 12, 822–838 (2013).
64. Albert, M. S. *et al.* The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7, 270–279 (2011).
65. McKhann, G. M. *et al.* The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7, 263–269 (2011).
66. Cronk, B. B., Johnson, D. K., Burns, J. M., & Alzheimer's Disease Neuroimaging Initiative. Body mass index and cognitive decline in mild cognitive impairment. *Alzheimer Dis Assoc Disord* 24, 126 (2010).
67. Guerin, O. *et al.* Nutritional status assessment during Alzheimer's disease: results after one year (the REAL French Study Group). *J Nutr Health Aging* 9, 81–84 (2005).
68. Andrieu, S. *et al.* Nutritional risk factors for institutional placement in Alzheimer's disease after one year follow-up. *J Nutr Health Aging* 5, 113–117 (2001).
69. Vellas, B. *et al.* Impact of nutritional status on the evolution of Alzheimer's disease and on response to acetylcholinesterase inhibitor treatment. *J Nutr Health Aging* 9, 75–80 (2005).
70. Kim, M. S. *et al.* Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model. *Gut* 69, 283–294 (2020).
71. Chen, T. & Guestrin, C. XGBoost: A scalable tree boosting system. *Proceedings of the ACM SIGKDD International Conference on Knowledge Discovery and Data Mining* 785–794 (2016). doi:10.1145/2939672.2939785.

PART I
HYPERTENSION

2

Associations between gut microbiota, faecal short chain fatty acids and blood pressure across ethnic groups: the HELIUS study

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Abstract

Background: Preliminary evidence from animal and human studies shows that gut microbiota composition and levels of microbiota-derived metabolites, including short chain fatty acids (SCFA), are associated with blood pressure (BP). We hypothesized that faecal microbiota composition and derived metabolites may be differently associated with BP across ethnic groups.

Methods: We included 4672 subjects (mean age 49.8±11.7 years, 52% women) from 6 different ethnic groups participating in the HELIUS study. The gut microbiota was profiled using 16S rRNA gene amplicon sequencing. Associations between microbiota composition and office BP were assessed using machine learning prediction models. In the subgroups with the largest associations, faecal SCFA levels were compared in 200 subjects with lower or higher systolic BP.

Results: Faecal microbiota composition explained 4.4% of the total systolic BP variance. Best predictors for systolic BP included *Roseburia* spp., *Clostridium* spp., *Romboutsia* spp., and *Ruminococcaceae* spp. Explained variance of the microbiota composition was highest in Dutch subjects (4.8%), but very low in African Surinamese, Ghanaians, and Turkish descent groups (explained variance <0.8%). Faecal SCFA levels, including acetate ($p<0.05$) and propionate ($p<0.01$), were lower in young Dutch participants with low systolic BP.

Conclusions: Faecal microbiota composition is associated with BP, but with strongly divergent associations between ethnic groups. Intriguingly, while Dutch participants with lower BP had higher abundances of several SCFA-producing microbes, they had lower faecal SCFA levels. Intervention studies with SCFAs could provide more insight in the effects of these metabolites on BP.

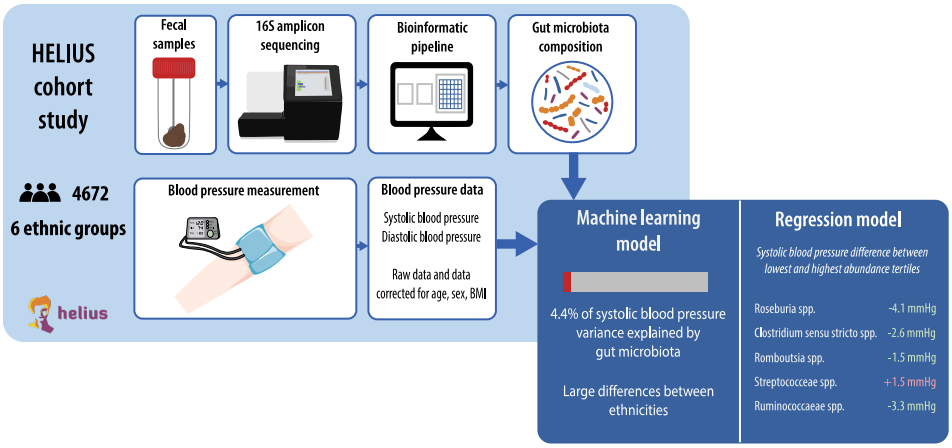
Introduction

Hypertension is the leading modifiable risk factor for cardiovascular morbidity and mortality, and thereby the most important risk factor for preventable death worldwide. The pathogenesis of essential hypertension remains incompletely understood, and is currently attributed to a complex interplay of genetic and cardiovascular risk factors.[1] However, recent studies have shown that only 3-4% of the variance in systolic blood pressure (SBP) can be explained by common genetic risk variants.[2] Lifestyle factors such as diet and obesity are known to be important for the pathogenesis of hypertension.[3] Analysis of population data from the UK biobank revealed that lifestyle factors can modify blood pressure (BP) by up to 4-5 mmHg depending on genetic risk.[4]

The gut microbiota is a reflection of both genetic make-up and life-long exposure to dietary risk factors, and could play a key role in mediating the development of essential hypertension.[5] Key metabolites produced by gut microbiota are short chain fatty acids (SCFAs), which are end-products of intestinal fermentation of otherwise indigestible dietary fibres.[6] Animal studies point towards a direct link between faecal SCFAs and BP, mediated by SCFAs receptors in kidneys and blood vessels.[7] In humans, evidence of the relation between faecal SCFA levels and BP is scarce and conflicting. Both higher and lower faecal SCFAs have been associated with higher BP.[8–10] Assuming that the gut microbiota and SCFAs are indeed associated with BP, this would provide new perspectives on both the pathogenesis and treatment of hypertension.

Earlier studies have identified important differences in both the prevalence, pathogenesis and treatment responses of hypertension among ethnic groups.[11–13] In addition, we found substantial differences in gut microbiota composition between ethnic groups within the population-based HELIUS cohort that were only partly explained by sociodemographic, lifestyle, or dietary influences.[14] Therefore, ethnic differences should be taken into account when studying associations between the gut microbiota composition and BP. Hence, in this cross-sectional study, we aim to investigate associations between the gut microbiota, faecal SCFA levels, and BP across different ethnic groups using data from the HELIUS cohort study.

Graphical abstract



Methods

Study population

We used cross-sectional data obtained during baseline visits between 2011 and 2015 of the ongoing HEalthy Life in an Urban Setting (HELIUS) prospective cohort study. The aims and design of this study have been described previously.[14] In brief, based on the municipality registry of Amsterdam, people aged between 18-70 were randomly sampled, stratified by ethnicity (Dutch, South-Asian Surinamese, African-Surinamese, Ghanaian, Turkish or Moroccan). For the present analysis, we included participants with available BP measurements, BMI, and faecal samples. All participants provided written informed consent and the study was approved by the medical ethical review board of the Amsterdam UMC, location AMC. This study followed the principles of the Declaration of Helsinki.

Data were collected during morning study visits at local research sites. Prior to these visits, all participants were asked to refrain from using any vasoactive medication and smoking. BMI was calculated from height and weight. BP was measured after at least 5 minutes of rest in the supine position, using the average of two consecutive measurements obtained with a validated semi-automatic oscillometric device (Microlife WatchBP Home; Microlife AG, Switzerland). Fasting glucose and creatinine levels were measured in venous blood samples, and estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI formula. In addition, urinary albumin-to-creatinine ratio was determined from early morning spot urine samples. Albuminuria was defined as a ratio ≥30 mg/mmol.[15]

Participants were asked to bring all current medication, from which current use of BP-lowering and glucose-lowering medication was determined. Diabetes was defined based on elevated fasting glucose levels (≥7 mmol/L) or the use of glucose-lowering medication. Hypertension was defined according to guidelines as an elevated SBP >140 mmHg or diastolic BP (DBP) >90 mmHg or self-reported use of BP-lowering medication.[15]

Gut microbiota composition

Participants received faecal collection tubes either prior or during the study visit. They were asked to bring a fresh faecal sample within 6 hours after collection, or, if not possible, to store the sample overnight in a freezer. Samples were stored at −20°C at the study visit location for a maximum of 1 day before transportation to the central freezer (-80°C). Samples obtained from participants who either had diarrhoea in the week prior to collection or used antibiotics within three weeks prior to collection were excluded. Samples were shipped to the Wallenberg Laboratory (Sahlgrenska Academy at University of Gothenburg, Sweden) for sequencing. DNA was extracted from 150 mg aliquot of faecal samples using a repeated bead-beating protocol.[16] Faecal microbiota composition was determined by sequencing the V4 region of the 16S rRNA gene on an Illumina MiSeq (llumina RTA v1.17.28; MCS v2.5, San Diego, CA, USA) using 515F and 806R primers designed for dual-indexing[17] and the V2 Illumina kit (2x250 bp paired-end reads). PCR was performed in duplicate reactions as previously described.[14] Preprocessing of the raw sequencing data, as described in **Supplement 1** resulted in a dataset containing 4672 samples.

Faecal SCFA measurements

Faecal SCFA levels were measured using high performance liquid chromatography (HPLC) with UV detection according to the method of De Baere *et al.*[18] In addition, for all samples, dry weights were determined after freeze-drying a homogenized faecal aliquot for 24 hours. All concentrations resulting from HPLC measurements were corrected for the difference in the wet and dry weight per sample.

Statistical analysis

We used machine learning models to assess the association between gut microbiota composition and BP. Analyses were performed for the total study population and for subgroups stratified by age (≤ 50 years, >50 years), sex, and ethnicity. A

separate set of models was performed using adjusted SBP and DBP values. Adjustments were made by determining the residuals after fitting a linear regression model for each of the subgroups with SBP/DBP as the dependent variable and age, sex, and BMI as independent variables. For age, we used sex-specific restricted cubic splines, of which the order was chosen based on the Akaike Information Criterion. Machine learning models were built aiming to predict SBP, corrected SBP, DBP, and corrected DBP from the gut microbiota composition (i.e. from the relative abundance of microbial 16s rRNA amplicon sequence variants; ASVs). Gradient boosted tree models were used in a nested-cross validation structure to prevent overfitting and ensure robustness of results (**Supplement 2**). Models were built using an iterative flow. In each iteration, the dataset was randomly split into a test set containing 20% of the participants and a training set containing the remaining 80%. Thereafter, 5-fold cross-validation was performed strictly within the train set in order to fit and optimize the model hyperparameters. The resulting model was finally evaluated on the test set. Two random variables were added to the predictor data during each iteration to serve as a benchmark. Explained

$$explained\ variance\ (y, \hat{y}) = 1 - \frac{Var\{y - \hat{y}\}}{Var\{y\}} * 100\%$$

variance was determined as the proportion of variance of the outcome (SBP or DBP) explained by the model-predicted values ŷ (predicted SBP or DBP), using: Explained variance and the ranked list of predictor importance were recorded for each iteration and were averaged across 100 iterations. If the explained variance was negative, we concluded that the model did not have any predictive power.

Spearman rank correlation coefficients were calculated between the top 10 best SBP-predicting ASVs found by the machine learning models and both SBP and DBP. Furthermore, participants were categorized into tertiles of the relative abundance of each of the ASVs. Effect sizes for the effect of each ASV on SBP were estimated for every tertile using linear regression in a crude model correcting only for age and sex, and in a full model with additional correction for BMI, smoking, use of antihypertensive medication, and history of diabetes.

For the analyses of faecal SCFAs, we used a subgroup of 200 participants selected from Dutch participants aged ≤50 years, as the explained variance was highest in the Dutch and the young subgroups. Based on age-specific percentiles (<30 years, 30-40, 40-50 years), 50 men and 50 women with the highest SBP were selected. Using sex, age and BMI, these 100 participants were matched to 100 other participants from the lowest 35th percentile of SBP. Faecal SCFA concentrations and abundance of the top predicting ASV's were compared between the high and

low BP group using Mann-Whitney U-tests. In addition, the relation with microbiota composition was examined using a correlation matrix of SCFA concentration, BP, and the top 10 predicting ASVs.

Machine learning was implemented in Python (v.3.7.4) using the XGBoost (v.0.90), numpy (v.1.16.4), pandas (v.0.25.1), and scikit-learn (v.0.21.2) packages. Statistical analyses were performed using R (v.3.6.2), using the Regression Modeling Strategies (rms, v.5.1-4) and Nonparametric Preprocessing for Parametric Causal Interference (MatchIt v.3.0.2) packages. Figures were created using R with the corrplot package (v.0.84), and Graphpad (v.8.3.0).

Data availability

The 16S rRNA gene amplicon raw sequence data and associated metadata have been deposited at the European Genome-phenome Archive under study number EGAD00001004106. The HELIUS data are owned by the Amsterdam UMC, location AMC in Amsterdam, The Netherlands. Any researcher can request the data by submitting a proposal as outlined at http://www.heliusstudy.nl/.

Results

Population characteristics

Characteristics of the included 4672 participants are shown in **Table 1**. Younger participants (≤50 years) had a lower prevalence of hypertension (24.2%) compared to older participants (57.1%), and a lower use of antihypertensive medication (8.8% vs 33.4%). Dutch and Moroccan groups were younger and contained more men than other groups. South Asian Surinamese, African Surinamese, and Ghanaian groups had higher BP and higher proportions of participants with hypertension than other ethnic groups. BMI was lowest in Dutch (25.5±4.4 kg/m^2) and in the South Asian Surinamese groups (26.6±4.5 kg/m^2). Diabetes prevalence was highest in South Asian Surinamese participants (23.9%) and lowest in Dutch participants (4.8%).

Microbiota composition and BP

The BP variance that was explained by gut microbiota composition is shown in **Table 2,** stratified for the different subgroups. In the total population, the explained variance of BP levels by microbiota composition was 4.4% for SBP and

Table 1: Population characteristics

	Overall	Younger (≤50)	Older (>50)	Female	Male
n	4672	2217	2455	2429	2243
Female	2429 (52.0)	1220 (55.0)	1209 (49.2)	-	-
Age (years)	49.8±11.7	39.9±8.3	58.8±5.2	49.2±11.7	50.5±11.6
SBP (mmHg)	129.9±18.2	123.7±16.0	135.4±18.1	127.2±18.5	132.7±17.3
DBP (mmHg)	81.1±10.6	79.1±10.5	83.0±10.4	78.6±10.4	83.9±10.2
BMI (kg/m^2)	27.2±4.9	26.7±5.0	27.7±4.8	27.8±5.4	26.6±4.2
eGFR (ml/ min/1.73m^2)	97.1±17.0	105.6±14.8	89.3±15.0	98.7±17.2	95.3±16.5
Hypertension	1937 (41.5)	536 (24.2)	1401 (57.1)	924 (38.0)	1013 (45.2)
Antihypertensive drugs	1016 (21.7)	196 (8.8)	820 (33.4)	559 (23.0)	457 (20.4)
Lipid lowering drugs	580 (12.4)	86 (3.9)	494 (20.1)	240 (9.9)	340 (15.2)
Albuminuria	196 (4.2)	67 (3.0)	129 (5.3)	91 (3.8)	105 (4.7)
Diabetes	507 (10.9)	96 (4.3)	411 (16.8)	218 (9.0)	289 (12.9)
Antidiabetic drugs	367 (7.9)	61 (2.8)	306 (12.5)	178 (7.3)	189 (8.4)
Smoking	941 (20.1)	456 (20.6)	485 (19.8)	349 (14.4)	592 (26.4)

	Dutch	SAS	Afr Sur	Ghanaian	Turkish	Moroccan
n	1328	575	1128	462	436	605
Female	633 (47.7)	300 (52.2)	672 (59.6)	255 (55.2)	224 (51.4)	281 (46.4)
Age (years)	51.43±12.7	51.6±11.2	51.9±10.5	48.2±9.0	44.2±11.0	45.5±11.4
SBP (mmHg)	127.6±17.2	132.4±19.6	132.9±17.9	137.2±18.2	124.1±16.2	125.3±17.6
DBP (mmHg)	79.5±10.2	81.6±10.4	83.4±10.4	85.8±11.0	79.5±10.3	77.6±9.8
BMI (kg/m^2)	25.5±4.4	26.6±4.5	28.2±5.4	28.2±4.5	28.9±4.8	27.9±4.7
eGFR (ml/ min/1.73m^2)	91.2±14.9	91.8±16.8	99.1±18.3	100.4±17.7	104.6±13.4	104.5±14.1
Hypertension	455 (34.3)	280 (48.7)	598 (53.0)	272 (58.9)	127 (29.1)	146 (24.1)

Antihypertensive drugs	210 (15.8)	174 (30.3)	353 (31.3)	136 (29.4)	64 (14.7)	49 (8.1)
Lipid lowering drugs	135 (10.2)	160 (27.8)	130 (11.5)	40 (8.7)	53 (12.2)	44 (7.3)
Albuminuria	26 (2.0)	45 (7.8)	42 (3.7)	27 (5.9)	19 (4.4)	28 (4.6)
Diabetes	63 (4.8)	137 (23.9)	143 (12.7)	47 (10.2)	39 (9.0)	64 (10.6)
Antidiabetic drugs	25 (1.9)	116 (20.2)	104 (9.2)	35 (7.6)	30 (6.9)	45 (7.4)
Smoking	263 (19.8)	139 (24.2)	300 (26.6)	17 (3.7)	117 (26.8)	74 (12.2)

Data is presented as mean±SD or n (%). SBP = systolic blood pressure; DBP = diastolic blood pressure, BMI = body mass index, eGFR = estimated glomerular filtration rate (CKD-EPI), SAS = South Asian Surinamese, Afr Sur = African Surinamese.

4.3% for DBP. Explained variance was higher in younger subjects (5.3% for both SBP and DBP) than in older subjects (2.5% for SBP; 1.4% for DBP), and higher in women (3.9% for SBP, 2.2% for DBP) than in men (1.8% for SBP; 0.3% for DBP). There was a clear difference between Dutch (4.8% for SBP, 0.4% for DBP) and other ethnic groups (range 0-0.8% for SBP, 0.48% for DBP). The correlations between alpha diversity of gut microbiota and BP (**Supplement 3**) showed the same pattern as the explained variance with stronger correlations in young, female and Dutch subgroups.

In the total study population, the best predicting ASVs were *Roseburia* spp., *Clostridium sensu stricto* spp., *Roseburia hominis*, *Romboutsia* spp., *Streptococcus* spp., and *Ruminococcaceae* NK4A214 spp. (**Supplement 4**). The abundance of the best predicting ASVs was negatively associated with both SBP and DBP, except for *Streptococcus* spp. and *Klebsiella* spp., as shown in **Figure 1**. In addition, the correlation plot showed significant collinearity between the best predicting ASVs. In the regression analyses, the effect of these ASVs on BP ranged between -6 mmHg and 2 mmHg (**Figure 2, Supplement 5)**, with increasing effect sizes for higher abundance in most of the ASVs. *Roseburia* spp. was both the best predictor from the machine learning model and had the largest absolute effect on BP: the middle and highest tertile were associated with a lower SBP of respectively 2.3 (95%CI 1.2–3.5) and 6.0 mmHg (95%CI 4.9–7.1). The effect of the ASV abundance on BP was attenuated when adjusting for confounders, including use of medication, but remained significant for most predictors, ranging between -4 and 2 mmHg. In this model, we found for the second tertile of *Roseburia* spp., a 1.9 (95%CI 0.8–3.0) lower SBP, while participants in the upper tertile had a 4.1 (95%CI 3.0–5.1) mmHg lower SBP.

Table 2: Explained variance of BP by microbiota composition for different subgroups

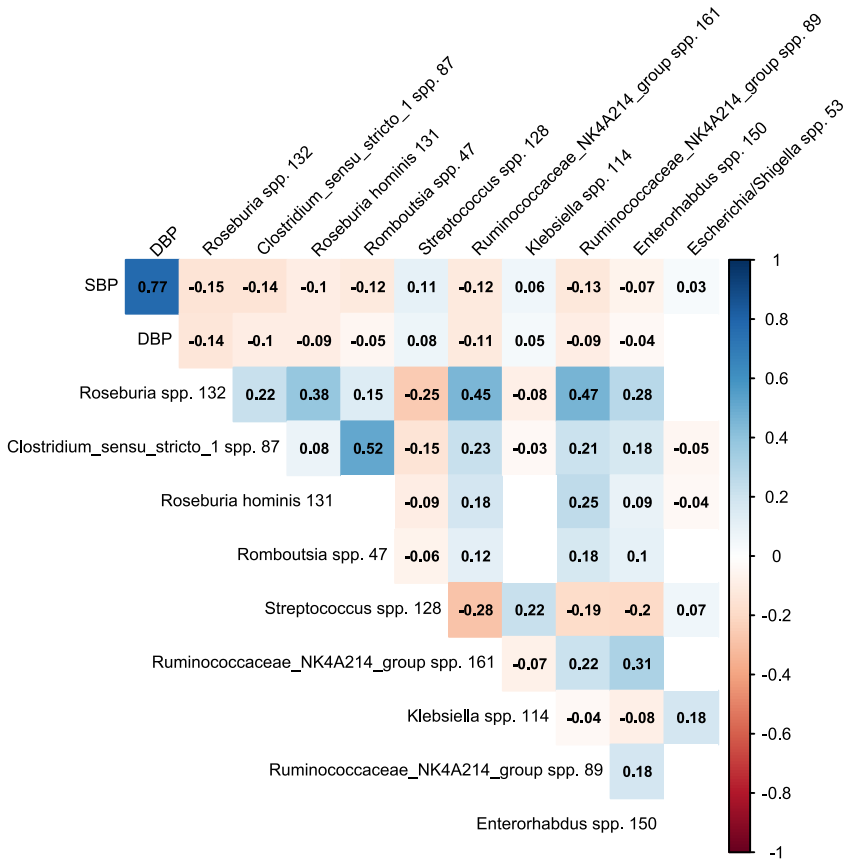
	Microbiota composition (explained variance in %)			
Group	**SBP**	**Res SBP**	**DBP**	**Res DBP**
All subjects	4.44	2.22	4.30	2.05
Younger (≤50)	5.31	3.11	5.34	2.81
Older (>50)	2.51	1.75	1.40	0.87
Men	1.82	1.41	0.32	1.41
Women	3.89	1.90	2.23	1.30
Dutch	4.76	0.60	0.40	n.a.
SA Surinamese	n.a.	0.64	n.a.	0.09
Afr Surinamese	n.a.	0.74	n.a.	0.08
Ghanaian	n.a.	0	n.a.	n.a.
Moroccan	0.77	0.42	n.a.	0.64
Turkish	n.a.	n.a.	0.48	0.62

Explained variance in % of the gut microbiome composition for blood pressure. SA Surinamese = South Asian Surinamese; Afr Surinamese = African Surinamese; SBP = systolic blood pressure; DBP = diastolic blood pressure; res = residuals adjusted for age, sex, BMI. N.a. = explained variance in these models was negative.

Faecal SCFA levels and BP

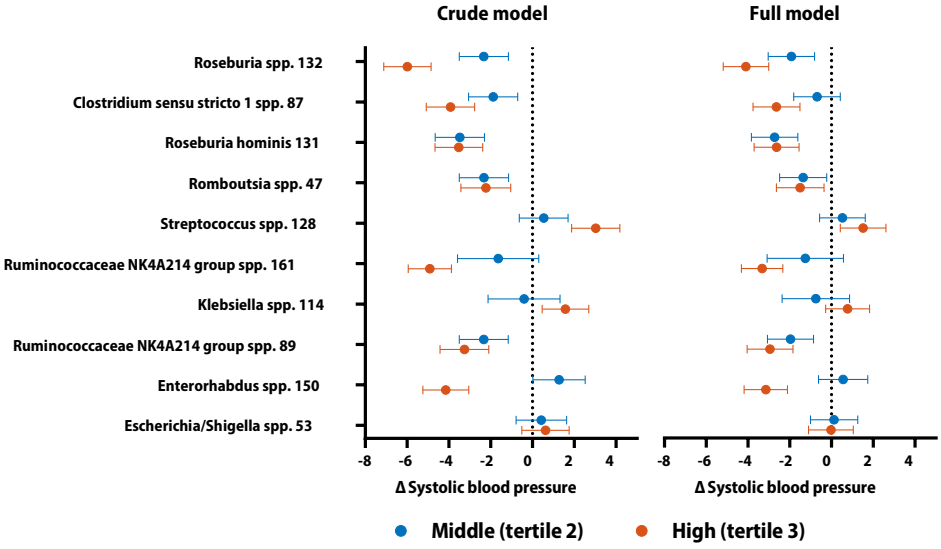
Matching of Dutch subjects on age, sex and BMI resulted in 100 subjects with low SBP and 100 subjects with high SBP (**Supplement 6**). Consistent with the data from the full cohort, subjects with low BP had higher abundance of *Roseburia* spp. (p=0.0047), *Roseburia hominis* (p=0.047) and *Ruminococcaceae* spp. (p=0.045). Differences of faecal SCFA levels are shown in **Figure 3**. Low SBP subjects had significantly lower faecal levels of acetate (p=0.022) and propionate (p=0.006), and there was a trend of lower butyrate levels (p=0.077). In addition, faecal SCFA levels were negatively correlated with the top 10 ASVs, and positively with SBP and DBP (**Supplement 7**).

Figure 1: Correlations of top predictors for systolic blood pressure



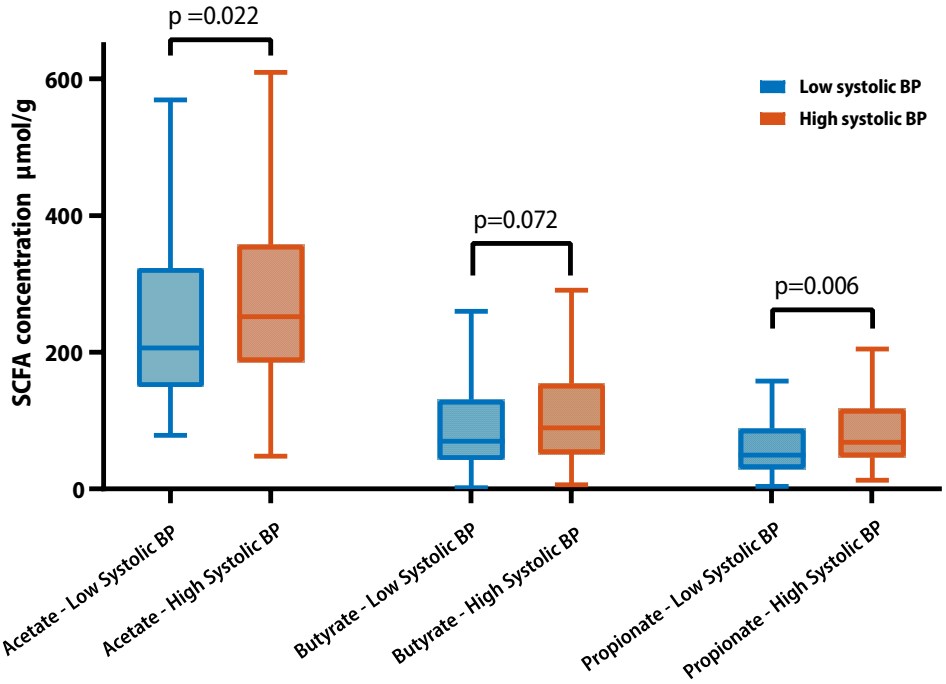
Correlation plot for top 10 predictors of systolic blood pressure from gut microbiota composition. Only significant ($p<0.05$) Spearman correlation coefficients between the relative abundance of each of the microbes, systolic (SBP) and diastolic (DBP) blood pressure are shown. Colours indicate direction and strength of each correlation.

Figure 2: Linear regression models



Linear regression coefficients with 95%-confidence intervals per tertile of ASV counts for top 10 predictors of systolic blood pressure (SBP) from gut microbiota composition, with the lowest tertile as reference. Left side: crude model (correcting for age and sex); right side: additional correction for BMI, smoking, use of antihypertensive medication and history of diabetes.

Figure 3: Faecal SCFA levels



Comparison of short chain fatty acid (SCFA) levels between high versus low blood pressure (BP) subgroups (boxes: median with interquartile range; bars: minimum and maximum). Differences tested with Mann-Whitney U tests.

Discussion

Our main finding is that gut microbiota composition is associated with BP and that the explained BP variance was widely divergent between ethnic groups. Associations between the gut microbiota and BP remained essentially unchanged after correcting for possible confounders, including BMI. Remarkably, while SCFA-producing microbes were associated with lower BP, increased faecal SCFA levels were associated with higher BP. In line with this finding, SCFA-producing microbes were negatively correlated with faecal SCFA levels. The current study extends previous findings in cohort studies by evaluating the association between gut microbiota composition and BP in a large multi-ethnic cohort using machine learning prediction models.[19,20]

We found that, in the complete cohort, machine learning models based on gut microbiota composition explained 4.2% and 4.3% of the variance in SBP and DBP. Regression models showed that in the top tertiles of microbiota predictors SBP was 4 to 6 mmHg lower compared to the lowest tertile. This is a similar effect size compared to the effect of genetic or lifestyle risk factors on SBP in the UK biobank.[4] Based on recent meta-analyses of randomised controlled trials, this corresponds to an overall cardiovascular risk reduction of 8-12%.[21] After correction for BMI, explained variance of SBP and DBP was attenuated to 2.2% and 2.1%. In line with these findings, in the regression analyses the effects were attenuated towards a difference of 2 to 4 mmHg after correction for BMI and other covariates, suggesting that the effect is only partly driven by BMI.

In the analysis of the top predictors, the finding that SCFA-producing microbes are associated with lower BP is in line with two studies of microbiota composition and hypertension that found lower abundances of either *Ruminococcaceae* spp. or *Roseburia* spp. in subjects with higher BP.[10,20] Moreover, comparable to our results, higher abundances of *Klebsiella* spp. and *Streptococcaceae* spp. have been previously associated with higher BP.[10,22]

Previous analyses of HELIUS and other cohorts have shown significant ethnic differences in gut microbiota composition.[14,23] We add that there are substantial differences in the association of gut microbiota and BP between ethnicities, sexes and ages, as we observed the highest explained variance in the young, female and Dutch subgroups. In addition to differences in microbiota composition, this could relate to age, sex and ethnic specific effects in the underlying aetiology of hypertension. At younger age, lifestyle and genetic factors are important determinants, while at older age SBP increases and DBP decreases as a consequence of arterial stiffness.[24] In addition, multiple studies have shown that older individuals and individuals of African descent are more salt-sensitive, suggesting that they have a more volume-dependent hypertension phenotype.[25] Earlier findings from animal models pointed towards a relation between the gut microbiota and salt-sensitive BP driven by abundance of *Lactobacillus* spp.[26] In contrast, we observed a lower explained variance in older, Ghanaian, and African Surinamese subjects, and *Lactobacillus* spp. was not among the top predictors in these models nor in the model with all subjects. We therefore could not confirm the association between gut microbiota composition and salt-sensitivity in susceptible populations.

Faecal SCFA levels were higher in subjects with higher BP, which is in line with previous results from other cohort studies that examined the relation between

faecal SCFAs and BP.[8,9] These positive associations between BP and faecal SCFA levels in our cohort seem to conflict with the negative associations found between BP and SCFA-producing microbes. However, faecal SCFA levels are not a direct measure of intestinal SCFA production but rather a net result of SCFA production after subtracting SCFA absorption.[27] We found consistent correlations in the subgroup with faecal SCFA levels in which SCFA-producing bacteria were both negatively correlated with BP and with faecal SCFA levels. Therefore, we hypothesize that higher microbial SCFA production upregulates intestinal SCFA absorption resulting in relatively lower levels of SCFAs excretion in faeces.[28]

The observed differences in SCFA levels between subjects with high and low BP and the multiple SCFA-producing microbes provide further evidence for the hypothesis that SCFAs have a role in BP regulation. Animal studies have shown that SCFAs can have disparate effects on BP depending on the receptors involved. Free fatty acid receptors (FFAR) are G-protein coupled receptors that can be found in a variety of tissues, including the kidney and renal artery, and causes arterial vasodilation in response to propionate, acetate and butyrate.[7] In contrast, a BP elevating effect is mediated by the SCFA receptor Olfr78 in mice through renin release from granules in the renal juxtaglomerular apparatus.[29] The human analogue of this olfactory receptor is OR51E2, which responds to propionate and acetate, but not butyrate.[7] It has been suggested that Olfr78 and OR51E2 serve as a negative feedback loop for the BP lowering effects of the FFARs, specifically FFAR3.[29] Future intervention studies with oral SCFAs could further unravel the cross-talk between the different SCFA and BP regulation in humans.

To our knowledge, this is the first study to assess the relation between gut microbiota composition and BP across different ethnic groups. We used a large population-based sample with standardized BP measurements for our analysis. Faecal samples were obtained using a standardized protocol from participants without diarrhoea and prior antibiotics use, and analysed using 16S rRNA sequencing, which is a widely used and reproducible method to determine microbiota composition.[30] For the main analysis, we used machine learning prediction models with nested cross-validation, which enabled us to simultaneously include the complete processed sequencing results in the models while minimizing the risk of overfitting. We corrected for BMI using residuals after fitting a regression model, which could lead to an additional random error in the corrected values. However, both correction for covariates in the regression analyses and the correlations between alpha diversity and BP yielded similar results. While the machine learning results could be hampered by the use of BP-lowering drugs or glucose-lowering medica-

tion, effects remained significant after correction in the regression model. In the analyses of SCFA levels, we matched the low and high BP groups for age, sex and BMI. However, significant differences in BMI remained after matching, that could have affected our results. Lastly, the cross-sectional design of this study complicates causal interpretation of the observed associations. In that regard, we expect that prospective data from the HELIUS cohort study will enable us to assess the longitudinal relation between gut microbiota composition and the development of hypertension. If a longitudinal relation can be confirmed such that changes in microbiota are found to precede and be proportional to changes in BP, potential therapeutic strategies that could be considered include supplementation of (a combination of) specific bacterial strains, modulating gut metabolites such as SCFAs, faecal microbiota transplantation or antibiotic treatment.

In conclusion, we found a consistent association between gut microbiota composition and BP, with large differences in explained variance between age and ethnic subgroups. Future studies should take ethnic differences into account when studying the gut microbiota in relation to BP. The observed associations between SCFA-producing microbes and BP provide further evidence for the hypothesis that SCFAs play a role in BP regulation. Intervention studies with SCFAs could provide more insight in the underlying mechanism of these metabolites on BP.

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Conflicts of interests

M.N. is on the scientific advisory board of Caelus Health, the Netherlands. F.B. is on the scientific advisory board of MetaboGen AB, Sweden, and received grants from BioGaia AB, Sweden. None of these conflicts of interest bear direct relation to the outcomes of this study. All other authors declare that they have no competing interests.

References

1. Poulter NR, Prabhakaran D, Caulfield M. Hypertension. *Lancet* 2015;386:801–812.
2. Giri A, Hellwege JN, Keaton JM, Park J, Qiu C, Warren HR, et al. Trans-ethnic association study of blood pressure determinants in over 750,000 individuals *Nat Genet* 2019;51:51–62.
3. Forman JP, Stampfer MJ, Curhan GC. Diet and lifestyle risk factors associated with incident hypertension in women. *JAMA* 2009;302:401–411.
4. Pazoki R, Dehghan A, Evangelou E, Warren H, Gao H, Caulfield M, Elliott P, Tzoulaki I. Genetic predisposition to high blood pressure and lifestyle factors: Associations with midlife blood pressure levels and cardiovascular events. *Circulation* 2018;137:653–661.
5. Marques FZ, Mackay CR, Kaye DM. Beyond gut feelings: How the gut microbiota regulates blood pressure. *Nat Rev Cardiol* 2018;15:20–32.
6. Cummings JH, Pomare EW, Branch HWJ, Naylor CPE, MacFarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 1987;28:1221–1227.
7. Pluznick JL. Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. *Curr Hypertens Rep* 2017;19:25.
8. Huart J, Leenders J, Taminiau B, Descy J, Saint-Remy A, Daube G, Krzesinski JM, Melin P, Tullio P De, Jouret F. Gut Microbiota and Fecal Levels of Short-Chain Fatty Acids Differ Upon 24-Hour Blood Pressure Levels in Men. *Hypertension* 2019;74:1005–1013.
9. la Cuesta-Zuluaga J de, Mueller NT, Álvarez-Quintero R, Velásquez-Mejía EP, Sierra JA, Corrales-Agudelo V, Carmona JA, Abad JM, Escobar JS. Higher fecal short-chain fatty acid levels are associated with gut microbiome dysbiosis, obesity, hypertension and cardiometabolic disease risk factors. *Nutrients* 2019;11:51.
10. Yan Q, Gu Y, Li X, Yang W, Jia L, Chen C, Han X, Huang Y, Zhao L, Li P, Fang Z, Zhou J, Guan X, Ding Y, Wang S, Khan M, Xin Y, Li S, Ma Y. Alterations of the Gut Microbiome in Hypertension. *Front Cell Infect Microbiol* 2017;7.
11. Laer SD van, Snijder MB, Agyemang C, Peters RJG, Born BJH van den. Ethnic differences in hypertension prevalence and contributing determinants – the HELIUS study. *Eur J Prev Cardiol* 2018;25:1914–1922.
12. Tu W, Eckert GJ, Hannon TS, Liu H, Pratt LM, Wagner MA, DiMeglio LA, Jung J, Pratt JH. Racial differences in sensitivity of blood pressure to aldosterone. *Hypertension* 2014;63:1212–1218.
13. Gupta AK, Poulter NR, Dobson J, Eldridge S, Cappuccio FP, Caulfield M, Collier D, Cruickshank JK, Sever PS, Feder G. Ethnic differences in blood pressure response to first and second-line antihypertensive therapies in patients randomized in the ASCOT trial. *Am J Hypertens* 2010;23:1023–1030.
14. Deschasaux M, Bouter KE, Prodan A, Levin E, Groen AK, Herrema H, Tremaroli

V, Bakker GJ, Attaye I, Pinto-Sietsma SJ, Raalte DH van, Snijder MB, Nicolaou M, Peters R, Zwinderman AH, Bäckhed F, Nieuwdorp M. Depicting the composition of gut microbiota in a population with varied ethnic origins but shared geography. *Nat Med* 2018;24:1526–1531.
15. The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J* 2018;39:3021–3104.
16. Mobini R, Tremaroli V, Ståhlman M, Karlsson F, Levin M, Ljungberg M, Sohlin M, Bertéus Forslund H, Perkins R, Bäckhed F, Jansson PA. Metabolic effects of Lactobacillus reuteri DSM 17938 in people with type 2 diabetes: A randomized controlled trial. *Diabetes Obes Metab* 2017;19:579–589.
17. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl Environ Microbiol* 2013;79:5112–5120.
18. Baere S De, Eeckhaut V, Steppe M, Maesschalck C De, Backer P De, Immerseel F Van, Croubels S. Development of a HPLC-UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation. *J Pharm Biomed Anal* 2013;80:107–115.
19. Jackson MA, Verdi S, Maxan ME, Shin CM, Zierer J, Bowyer RCE, Martin T, Williams FMK, Menni C, Bell JT, Spector TD, Steves CJ. Gut microbiota associations with common diseases and prescription medications in a population-based cohort. *Nat Commun* 2018;9:1–8.
20. Sun S, Lulla A, Sioda M, Winglee K, Wu MC, Jacobs DR, Shikany JM, Lloyd-Jones DM, Launer LJ, Fodor AA, Meyer KA. Gut microbiota composition and blood pressure: The CARDIA study. *Hypertension* 2019;73:998–1006.
21. Ettehad D, Emdin CA, Kiran A, Anderson SG, Callender T, Emberson J, Chalmers J, Rodgers A, Rahimi K. Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. *Lancet* 2016;387:957–967.
22. Kim S, Goel R, Kumar A, Qi Y, Lobaton G, Hosaka K, Mohammed M, Handberg EM, Richards EM, Pepine CJ, Raizada MK. Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clin Sci* 2018;132:701–718.
23. Vangay P, Johnson AJ, Ward TL, Al-Ghalith GA, Shields-Cutler RR, Hillmann BM, Lucas SK, Beura LK, Thompson EA, Till LM, Batres R, Paw B, Pergament SL, Saenyakul P, Xiong M, Kim AD, Kim G, Masopust D, Martens EC, Angkurawaranon C, McGready R, Kashyap PC, Culhane-Pera KA, Knights D. US Immigration Westernizes the Human Gut Microbiome. *Cell* 2018;175:962-972.e10.
24. Gurven M, Blackwell AD, Rodríguez DE, Stieglitz J, Kaplan H. Does blood pressure inevitably rise with age? *Hypertension* 2012;60:25–33.
25. Brown MJ. Hypertension and ethnic group. *Br Med J* 2006;332:835–836.
26. Wilck N, Matus MG, Kearney SM, Olesen SW, Forslund K, Bartolomaeus H, Haase S, Mahler A, Balogh A, Marko L, Vvedenskaya O, Kleiner FH, Tsvetkov D, Klug L, Costea PI, Sunagawa S, Maier L, Rakova N, Schatz V, Neubert P, Fratzer C, Krannich A, Gollasch M, Grohme DA, Corte-Real BF, Gerlach RG, Basic M, Typas A, Wu C, Titze JM, Jantsch J, Boschmann M, Dechend R, Kleinewietfeld M, Kempa

S, Bork P, Linker RA, Alm EJ, Müller DN Salt-responsive gut commensal modulates TH17 axis and disease. *Nature* 2017;551:585–589.
27. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat Rev Endocrinol* 2015;11:577–591.
28. Yang T, Magee KL, Colon-Perez LM, Larkin R, Liao YS, Balazic E, Cowart JR, Arocha R, Redler T, Febo M, Vickroy T, Martyniuk CJ, Reznikov LR, Zubcevic J. Impaired butyrate absorption in the proximal colon, low serum butyrate and diminished central effects of butyrate on blood pressure in spontaneously hypertensive rats. *Acta Physiol* 2019;226.
29. Pluznick JL, Protzko RJ, Gevorgyan H, Peterlin Z, Sipos A, Han J, Brunet I, Wan LX, Rey F, Wang T, Firestein SJ, Yanagisawa M, Gordon JI, Eichmann A, Peti-Peterdi J, Caplan MJ. Olfactory receptor responding to gut microbiotade rived signals plays a role in renin secretion and blood pressure regulation. *PNAS* 2013;110:4410–4415.
30. Bender JM, Li F, Adisetiyo H, Lee D, Zabih S, Hung L, Wilkinson TA, Pannaraj PS, She RC, Bard JD, Tobin NH, Aldrovandi GM. Quantification of variation and the impact of biomass in targeted 16S rRNA gene sequencing studies. *Microbiome* 2018;6.

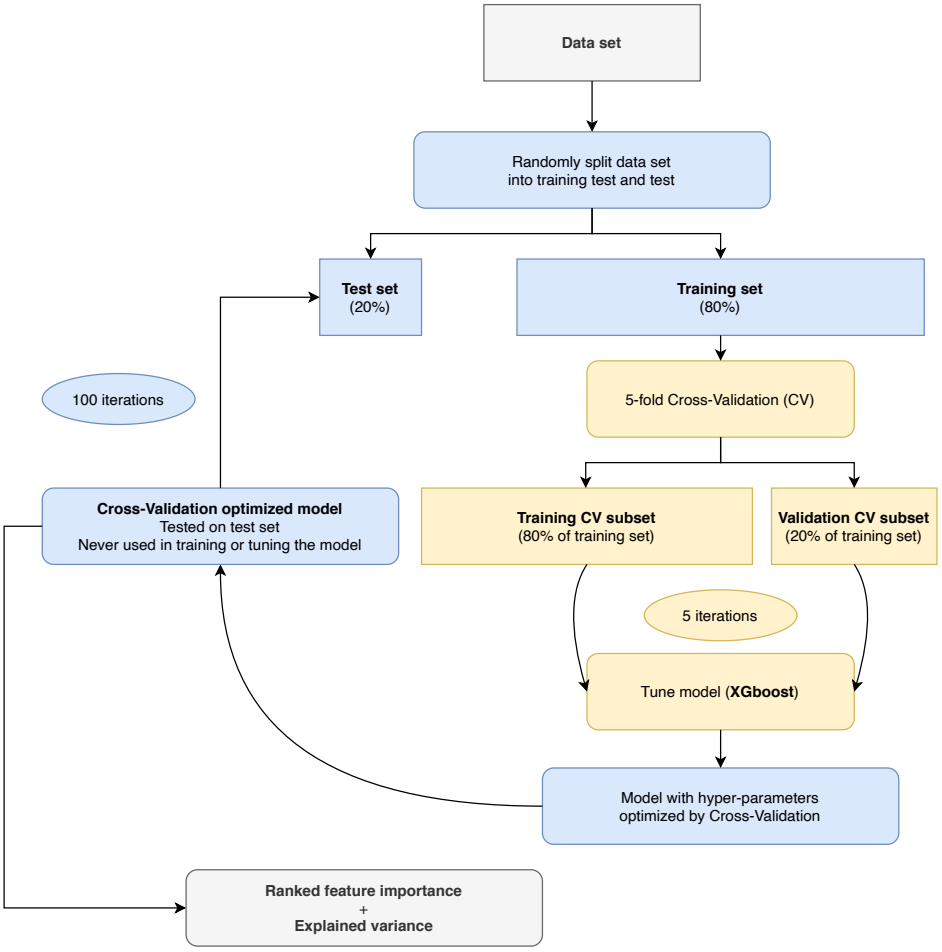
Supplements

Supplement 1: Bioinformatic pipeline

Raw sequencing reads were processed using USEARCH (v. 11.0.667).[1] Paired-end reads were merged allowing a maximum of 30 differences in the overlapping region and a maximum of 1 expected error in the merged contig. Expected error-based read quality filtering was performed as described in Edgar et al.[2] Remaining contigs were dereplicated and unique sequences were denoised using the UNOISE3 algorithm to infer Amplicon Sequence Variants (ASVs).[2] All merged reads were subsequently mapped against the resulting ASVs to produce a count table. ASVs not matching expected amplicon length were removed (i.e. ASV sequences longer than 260 bp or shorter than 250 bp). Taxonomy was assigned with the 'assignTaxonomy' function from the 'DADA2' R package (v. 1.12.1) using the SILVA (v. 132) reference database.[3,4] ASVs sequences were then aligned using MAFFT (v. 7.427) using the auto settings.[5] A phylogenetic tree was constructed from the resulting multiple sequence alignment with FastTree (v. 2.1.11 Double Precision) using a generalized time-reversible model.[6] The ASV table, taxonomy, and tree were integrated using the 'phyloseq' R package (v. 1.28.0). The ASV table was rarefied to 14932 counts per sample.[7] Of 6056 sequenced samples, 24 had insufficient counts (<5000 counts per sample) and were excluded at the rarefying stage.

1. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* (2010) doi:10.1093/bioinformatics/btq461.
2. Edgar, R. C. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv* (2016) doi:10.1101/081257.
3. Callahan, B. J. *et al*. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* (2016) doi:10.1038/nmeth.3869.
4. Quast, C. *et al*. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* (2013) doi:10.1093/nar/gks1219.
5. Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* (2013) doi:10.1093/molbev/mst010.
6. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS ONE* (2010) doi:10.1371/journal.pone.0009490.
7. McMurdie, P. J. & Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* (2013) doi:10.1371/journal.pone.0061217.

Supplement 2: Design of machine learning model



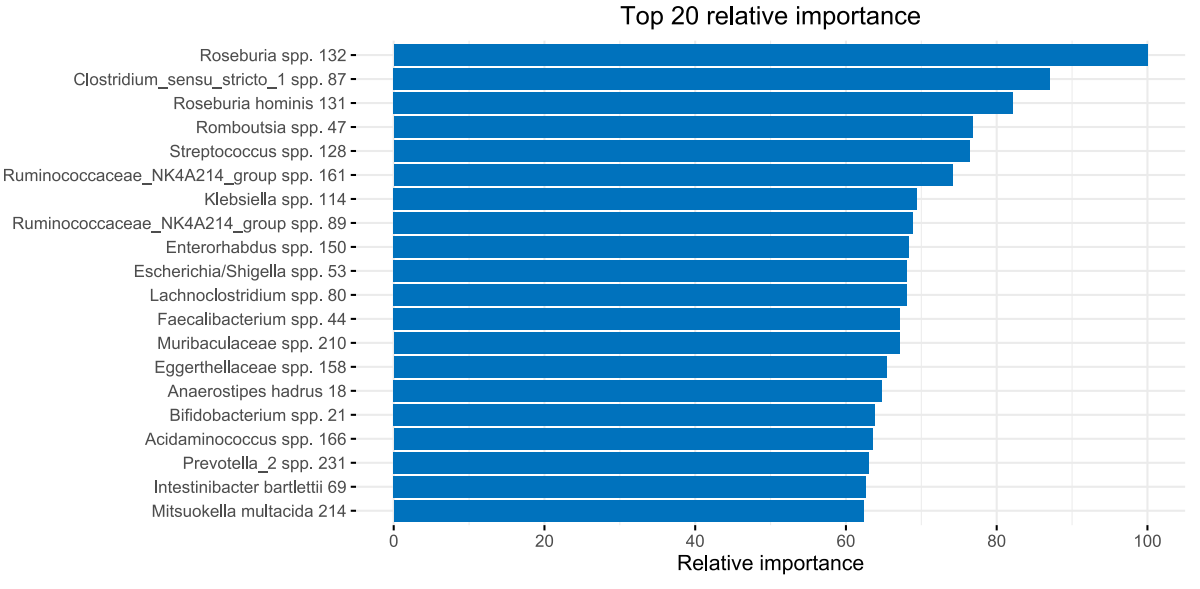
Schematic overview of the models used for determining the association between the gut microbiota composition and blood pressure.

Supplement 3: Correlations Shannon index and blood pressure

	Alpha diversity			
Group	**SBP (ρ)**	**p-value**	**DBP (ρ)**	**p-value**
All subjects	-0.10	<0.001	-0.10	<0.001
Younger (≤50)	-0.12	<0.001	-0.12	<0.001
Older (>50)	-0.12	<0.001	-0.11	<0.001
Men	-0.05	0.03	-0.07	0.033
Women	-0.13	<0.001	-0.11	<0.001
Dutch	-0.08	<0.001	-0.05	0.005
SA Surinamese	-0.07	0.10	-0.07	0.101
Afr Surinamese	-0.08	0.01	-0.06	0.006
Ghanaian	0.00	0.99	0.05	0.990
Morrocan	-0.08	0.06	-0.10	0.059
Turkish	-0.07	0.16	-0.08	0.164

Spearman correlation coefficients with corresponding p-values for the alpha diversity and both systolic and diastolic blood pressure. SA Surinamese = South Asian Surinamese; Afr Surinamese = African Surinamese; SBP = systolic blood pressure; DBP = diastolic blood pressure.

Supplement 4: Relative feature importance of systolic blood pressure predictors



Relative feature importance of top 20 predictors for systolic blood pressure from gut microbiota composition. Relative importance is determined with respect to the most important predictor of the model. The top 10 predictors were selected for further analyses in the regression models.

Supplement 5: Linear regression coefficients ASVs and blood pressure

All subjects		Crude model			Full model		
ASV	**Tertile**	**Coefficient**	**lower**	**upper**	**Coefficient**	**lower**	**upper**
Roseburia spp.	2	-2.33	-3.50	-1.16	-1.92	-3.03	-0.82
	3	-5.99	-7.11	-4.86	-4.10	-5.19	-3.01
Clostridium sensu stricto 1 spp.	2	-1.88	-3.06	-0.71	-0.70	-1.81	0.42
	3	-3.92	-5.08	-2.76	-2.64	-3.76	-1.52
Roseburia hominis	2	-3.48	-4.66	-2.30	-2.73	-3.84	-1.62
	3	-3.52	-4.66	-2.39	-2.63	-3.71	-1.56
Romboutsia spp.	2	-2.32	-3.50	-1.15	-1.36	-2.49	-0.23
	3	-2.23	-3.42	-1.04	-1.50	-2.64	-0.36
Streptococcus spp.	2	0.54	-0.63	1.70	0.52	-0.58	1.61
	3	3.02	1.87	4.18	1.51	0.42	2.60
Ruminococcaceae NK4A214 group spp.	2	-1.64	-3.59	0.30	-1.26	-3.09	0.57
	3	-4.91	-5.94	-3.87	-3.32	-4.31	-2.33
Klebsiella spp.	2	-0.41	-2.12	1.31	-0.75	-2.36	0.86
	3	1.57	0.46	2.69	0.77	-0.28	1.82
Ruminococcaceae NK4A214 group spp.	2	-2.33	-3.51	-1.16	-1.97	-3.07	-0.86
	3	-3.26	-4.42	-2.09	-2.94	-4.04	-1.85
Enterorhabdus spp.	2	1.27	0.02	2.52	0.55	-0.63	1.73
	3	-4.15	-5.25	-3.05	-3.15	-4.18	-2.11
Escherichia/Shigella spp.	2	0.42	-0.78	1.63	0.12	-1.01	1.25
	3	0.62	-0.51	1.75	-0.03	-1.10	1.04

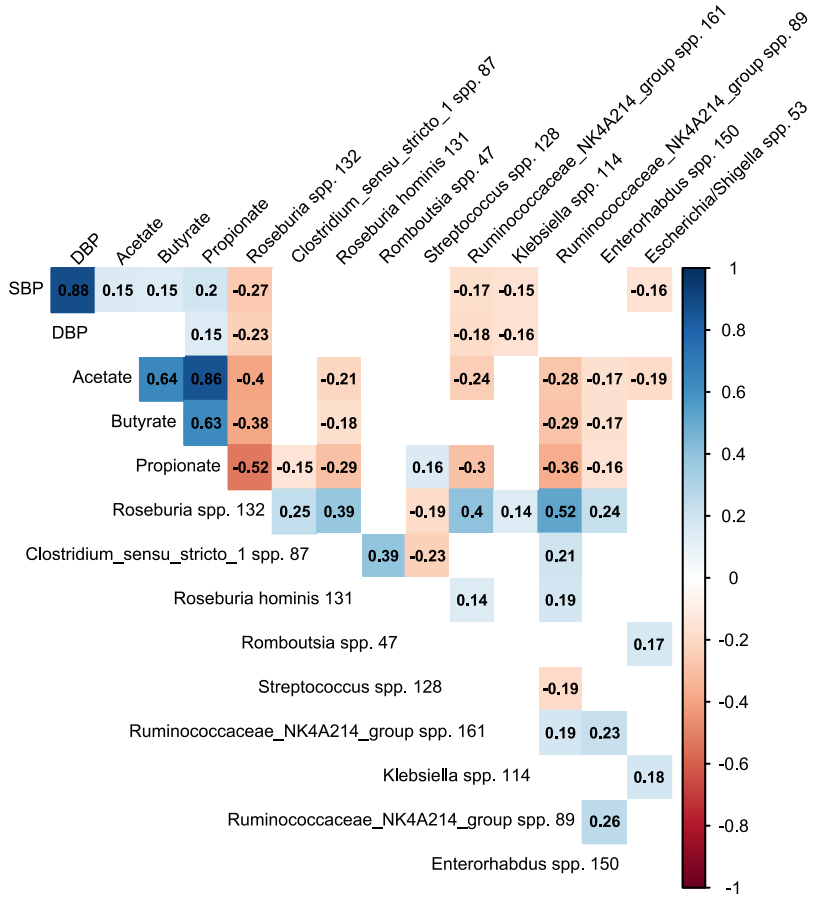
Linear regression coefficients per tertile of microbe counts for top 10 predictors of systolic blood pressure (SBP) from gut microbiota composition. Crude model: corrected for age and sex, full model: additional correction for BMI, smoking, use of antihypertensive medication and history of diabetes. ASV = amplicon sequencing variants.

Supplement 6: Population characteristics matched subjects

	Low SBP	High SBP	p
n	100	100	
Female	50	50	1.000
Age (years)	37.0±8.2	38.8±8.8	0.140
SBP (mmHg)	109.6±6.5	139.7±10.9	<0.001
DBP (mmHg)	71.6±6.4	87.4±8.6	<0.001
BMI (kg/m²)	24.0±2.6	27.4±5.2	<0.001
Hypertension	4 (4.0)	57 (57.0)	<0.001
Antihypertensive drugs	4 (4.0)	12 (12.0)	0.068
Lipid lowering drugs	1 (1.0)	4 (4.0)	0.365
eGFR (ml/min/1.73m2)	102.0 (12.7)	98.7 (14.1)	0.090
Albuminuria	2 (2.0)	4 (4.0)	0.678
Diabetes	1 (1.0)	2 (2.0)	1.000
Antidiabetic drugs	0 (0.0)	0 (0.0)	1.000
Smoking	22 (22.0)	22 (22.0)	1.000

Data is presented as mean±SD, n (%) or median [interquartile range]. Differences were tested with t-tests for continuous variables and chi-square tests for categorical variables. SBP = systolic blood pressure; DBP = diastolic blood pressure, BMI = body mass index.

Supplement 7: Correlation plot ASVs, faecal SCFA and blood pressure



Correlation plot for top 10 predictors of systolic blood pressure from gut microbiota composition in the subgroup of n=200 where fecal short chain fatty acid (SCFA) levels were determined. Only significant ($p<0.05$) Spearman correlation coefficients between the relative abundance of each of the microbes, systolic (SBP) and diastolic blood pressure (DBP) and levels of fecal SCFAs are shown. Colors indicate direction and strength of each correlation.

3

Gut microbiota in hypertension and atherosclerosis: a review

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Abstract

Gut microbiota and its metabolites such as short chain fatty acids (SCFA), lipopolysaccharides (LPS) and trimethylamine-N-oxide (TMAO) impact cardiovascular health. In this review, we discuss how gut microbiota and gut metabolites can affect hypertension and atherosclerosis. Hypertensive patients were shown to have lower alpha diversity, lower abundance of SCFA-producing microbiota and higher abundance of gram-negative bacteria, which are a source of LPS. Animal studies point towards a direct role for SCFAs in blood pressure regulation and show that LPS has pro-inflammatory effects. Translocation of LPS into the systemic circulation is a consequence of increased gut permeability. In atherosclerosis, a multifactorial disease, the pathways of gut microbiota effects are diverse. Many studies have focused on the pro-atherogenic role of TMAO, however, it is not clear if this is a causal factor. In addition, gut microbiota play a key role in bile acid metabolism and some interventions targeting bile acid receptors tend to decrease atherosclerosis. Concluding, gut microbiota affect hypertension and atherosclerosis through many pathways, providing a wide range of potential therapeutic targets. Challenges ahead include translation of findings and mechanisms to humans and development of therapeutic interventions that target cardiovascular risk by modulation of gut microbes and metabolites.

Introduction

Cardiovascular diseases, including atherosclerosis and hypertension, are public health care priorities of the World Health Organisation (WHO).[1] Cardiovascular disease is the leading cause of mortality, representing a third of global deaths, and disproportionally affects low- and middle- income countries.[2] Despite current preventive and therapeutic strategies, mortality due to cardiovascular disease is expected to further increase over the next decade.[2] Accumulating evidence describes the role of gut microbiota in cardiovascular disease, potentially providing novel therapeutic targets. The gut microbiome consists of more than 100 trillion micro-organisms, predominantly bacteria and viruses.[3] Due to the development of 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing, the understanding of the role of the gut microbiota in health and disease has increased tremendously over the past decade.[4] Gut microbiota composition is largely determined by exposure to dietary factors, but conversely, gut microbiota are needed for digestion of macronutrients and production of a wide range of metabolites.[5] Alterations in gut microbiota composition have been observed in a variety of health conditions, including type 2 diabetes, inflammatory bowel disease, asthma, psychiatric disorders, but also in cardiovascular disease.[6–10] In addition, several gut metabolites have been shown to interact with metabolism and the nervous system, affecting insulin sensitivity, energy balance and appetite regulation.[11–13]

Low-grade chronic inflammation contributes to the development of both atherosclerosis and hypertension.[14–17] Gut microbiota can induce systemic inflammation, as has been shown in patients with type 2 diabetes.[18] In addition, gut microbiota could affect cardiovascular risk indirectly, through metabolites such as short chain fatty acids (SCFA) and trimethylamine N-oxide (TMAO). The relation between gut microbiota and its key metabolites in hypertension and atherosclerosis could improve our understanding of differences in susceptibility for cardiovascular disease and provide potential therapeutic targets. In this narrative review, we will focus on the role of gut microbiota in hypertension and atherosclerosis. After summarizing the current evidence, we will discuss future perspectives in this field.

Gut microbiota in hypertension

Gut microbiota composition in hypertension

Hypertension is the most important modifiable risk factor for cardiovascular disease.[19] Although hypertension is thought to be driven by a combination of genetic and lifestyle factors, genome-wide association studies showed that only a small (<5%) proportion of the incidence of hypertension can be explained by genetics.[20] In contrast, lifestyle tends to have a much larger influence, with separate life style factors such as body mass index (BMI) and salt intake affecting blood pressure levels with 5 mmHg.[21] Several dietary interventions, including diets such as the Mediterranean diet and the DASH (Dietary Approaches to Stop Hypertension) diet have illustrated that higher intake of fruits, vegetables and fibers are associated with lower blood pressure.[22(p19),23] The Mediterranean diet has been shown to induce a rise in SCFAs, key metabolites produced by the gut microbiome.[24]

Several animal studies have reported compositional differences in the gut microbiota of animal models for hypertension, including Dahl-sensitive rats, spontaneous hypertensive rats, angiotensin-II induced hypertensive rats and deoxycorticosterone acetate (DOCA)-salt mice, when compared to wild-type animals.[25–28] These differences include a lower abundance of SCFA-producing bacteria, higher abundance of lactate-producing bacteria,[27] lower abundance of Bacteroidetes and higher abundance of Proteobacteria and Cyanobacteria,[28] compared to control animals. Intervention studies in animals showed that blood pressure levels in these animal models for hypertension can be modified by fecal microbiota transplants and antibiotic treatment.[27]

In humans, several cross-sectional studies have assessed associations between gut microbiota composition and blood pressure or hypertension (**Table 1**).[27,29–37] Despite differences in sequencing methods and downstream analyses, some results regarding microbial alpha diversity and microbiota composition are consistent across studies. Higher blood pressure was associated with lower gut microbiota alpha diversity in almost all studies.[27,30,32,34–37] Low alpha diversity is considered an adverse but nonspecific characteristic, since a decrease in diversity has also been observed in obesity, hyperinsulinemia and dyslipidemia. In addition, higher abundances of gram-negative microbiota including *Klebsiella*, *Parabacteroides*, *Desulfovibrio*, and *Prevotella* were associated with higher blood pressure. Gram-negative bacteria are a source of lipopolysaccharides (LPS), also known as endotoxins, that are pro-inflammatory. In contrast, SCFA-producing

Table 1. Cross-sectional studies on gut microbiota composition in hypertension in humans

Author	Population	Hypertension definition	Sequencing method	Higher abundance in HT or higher BP	Lower abundance in HT or higher BP	Alpha diversity in HT or higher BP	Covariates in analyses	
Dan et al. 2019	67 HT, 62 controls	SBP≥140 or DBP≥90 mmHg	16S	Acetobacteroides, Alistipes, Bacteroides, Christensenella, Clostridium sensu stricto, Desulfovibrio, Parabacteroides*	Acetobacteroides, Clostridium, Coprobacter, Enterococcus, Enterorhabdus, Lachnospiracea, Lactobacillus, Paraprevotella, Prevotella, Romboutsia, Ruminococcus, Veillonella*	No difference	Unadjusted	29
De la Cuesta-Zuluaga et al. 2019	441 subjects	No hypertension groups	16S	NR	NR	Lower	Unadjusted	30
Huart et al. 2019	38 HT, 7 pre-HT, 9 controls	Antihypertensive medication use, mean 24h BP SBP≥130 or DBP≥80 mmHg	16S	Clostridum sensu stricto	Ruminococcaceae, Clostridiales	NR	Unadjusted	31
Jackson et al. 2019	756 HT, 1790 controls	Self-report or antihypertensive medication use	16S	Lactobacillaceae, Streptococcaceae	Dehalobacteriaceae, Christensenellaceae, Oxalobacteraceae, Mollicutes, Rikenellaceae, Clostridia, Anaeroplasmataceae, Peptococcaceae	Lower	Age	32
Kim et al. 2018	22 HT, 18 controls	SBP≥140 mmHg	Shotgun	Parabacteroides johnsonii, Eubacterium siraeum, Alistipes finegoldii	Bacteroides thetaiotaomicron	NR	Unadjusted	33

Li et al. 2017	99 HT, 56 pre-HT, 41 controls	SBP≥140 or DBP≥90 mmHg	Shotgun	Prevotella, Klebsiella, Desulfovibrio	Faecalibacterium, Oscillibacter, Roseburia, Bifidobacterium, Coprococcus, Butyrivibrio	Lower	Unadjusted	34
Sun *et al.* 2019	529 subjects (183 HT)	Antihypertensive medication use or elevated office BP: SBP≥140 or DBP≥90 mmHg	16S	Anaerovorax, Butyricicoccus, Cellulosibacter, Clostridium IV, Methanobrevibacter, Mogibacterium, Oscillibacter, Oxalobacter, Papillobacter, Sporobacter, Vampirovibrio	Anaeroglobus, Atopobium, Lactobacillus, Megaspheara, Pseudocitrobacter, Rothia,	Lower	Age, ethnicity, sex, study center, sequencing run, education, smoking, physical activity, diet quality score	35
Verhaar *et al.* 2020	4672 subjects	No hypertension groups	16S	Streptococcus	Roseburia, Clostridium sensu stricto, Roseburia hominis, Romboutsia, Ruminococcaceae, Enterorhabdus		Age, sex, BMI, smoking status, antihypertensive medication, diabetes	36
Yan *et al.* 2017	60 HT, 60 controls	SBP≥140 or DBP≥90 mmHg	Shotgun	Klebsiella, Streptococcus, Parabacteroides	Roseburia, Faecalibacterium prausnitzii	Lower	Not adjusted, but age, sex-, and BMI-matched	37
Yang *et al.* 2015	7 HT, 10 controls	SBP≥125 mmHg	16S	NR	NR	Lower	Unadjusted	27

*BP = blood pressure, DBP = diastolic blood pressure, SBP = systolic blood pressure, HT = hypertensive, NR = not reported, * = selection of the microbiota listed by this paper*

bacteria, including *Ruminococcaceae*, *Roseburia* and *Faecalibacterium* spp. were less abundant in hypertensive compared to normotensive patients.[29,31,34,35,37] Of note, the majority of these studies did not adjust for important confounders such as age, BMI, or dietary factors in their analyses.

Dietary salt intake affects both the incidence of hypertension as well as gut microbiota composition. Higher salt intake has been associated with a shift in microbiota composition in several animal models, including an increase in *Lachnospiraceae*, *Ruminococcus* and *Parasutterella* spp. and decrease in *Lactobacillus* and *Oscillibacter*.[38–40] *Lactobacillus* abundance has been associated with salt sensitivity in hypertension, since supplementation of *Lactobacillus* spp. in a mice model has been shown to attenuate salt-sensitive hypertension, presumably by modulation of Th17-cells.[40] The blood pressure lowering effect of *Lactobacillus* was confirmed by several other animal models.[41–44] In humans, however, a decrease of *Lactobacillus* spp. was only reported by one of the cross-sectional studies in hypertensive subjects in **Table 1**.[29] A meta-analysis including nine randomized-controlled trials, predominantly with healthy controls, found a blood pressure lowering effect of probiotics with several *Lactobacillus* spp.[43] The blood pressure lowering effect tended to be stronger in the only included placebo-controlled intervention study with hypertensive subjects (17/13), although this study did not assess changes in gut microbiota composition.[45]

In summary, animal studies suggest a causal link between gut microbiota composition and blood pressure regulation. Cross-sectional studies in human subjects show specific differences in microbiota composition between hypertensive subjects and controls, including lower SCFA-producing bacteria and higher gram-negative species. These differences point to a role for SCFAs and LPS in hypertension, although the direction of this association is unclear.

Short chain fatty acids

SCFAs, including acetate, propionate and butyrate, are produced by specific gut microbes by fermentation of otherwise indigestible dietary fibers.[46] Fecal and plasma levels of SCFA are associated with the abundance of SCFA-producing microbiota in the gut and the intake of dietary fibers.[36,47,48] Butyrate-producing microbiota include bacteria from the families *Ruminococcaceae* and *Lachnospiraceae*, but also bacteria such as *Anaerobutyricum hallii* and *Anaerostipes* spp. Acetate and propionate are mainly produced by *Bifidobacterium* spp. and mucin-degrading bacteria such as *Akkermansia muciniphila*.[49] Most of the produced acetate and propionate is absorbed by the gut, while butyrate is used as a primary

energy source by colonocytes and only absorbed in very small proportions.[50,51] As a result, plasma concentrations of acetate and propionate are much higher than circulating butyrate levels.

Human studies on the role of SCFAs in blood pressure regulation are rather scarce. Intriguingly, fecal SCFA concentrations in humans have been associated with higher blood pressure,[30] while SCFA-producing microbiota are often associated with lower blood pressure.[31,35,37] Perhaps, increased SCFA availability in the intestines results in upregulation of absorption mechanisms, which could lead to relatively lower fecal concentrations and higher plasma availability, as was supported by a murine model.[52] There are no results from human intervention studies with SCFAs to target blood pressure. However, butyrate tended to lower blood pressure in intervention trials in subjects with metabolic syndrome.[53,54] Moreover, the Mediterranean diet, which induces a rise in SCFA levels, has been reported to have a blood pressure lowering effect.[24]

In animal models, SCFAs were associated with both higher and lower blood pressure, which might be explained by the differential effects of SCFA receptors.[55] Several SCFA receptors have been identified, including fatty acid receptor (FFAR)-2 and FFAR3 (formerly known as GPR43 and GPR41).[56] Animal studies have shown that SCFAs can have differential effects on blood pressure depending on the receptors involved. FFAR2 is expressed in a variety of tissues, including renal arteries, and causes vasodilation in response to SCFAs. In contrast, a blood pressure elevating effect is mediated by Olfr78 in mice through renin release from granules in the renal juxtaglomerular apparatus.[57,58] The potency of SCFAs is much lower for Olfr78 and the human analogue, OR51E2, than for FFAR2, and therefore, it was suggested that Olfr78 serves as a negative feedback loop for the blood pressure lowering effects of FFAR2.[59]

In addition, SCFAs, in particular butyrate, have anti-inflammatory effects that are primarily mediated by inhibition of histone deacetylase (HDAC).[60,61] Butyrate suppresses the production of pro-inflammatory cytokines, such as tumor-necrosis factor-α (TNFα), interleukin-12 (IL-12) and interferon-γ (IFN-γ), and upregulates the production of anti-inflammatory interleukin-10 (IL-10) by monocytes in vitro.[62] In addition, SCFAs have anti-inflammatory effects on epithelial cells that are partly mediated through HDAC.[63] In spontaneously hypertensive rats, HDAC activation has been associated with hypertension.[64] Conversely, butyrate administration to mice resulted in decreased blood pressure levels and reduced renal inflammation by HDAC inhibition.[65]

SCFAs have also been suggested to be implicated in gut-brain communication. Vagal afferents express receptors that can sense SCFAs, which provides another

pathway for the blood pressure modulating effects of SCFAs.[66] Animal studies showed that higher colonic levels of acetate could result in blood pressure lowering through parasympathetic activation. In addition, the blood pressure lowering effects of butyrate in rats were shown to be significantly reduced by vagotomy.[67] Another study with spontaneous hypertensive rats described a reduced central responsiveness to butyrate, as a result from reduced expression of butyrate receptors in the hypothalamus.[52] Thus, SCFAs could affect blood pressure through direct vascular and renal receptors, through HDAC inhibition, but also through colonic nerve signaling.

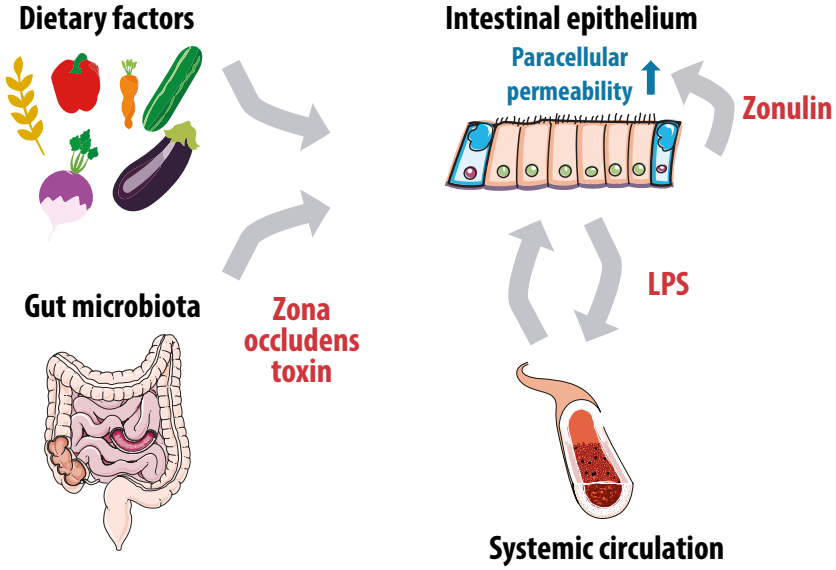
Gut permeability and lipopolysaccharides

Gut microbiota can also affect gut permeability and therefore influence the extent to which metabolites and endotoxins are absorbed (**Figure 1**). The barrier of the intestinal epithelium consists primarily of enterocyte brush borders and is more permeable for hydrophobic than for water soluble compounds. However, intercellular junctions on the enterocyte's lateral margins provide an alternative paracellular absorption route.[68] These intercellular junctions are dynamic structures that regulate paracellular permeability, and consist of tight junctions on the luminal side and adherens junctions on the laminal side. The level of permeability can be influenced by dietary factors, but also by the zonulin pathway. Zonulin is secreted by the basal lamina of the intestinal epithelium and binds enterocytes to initiate a complex intracellular signaling pathway that eventually phosphorylates the tight junction, resulting in permeability of the paracellular route.[69] Gut microbiota such as Vibrio cholerae appear to exploit this physiological pathway by excreting zona occludens toxin, a zonulin homologue that has similar effects.[70]

Animal models suggest that gut permeability is higher in the hypertensive state. Hypertensive rats had lower levels of mRNA of gap junction proteins, indicating higher gut permeability, which was restored after fecal microbiota transplantation from controls.[71] In a similar model, an increase in blood pressure in spontaneous hypertensive rats was associated with more permeability and lower levels of tight junction proteins.[72]

A consequence of higher gut permeability is increased translocation of certain metabolites and endotoxins in the portal and systemic circulation, which could cause further amplification of gut permeability.[73] Lipopolysaccharides (LPS), also known as endotoxins, can be found in the outer membrane of gram-negative bacteria, the most abundant bacteria in the gut microbiome.[74] The lipid A component of LPS is the main pathogen-associated molecular pattern (PAMP) that can inter-

Figure 1: Gut permeability



Gut microbiota, gut permeability and lipopolysaccharides (LPS) absorption. Paracellular permeability of the intestinal epithelium is affected by zonulin production of the basal lamina, dietary factors and gut microbiota that produce zone occludens toxin. Increased permeability leads to more LPS translocation to the systemic circulation, which has a pro-inflammatory effect and further increases gut permeability.

act with Toll-like receptor 4 (TLR4).[75,76] When translocated from the gut into the circulation, LPS forms a complex with LPS-binding protein (LBP) which can bind to CD14 on mononuclear cells.[77] This could lead to production of pro-inflammatory cytokines, such as TNF-α, interleukin-1 (IL-1) and interleukin-6 (IL-6), mediated by the MD2/TLR4 receptor complex.[76,78] Butyrate was shown to attenuate the pro-inflammatory effects of LPS-stimulation.[79]

LPS is known to induce systemic inflammation and has been shown to have both metabolic and cardiovascular effects. In mice, infusion of LPS to 2 to 3 fold higher plasma levels resulted in higher glucose and insulin levels and weight gain comparable to mice on a 4-week high-fat diet.[73] LPS-administration to rats increased heart rate and norepinephrine levels, decreased baroreflex sensitivity, and increased neuroinflammation, as indicated by increased TLR and TNF-alfa expression in the paraventricular nucleus (PVN) that plays a key role in blood pressure regulation.[80] The same effects were observed in a small (n=8) group of human subjects that showed a significant decrease in systolic and diastolic blood pressure after administration of LPS. Moreover, in this study, LPS increased brain microglial activation on positron emission tomography (PET)-scans.[81] Summa-

rizing, there is a limited number of studies suggesting that systemic LPS could have pro-inflammatory, sympathetic activating and neuroinflammatory effects, all of which are relevant in hypertension pathogenesis.

Gut-brain interactions

Increased sympathetic activation is considered one of the causal factors in the development of hypertension, and can already be observed in early stages.[82] The sympathetic nervous system modulates blood pressure levels through vasoconstriction in peripheral blood vessels, renal regulation of water and sodium balance and release of renin by juxtaglomerular cells.[83] Regions in the central nervous system that are involved in sympathetic activation include the PVN, the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM).[84] Hypertension is associated with neuroinflammation in these regions, which might be mediated by the renin-angiotensin aldosterone system, since prorenin was shown to cause microglial activation in mice and spontaneously hypertensive rats (SHR).[85,86]

Gut-brain communication could stimulate sympathetic activation and therefore play a role in the hypertension pathogenesis. The gut is innervated by the autonomic nervous system that signals physiological conditions such as acidity, osmolarity and pain.[87] Intrinsically, the enteric nervous system (ENS), consisting of the myenteric plexus and the submucosal plexus, controls intestinal motor and sensory functions.[88] The ENS is a complex system that is sometimes referred to as the 'second brain', because of the structural and functional similarities.[89] It communicates with the brain via the vagal nerve, which projects to the NTS, that is involved in sympathetic regulation. Gut microbiota interfere in ENS-brain interactions by stimulating enterochromaffin cells to produce serotonin, a neurotransmitter that affects gut secretion, motility and local nerve reflexes.[90] Conversely, central sympathetic activation can through a cascade of events lead to increased gut permeability and increased translocation of metabolites into the systemic circulation.[91]

Elevated sympathetic drive shifts bone marrow hemopoietic stem cells to a pro-inflammatory state, and the release of these immune cells contributes to further hypertension development.[72,92] An animal study with SHR showed that microbiota affect inflammation in brain regions crucial to sympathetic outflow. Microbiota composition in these rats was associated with reactive oxygen species (ROS) and proinflammatory cytokines in the PVN.[71] In addition, fecal transplantation in rat models from Wistar Kyoto (WKY) rats to SHR led to higher

sympathetic activity, independent of renin levels.[71,93] Taken together, this suggests that gut microbiota can stimulate sympathetic drive, possibly by direct ENS-brain interactions or by promoting neuroinflammation. This increased sympathetic activity can contribute to hypertension development directly or indirectly, by stimulating low-grade systemic inflammation.

Gut microbiota in atherosclerosis

Atherosclerosis and gut microbiota

Atherosclerosis is a multifactorial process, with lipid metabolism, inflammation, vascular ageing and blood pressure as key players. Atherosclerosis is closely related to arterial stiffness, which is caused by a loss of elastic fibers and thickening of arteriole walls. Arterial stiffness tends to increase with age and results in a less compliant arterial system and higher pulse wave velocity. The resulting increased shear stress has an aggravating effect on the formation of subsequent atherosclerotic plaques.[94,95] In this process, cholesterol accumulation in vessel walls leads to transformation of macrophages to foam cells after phagocytic uptake of lipid particles. Oxidation of lipids results in cholesterol crystallization, inflammasome activation and production of proinflammatory cytokines such as TNFα and IL-1β. Statins have been proven effective in preventing atherosclerotic events, not only by lowering low-density lipoprotein (LDL) cholesterol, but also through anti-inflammatory effects.[96] The Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS)-trial underlined the role of inflammation in atherosclerosis by demonstrating that treatment with canakinumab, a monoclonal inhibitor of IL-1β, lowers the incidence of cardiovascular events.[97]

An atherosclerotic plaque was shown to be a microbial environment on itself, containing microbes such as *Streptococcus*, *Pseudomonas*, *Klebsiella*, *Veillonella* spp., and *Chlamydia pneumoniae*.[98–100] Most studies could not relate plaque microbiota composition to outcomes such as plaque vulnerability, rupture or cardiovascular events.[101,102] It was suggested that pathogenic bacteria originating from oral or gut microbiomes make vessel walls more prone to plaque formation, either by direct infection of the vessel wall or by distant infections eliciting an auto-immune inflammatory reaction through molecular mimicry.[103,104] Interventions with antibiotic treatment as secondary prevention, targeted at eliminating plaque microbiota did not result in lower incidence of cardiovascular events.[105,106] Therefore, these studies did not provide evidence for direct vessel wall infection

as a causal factor, although some argue that not all microbes were targeted by the antibiotics used and that interventions were too short.[104,107]

In humans, cross-sectional studies showed that higher abundance of the *Collinsella* genus, *Enterobacteriaceae*, *Streptococcaceae* and *Klebsiella* spp., and lower abundance of SCFA-producing bacteria *Eubacterium*, *Roseburia* and *Ruminococcaceae* spp. in the gut microbiota of patients with symptomatic atherosclerosis compared to healthy controls.[108–110] Pulse wave velocity, a marker of arterial stiffness, was associated with a lower alpha diversity and lower number of SCFA-producing bacteria such as *Ruminococcaceae* spp. in middle aged women in the TwinUK cohort.[111] Hence, the compositional differences in atherosclerosis overlap with findings in hypertensive patients, which is not surprising considering the shared risk factors and pathogenesis. Causal evidence of gut microbiota composition in atherosclerosis is based on fecal microbiota transplantation (FMT) in animal studies. For example, mice transplanted with a more pro-inflammatory gut microbiota composition from Caspase1$^{-/-}$ mice had 29% larger plaque sizes than controls.[112] Alternatively, gut microbiota could have indirect proatherogenic effects, by production of pro-atherogenic metabolites. These metabolites could also very well include the metabolites that are described for hypertension, including SCFAs. For the scope of this review, we chose to focus on the role of trimethylaminoxide (TMAO) and bile acids in atherosclerosis.

Trimethylamine-N-oxide

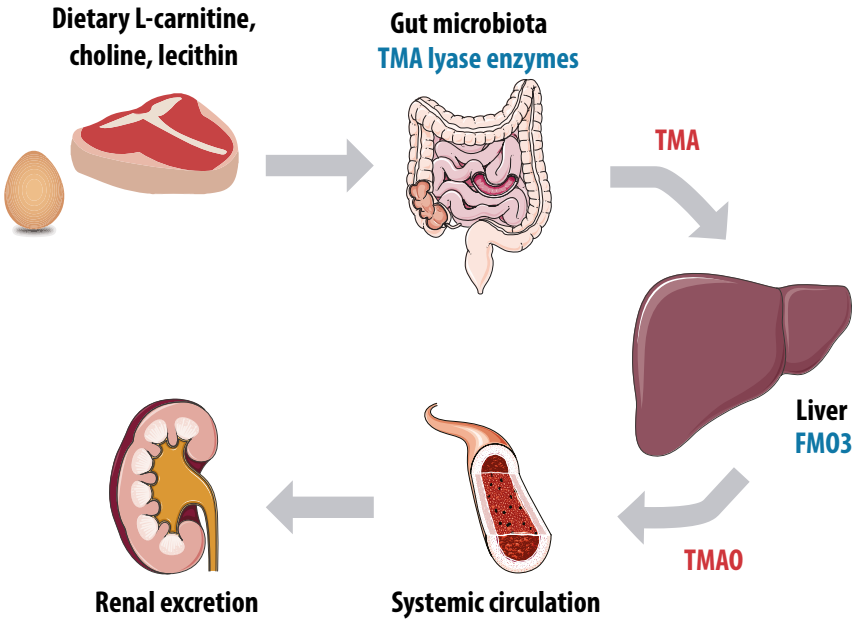
The role of trimethylamine (TMA) and TMAO in the development of atherosclerosis is an extensively researched topic. The role of gut microbiota in TMAO production is illustrated by **Figure 2**. TMA is produced by gut microbes, primarily those from the families *Clostridia* and *Enterobacteriaceae,* in the degradation of nutrients such as carnitine, choline and lecithin, that can be found in dietary products including meat and eggs.[113] After absorption, TMA is oxidized into TMAO by the hepatic enzyme flavin mono-oxygenase (FMO)-3.[114] Plasma levels of TMAO have both a high within-individual and inter-individual variability, which hampers comparison of studies.[115] In addition, TMAO levels are higher in women, presumably due to different expression of the converting enzyme FMO3 and higher excretion rates in men.[114] TMAO is primarily excreted by the kidneys through both glomerular filtration and tubular secretion, which is a reason for increasing TMAO levels with decreasing renal function.[116]

Several mechanisms for the role of TMAO in atherosclerosis have been proposed, including the effects TMAO has on inflammation, cholesterol metab-

olism and thrombosis. TMAO was shown to increase the production of pro-inflammatory cytokines such as TNF-α and IL-1β, and decrease anti-inflammatory cytokines such as IL-10.[117] In addition, the hepatic enzyme FMO3 appeared to have a regulating function in lipid metabolism. FMO3 knockdown in mice on a high cholesterol diet lowered intestinal lipid absorption and hepatic cholesterol production and stimulated reverse cholesterol transport, thereby restoring cholesterol balance.[118] Lastly, TMAO was reported to induce platelet hyperreactivity, which can facilitate thrombosis thus causing atherosclerotic thrombotic events.[119] Administration of TMAO indeed promoted atherosclerosis in several mouse models.[120,121] However, there are also several animal studies that could not confirm this association, or even found a protective effect of TMAO.[122–125] In humans, higher levels of TMAO have been associated with cardiovascular disease incidence in several prospective studies.[123,126,127] Two meta-analyses concluded that elevated TMAO levels were associated with a higher risk of cardiovascular events and a higher all-cause mortality with relative risks ranging between 55% and 62%.[122,128]

Nevertheless, a causal effect of TMAO on atherosclerosis has not yet been

Figure 2: Trimethylamine-N-oxide metabolism



Production of trimethylamine-N-oxide (TMAO). Gut microbiota enzymes, including trimethylamine (TMA) lyase, convert dietary L-carnitine, choline, and lecithin into TMA. The hepatic enzyme flavin mono-oxygenase 3 (FMO3) converts TMA into TMAO, and TMAO is primarily excreted by the kidneys.

proven. An elegant way to assess causality is Mendelian randomization, using genetic variants known to modify the exposure to examine the effect on disease.[129] In this case, the prevalence of cardiovascular disease in individuals with single nucleotide polymorphisms (SNPs) known to cause higher levels of TMAO was compared to individuals without these SNPs.[130] Interestingly, in this study, atherosclerotic cardiovascular disease was not more prevalent in the group with genetically predicted higher TMAO levels. Another way to prove causality is to lower TMAO levels with interventions, such as with TMA lyases that lower TMAO by degrading TMA before oxidization.[131] However, results of human intervention studies have not yet been published.

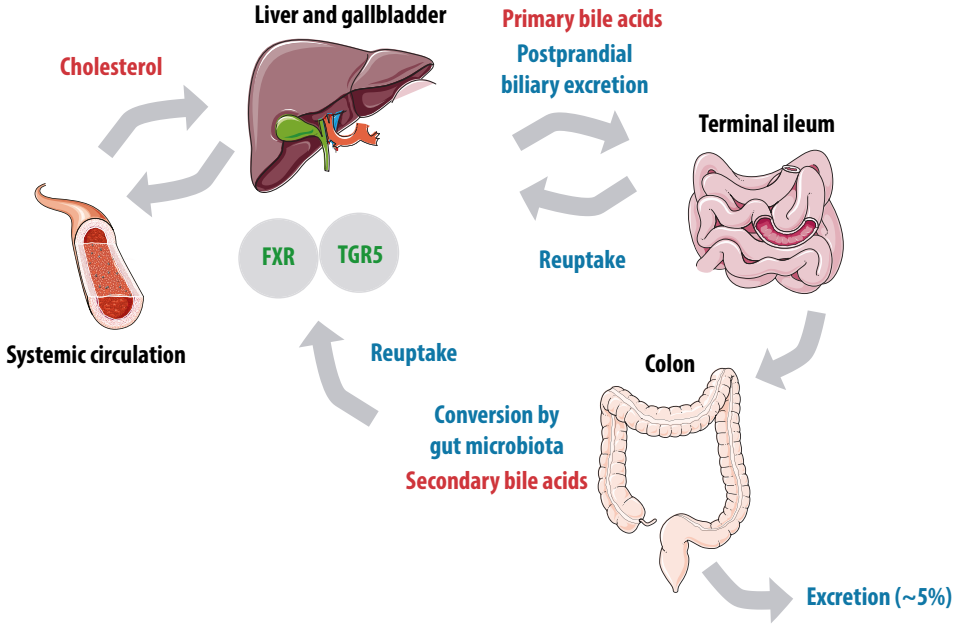
Bile acids

Bile acid metabolism is dependent on microbial modifications in the gut (**Figure 3**) and this interaction was previously shown to affect inflammatory bowel disease and hyperinsulinemia.[132,133] Primary bile acids are synthesized by the liver, which converts hydrophobic cholesterol to hydrophilic primary bile acids.[134] These bile acids are excreted by the gall bladder and reabsorbed in the terminal ileum by sodium-dependent bile acid transporters.[135] Bile acids affect gut microbiota composition and inhibit microbial growth in the small intestines.[136] A small proportion of bile acids reaches the colon, where microbiota convert primary bile acids to secondary bile acids by several modifications, including deconjugation, 7α-dehydroxylation and 7α-hydrogenation.[137] Secondary bile acids are hydrophobic and therefore easily absorbed by colonocytes and taken up into the systemic circulation. Only an estimated proportion of 5% of bile acids escape the enterohepatic cycle and are excreted.[138] Bile acids also affect diverse metabolic pathways through Takeda G-protein coupled receptor 5 (TGR5) and the nuclear farnesoid X receptor (FXR), both of which have a preference for secondary bile acids. The composition of the microbiota and the microbial community's enzymatic repertoire determine the secondary bile acid profile.[139] The impact of gut microbiota on the bile acid pool was illustrated by a study showing that germ-free mice had a 71% decreased bile acid pool compared to controls.[140] Interestingly, the bile acid metabolism interacts with the TMAO pathway, as FXR has been shown to regulate FMO3, the hepatic enzyme that converts TMA in TMAO.[114]

TGR5 is expressed in a variety of tissues, including liver, gall bladder, intestines, kidneys, pancreas, muscle and adipose tissue, but can also be found on leukocytes, macrophages and endothelial cells.[141] A TGR5 agonist (INT-777) was shown to have immunosuppressive effects, including reduced pro-inflammatory

cytokine production by macrophages and attenuation of atherosclerotic plaque formation in LDLr$^{-/-}$ mice.[142,143] Translation of findings from animal studies on TGR5 to humans in other contexts has not always been successful. Despite beneficial metabolic effects of TGR5 agonists in mice, including lower glucose levels and improved lipid profiles, the TGR5 agonist SB-756050 increased fasting glucose levels compared to placebo in human subjects with type 2 diabetes.[144] TGR5 agonists had limited adverse effects in this trial, which is surprising considering the number of tissues that express this receptor. In animal models, TGR5 agonists have been associated with increased gastrointestinal motility, a potential higher incidence of biliary stones, lower vascular tone and blood pressure, and itching.[145] Atherogenic mice models with *FXR* knock-out showed conflicting findings, with both increased and decreased atherosclerosis.[146–148] However, administration of synthetic FXR agonists to atherogenic mice prevented plaque formation in three studies, presumably by lipid-lowering and anti-inflammatory effects.[149–151] Although the FXR agonist obeticholic acid (OCA) lowered hepatic fat in human

Figure 3: Enterohepatic cycle of bile acids



Enterohepatic cycle of bile acids. Hepatic conversion of cholesterol results in primary bile acids, that are excreted postprandially by the gallbladder. Active reuptake takes place in the terminal ileum. In the colon, primary bile acids are converted to secondary bile acids by gut microbiota, and passively reabsorbed. Farnesoid X receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5) have a preference for secondary bile acids.

subjects with non-alcoholic steatohepatitis (NASH), it had paradoxical effects on cholesterol levels, increasing LDL and decreasing high-density lipoprotein (HDL) cholesterol.[152]

Dual agents that target both TGR5 and FXR might have more therapeutic potential. Animal studies on the effect of dual agonists reported beneficial effects on metabolic syndrome, NASH, cholangiopathy, progression of diabetic nephropathy, and atherosclerosis.[153–157] In a mouse model for atherosclerosis, dual targeting with INT-767 seemed to be more effective in attenuating atherosclerosis than separate effects on TGR5 and FXR.[153] All in all, although findings in animal studies are promising, it remains to be seen whether these results can be translated to humans, especially considering the substantial differences in atherosclerosis pathogenesis and bile acid metabolism between men and mice.

Therapeutic strategies

The changes in gut microbiota composition and gut metabolites discussed in this review could all be potential therapeutic targets in the treatment of atherosclerosis and hypertension. The most direct ways of altering gut microbiota composition are oral supplementation of specific microbial strains and fecal microbiota transplantation (FMT).

Probiotics containing SCFA-producing microbes including *Bifidobacterium*, *Enterococcus* and *Lactobacillus* were suggested to have a variety of health benefits including anti-inflammatory and beneficial metabolic effects.[158] In addition, oral treatment with specific *Bifidobacterium*, *Lactobacillus* and SCFA-producing *Anaerobutyricum soehngenii* species had modest blood pressure lowering effects in humans.[43,159] However, our understanding of mechanisms is based on animal research. Evidence in humans is limited and inconclusive due to heterogeneity in investigational products and study designs.[160] Therefore, the effect of specific strains is often unclear, which is one of the reasons that probiotics are marketed as nutritional supplements rather than medication.[160]

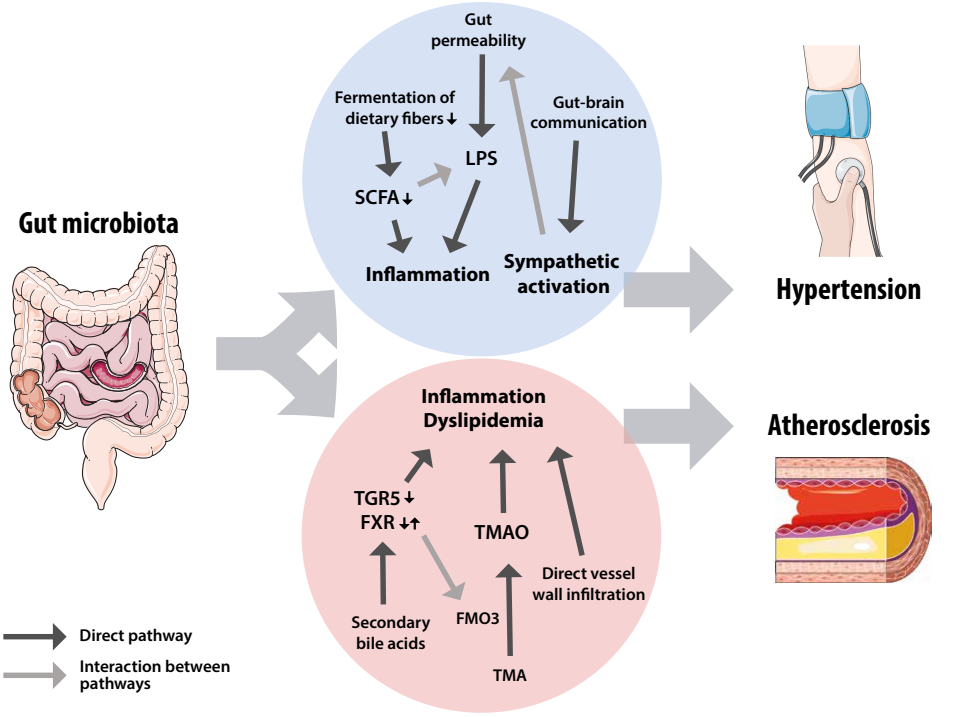
Probiotic efficacy is both disease-specific and strain-specific,[161] underlining the need for well-designed trials that survey gut microbiota composition before and after the intervention. Preferably, this should be measured with metagenomic sequencing (as opposed to 16S rRNA sequencing) in order to provide species-level resolution to compositional data. Another advantage of this technique is the potential to assess differences in gut microbiota functionality, as differences in microbiota composition do not always match differences in function. In addition, the gut microbiome has a spatial dimension, with composition gradients along

the different parts of the intestinal tract, yet due to sampling difficulties, fecal samples are used as a proxy for the entire extent of the intestinal tract lumen. Localized sampling would aid in deciphering the actual biology in the intestine. Alternatively, FMT could be used to optimize microbiota composition in individuals at risk for cardiovascular disease. FMT has been shown to be efficacious with limited adverse effects.[162] However, optimal FMT approaches, including donor selection, screening and preparation, have yet to be defined.[163,164] In addition, the long-term effects of FMT are not clear, since the follow-up in most studies is less than a year. As our understanding of the gut microbiome progresses, so does our knowledge of potential risks of FMT. To illustrate, bacteriophages – long understudied yet now known to play an important role in the microbiome - were shown to be transferred from donor to host by FMT, with uncertain implications.[165]

To date, only one FMT trial targeted cardiovascular risk by transplantation from lean vegan donors to meat-consuming subjects with metabolic syndrome in order to lower TMAO levels.[166] Despite alterations in gut microbiota composition, TMAO levels did not change upon this intervention. Other FMT trials in obesity and metabolic syndrome also showed that effects on microbiota composition and glucose metabolism are small and transient, underlining the importance of pre-screening in order to select recipients most likely to respond.[167,168] To that end, a better understanding of the structural and functional aspects of the microbiota that affect hypertension and atherosclerosis incidence is needed.

Prebiotics and dietary interventions target gut microbiota composition indirectly. Prebiotics selectively stimulate specific microbes in the colon. Prebiotics are often fibers, although not all fibers are prebiotics.[169] Prebiotics were shown to stimulate growth of SCFA-producing microbes such as *Bifidobacterium* and *Lactobacillus*. Diet also has a substantial influence on gut microbiota composition. Dietary interventions such as the DASH and the Mediterranean diet were shown to lower cardiovascular risk.[170,171] However, since dietary interventions are multifaceted, it is difficult to point out what mechanisms explain the beneficial effects. In summary, multiple interventions could target gut microbiota composition and its associated metabolites, ranging from targeted approaches to more accessible but non-specific interventions. However, translation of findings from animal studies to humans is needed, preferably by prospective cohort studies using metagenomic sequencing that can also assess microbiome functionality. In addition, adjusting for confounders when assessing associations between microbiota and cardiovascular disease is vital, since microbiota composition is shaped by a combination of lifestyle factors, health conditions and medication use.

Figure 4: Graphical abstract



Summary of hypothesized pathways for the effects of gut microbiota on hypertension and atherosclerosis. Gut microbiota could affect hypertension through inflammatory factors, influenced by short chain fatty acids (SCFAs) and lipopolysaccharides (LPS), and through sympathetic activation by gut–brain interactions. The effects on inflammation and dyslipidemia in atherosclerosis could be mediated by bile acid receptors Takeda G-protein-coupled receptor 5 (TGR5) and farnesoid X receptor (FXR), trimethylamine-N-oxide (TMAO) and trimethylamine (TMA), and direct vessel infiltration of microbiota. The grey arrows indicate interactions between pathways: FXR regulates the TMAO-converting enzyme flavin mono-oxygenase 3 (FMO3), sympathetic activation increases gut permeability, and short chain fatty acids can attenuate the inflammatory effects of LPS.

Conclusion

The pathways by which gut microbiota affect hypertension and atherosclerosis are diverse and often interact, as shown in **Figure 4**. Gut microbiota produce or convert metabolites, produce substrates needed for production of metabolites elsewhere and are involved in regulating local intestinal homeostasis, resulting in a wide range of potential therapeutic targets. However, our understanding of mechanisms is mainly based on animal research and translation to humans remains challenging, as illustrated by developments in bile acid receptors research. Lon-

gitudinal studies in human subjects are needed to identify beneficial or adverse characteristics of gut microbiota structure and functionality, in order to better target potential therapeutic strategies.

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Conflicts of interest

M.N. is in the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands and Kaleido, USA. None of these are directly relevant to the current paper. The other authors declare no conflicts of interest.

Author contributions

B.V. drafted the manuscript; A.P., M.N. and M.M. critically revised the manuscript.

References

1. Global Action Plan for the Prevention and Control of Noncommunicable Diseases 2013–2020. World Health Organization; 2013. Accessed September 28, 2020. https://www.who.int/nmh/events/ncd_action_plan/en/
2. Cardiovascular Diseases (CVDs) Fact Sheet. World Health Organization; 2016. Accessed September 28, 2020. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-
3. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*. 2006;124(4):837-848. doi:10.1016/j.cell.2006.02.017
4. Lagier JC, Khelaifia S, Alou MT, *et al.* Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat Microbiol.* Published online 2016. doi:10.1038/nmicrobiol.2016.203
5. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. *Pharmacol Res.* 2013;69(1):52-60. doi:10.1016/j.phrs.2012.10.020
6. Wang J, Qin J, Li Y, *et al.* A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*. Published online 2012. doi:10.1038/nature11450
7. Zuo T, Ng SC. The Gut Microbiota in the Pathogenesis and Therapeutics of Inflammatory bowel disease. *Front Microbiol.* Published online 2018. doi:10.3389/fmicb.2018.02247
8. Wang Q, Li F, Liang B, *et al.* A metagenome-wide association study of gut microbiota in asthma in UK adults. *BMC Microbiol.* Published online 2018. doi:10.1186/s12866-018-1257-x
9. Valles-Colomer M, Falony G, Darzi Y, *et al.* The neuroactive potential of the human gut microbiota in quality of life and depression. *Nat Microbiol.* Published online

2019. doi:10.1038/s41564-018-0337-x
10. Tang WHW, Kitai T, Hazen SL. Gut Microbiota in Cardiovascular Health and Disease. *Circ Res.* 2017;120(7):1183-1196. doi:10.1161/CIRCRESAHA.117.309715
11. Li Z, Yi CX, Katiraei S, *et al.* Butyrate reduces appetite and activates brown adipose tissue via the gut-brain neural circuit. *Gut*. Published online 2018. doi:10.1136/gutjnl-2017-314050
12. Gao Z, Yin J, Zhang J, *et al.* Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes*. Published online 2009. doi:10.2337/db08-1637
13. De Vadder F, Kovatcheva-Datchary P, Goncalves D, *et al*. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell*. Published online 2014. doi:10.1016/j.cell.2013.12.016
14. Ridker PM. Inflammation, atherosclerosis, and cardiovascular risk: an epidemiologic view. *Blood coagulation & fibrinolysis*. 1999;10 Suppl 1:S9-12.
15. Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM. C-Reactive Protein and the Risk of Developing Hypertension. *JAMA*. 2003;290(22):2945-2951. doi:10.1001/jama.290.22.2945
16. Vlachopoulos C, Ioakeimidis N, Aznaouridis K, *et al.* Association of Interleukin-18 Levels With Global Arterial Function and Early Structural Changes in Men Without Cardiovascular Disease. *Am J Hypertension.* 2010;23(4):351-357. doi:10.1038/ajh.2009.256
17. Mahmud A, Feely J. Arterial stiffness is related to systemic inflammation in essential hypertension. *Hypertension*. 2005;46(5):1118-1122. doi:10.1161/01.HYP.0000185463.27209.b0
18. Cani PD, Osto M, Geurts L, Everard A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. *Gut microbes*. 2012;3(4):279-288.
19. Stanaway JD, Afshin A, Gakidou E, *et al*. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Stu. *The Lancet*. 2018;392(10159):1923-1994. doi:10.1016/S0140-6736(18)32225-6
20. Giri A, Hellwege JN, Keaton JM, *et al*. Trans-ethnic association study of blood pressure determinants in over 750,000 individuals. *Nature Genetics*. 2019;51(1):51-62. doi:10.1038/s41588-018-0303-9
21. Pazoki R, Dehghan A, Evangelou E, *et al*. Genetic predisposition to high blood pressure and lifestyle factors: Associations with midlife blood pressure levels and cardiovascular events. *Circulation*. 2018;137(7):653-661. doi:10.1161/CIRCULATIONAHA.117.030898
22. Appel LJ, Moore TJ, Obarzanek E, *et al.* A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. Published online 1997. doi:10.1056/NEJM199704173361601
23. Estruch R, Ros E, Salas-Salvadó J, *et al*. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. Published online 2013. doi:10.1056/NEJMoa1200303
24. De Filippis F, Pellegrini N, Vannini L, *et al*. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated

metabolome. *Gut*. Published online 2016. doi:10.1136/gutjnl-2015-309957
25. Mell B, Jala VR, Mathew AV, *et al*. Evidence for a link between gut microbiota and hypertension in the Dahl rat. *Physiol Genomics*. Published online 2015. doi:10.1152/physiolgenomics.00136.2014
26. Adnan S, Nelson JW, Ajami NJ, *et al*. Alterations in the gut microbiota can elicit hypertension in rats. *Physiol Genomics*. Published online 2017. doi:10.1152/physiolgenomics.00081.2016
27. Yang T, Santisteban MM, Rodriguez V, *et al*. Gut Dysbiosis is Linked to Hypertension. *Hypertension*. 2015;65(6):1331-1340. doi:10.1161/HYPERTENSIONAHA.115.05315
28. Marques FZ, Nelson E, Chu PY, *et al*. High-fiber diet and acetate supplementation change the gut microbiota and prevent the development of hypertension and heart failure in hypertensive mice. *Circulation*. Published online 2017. doi:10.1161/CIRCULATIONAHA.116.024545
29. Dan X, Mushi Z, Baili W, *et al*. Differential analysis of hypertension-associated intestinal microbiota. *Int J Med Sci*. Published online 2019. doi:10.7150/ijms.29322
30. de la Cuesta-Zuluaga J, Mueller NT, Álvarez-Quintero R, *et al.* Higher Fecal Short-Chain Fatty Acid Levels Are Associated with Gut Microbiome Dysbiosis, Obesity, Hypertension and Cardiometabolic Disease Risk Factors. *Nutrients*. 2018;11(1):51. doi:10.3390/nu11010051
31. Huart J, Leenders J, Taminiau B, *et al*. Gut Microbiota and Fecal Levels of Short-Chain Fatty Acids Differ Upon 24-Hour Blood Pressure Levels in Men. *Hypertension*. 2019;74(4):1005-1013. doi:10.1161/HYPERTENSIONAHA.118.12588
32. Jackson MA, Verdi S, Maxan ME, *et al*. Gut microbiota associations with common diseases and prescription medications in a population-based cohort. *Nat Commun*. 2018;9(1):2655. doi:10.1038/s41467-018-05184-7
33. Kim S, Goel R, Kumar A, *et al.* Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clinical Science*. 2018;132(6):701-718. doi:10.1042/CS20180087
34. Li J, Zhao F, Wang Y, *et al.* Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome*. Published online 2017. doi:10.1186/s40168-016-0222-x
35. Sun S, Lulla A, Sioda M, *et al*. Gut microbiota composition and blood pressure: The CARDIA study. *Hypertension*. Published online 2019. doi:10.1161/HYPERTENSIONAHA.118.12109
36. Verhaar BJH, Collard D, Prodan A, *et al.* Associations between gut microbiota, faecal short-chain fatty acids, and blood pressure across ethnic groups: the HELIUS study. *Eur Heart J*. 2020;(ehaa704). doi:10.1093/eurheartj/ehaa704
37. Yan Q, Gu Y, Li X, *et al.* Alterations of the Gut Microbiome in Hypertension. *Front Cell Infect Microbiol*. 2017;7(AUG). doi:10.3389/fcimb.2017.00381
38. Bier A, Braun T, Khasbab R, *et al.* A High Salt Diet Modulates the Gut Microbiota and Short Chain Fatty Acids Production in a Salt-Sensitive Hypertension Rat Model. *Nutrients*. 2018;10(9):1154. doi:10.3390/nu10091154
39. Miranda PM, Serkis V, De Palma G, *et al.* High salt diet increases susceptibility to experimental colitis: A putative role of gut microbiota. *Gastroenterology*. 2016;150(4):S583.
40. Wilck N, Matus MG, Kearney SM, *et al.* Salt-responsive gut commensal modulates

TH17 axis and disease. *Nature*. 2017;551(7682):585-589. doi:10.1038/nature24628
41. Tanida M, Yamano T, Maeda K, Okumura N, Fukushima Y, Nagai K. Effects of intraduodenal injection of Lactobacillus johnsonii La1 on renal sympathetic nerve activity and blood pressure in urethane-anesthetized rats. *Neuroscience Letters*. Published online 2005. doi:10.1016/j.neulet.2005.07.036
42. Gómez-Guzmán M, Toral M, Romero M, *et al.* Antihypertensive effects of probiotics Lactobacillus strains in spontaneously hypertensive rats. *Mol Nutr Food Res*. 2015;59(11):2326-2336.
43. Khalesi S, Sun J, Buys N, Jayasinghe R. Effect of probiotics on blood pressure: a systematic review and meta-analysis of randomized, controlled trials. *Hypertension* 2014;64(4):897-903. doi:10.1161/HYPERTENSIONAHA.114.03469
44. Kawase M, Hashimoto H, Hosoda M, Morita H, Hosono A. Effect of administration of fermented milk containing whey protein concentrate to rats and healthy men on serum lipids and blood pressure. *Journal of Dairy Science*. Published online 2000. doi:10.3168/jds.S0022-0302(00)74872-7
45. Hata Y, Yamamoto M, Ohni M, Nakajima K, Nakamura Y, Takano T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *American Journal of Clinical Nutrition*. Published online 1996. doi:10.1093/ajcn/64.5.767
46. Cummings JH, Pomare EW, Branch HWJ, Naylor CPE, MacFarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*. Published online 1987. doi:10.1136/gut.28.10.1221
47. Calderón-Pérez L, Gosalbes MJ, Yuste S, *et al.* Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study. *Scientific Reports*. Published online 2020. doi:10.1038/s41598-020-63475-w
48. Cuervo A, Salazar N, Ruas-Madiedo P, Gueimonde M, González S. Fiber from a regular diet is directly associated with fecal short-chain fatty acid concentrations in the elderly. *Nutrition Research*. Published online 2013. doi:10.1016/j.nutres.2013.05.016
49. Parada Venegas D, De la Fuente MK, Landskron G, *et al.* Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol*. 2019;10:277. doi:10.3389/fimmu.2019.00277
50. Boets E, Gomand SV, Deroover L, *et al.* Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. *Journal of Physiology*. Published online 2017. doi:10.1113/JP272613
51. Roediger WEW. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut*. Published online 1980. doi:10.1136/gut.21.9.793
52. Yang T, Magee KL, Colon-Perez LM, *et al.* Impaired butyrate absorption in the proximal colon, low serum butyrate and diminished central effects of butyrate on blood pressure in spontaneously hypertensive rats. *Acta Physiologica*. 2019;226(2):e13256. doi:10.1111/apha.13256
53. Bouter K, Bakker G, Levin E, *et al.* Differential metabolic effects of oral butyrate treatment in lean versus metabolic syndrome subjects. *Clin Transl Gastroenterol.* 2018;9(5):155. doi:10.1038/s41424-018-0025-4
54. Roshanravan N, Mahdavi R, Alizadeh E, *et al.* Effect of Butyrate and Inulin Supplementation on Glycemic Status, Lipid Profile and Glucagon-Like Peptide 1

Level in Patients with Type 2 Diabetes: A Randomized Double-Blind, Placebo-Controlled Trial. *Hormone and Metabolic Research.* Published online 2017. doi:10.1055/s-0043-119089
55. Pluznick JL. Gut microbiota in renal physiology: focus on short-chain fatty acids and their receptors. *Kidney international.* 2016;90(6):1191-1198. doi:10.1016/j.kint.2016.06.033
56. Le Poul E, Loison C, Struyf S, *et al.* Functional Characterization of Human Receptors for Short Chain Fatty Acids and Their Role in Polymorphonuclear Cell Activation. *J Biol Chem.* 2003;278(28):25481-25489. doi:10.1074/jbc.M301403200
57. Pluznick JL, Protzko RJ, Gevorgyan H, *et al.* Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. *Proc Natl Acad Sci USA*. 2013;110(11):4410-4415. doi:10.1073/pnas.1215927110
58. Wang L, Zhu Q, Lu A, *et al.* Sodium butyrate suppresses angiotensin II-induced hypertension by inhibition of renal (pro)renin receptor and intrarenal renin–angiotensin system. *J Hypertension*. 2017;35(9):1899. doi:10.1097/HJH.0000000000001378
59. Pluznick JL. Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. *Curr Hypertens Rep*. 2017;19(4):25. doi:10.1007/s11906-017-0722-5
60. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA*. 2014;111(6):2247-2252. doi:10.1073/pnas.1322269111
61. Aguilar EC, Leonel AJ, Teixeira LG, *et al.* Butyrate impairs atherogenesis by reducing plaque inflammation and vulnerability and decreasing NFκB activation. *Nutr Metab Cardiovasc Dis*. Published online 2014. doi:10.1016/j.numecd.2014.01.002
62. Säemann MD, Böhmig GA, Österreicher CH, *et al.* Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J*. Published online 2000. doi:10.1096/fj.00-0359fje
63. Li M, van Esch BCAM, Henricks PAJ, Folkerts G, Garssen J. The anti-inflammatory effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-stimulated endothelial cells via activation of GPR41/43 and inhibition of HDACs. *Front Pharmacol*. Published online 2018. doi:10.3389/fphar.2018.00533
64. Cardinale JP, Sriramula S, Pariaut R, *et al.* HDAC inhibition attenuates inflammatory, hypertrophic, and hypertensive responses in spontaneously hypertensive rats. *Hypertension*. Published online 2010. doi:10.1161/HYPERTENSIONAHA.110.154567
65. Kumar P, Gogulamudi VR, Periasamy R, Raghavaraju G, Subramanian U, Pandey KN. Inhibition of HDAC enhances STAT acetylation, blocks NF-kappaB, and suppresses the renal inflammation and fibrosis in Npr1 haplotype male mice. *Am J Physiol Renal Physiol*. 2017;313(3):F781-F795. doi:10.1152/ajprenal.00166.2017
66. Lal S, Kirkup AJ, Brunsden AM, Thompson DG, Grundy D. Vagal afferent responses to fatty acids of different chain length in the rat. *Am J Physiol Gastrointest Liver Physiol*. 2001;281(4 44-4). doi:10.1152/ajpgi.2001.281.4.g907
67. Onyszkiewicz M, Gawrys-Kopczynska M, Konopelski P, *et al.* Butyric acid, a gut bacteria metabolite, lowers arterial blood pressure via colon-vagus nerve signaling

and GPR41/43 receptors. *Eur J Physiol*. 2019;471(11):1441-1453. doi:10.1007/s00424-019-02322-y
68. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. *Gut*. 2006;55(10):1512-1520.
69. Wang W, Uzzau S, Goldblum SE, Fasano A. Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci*. 2000;113(24).
70. Fasano A, Not T, Wang W, et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet*. 2000;355(9214):1518-1519. doi:10.1016/S0140-6736(00)02169-3
71. Toral M, Robles-Vera I, de la Visitación N, *et al*. Critical Role of the Interaction Gut Microbiota – Sympathetic Nervous System in the Regulation of Blood Pressure. *Front Physiol*. 2019;10:231. doi:10.3389/fphys.2019.00231
72. Santisteban MM, Ahmari N, Carvajal JM, *et al*. Involvement of bone marrow cells and neuroinflammation in hypertension. *Circ Res*. 2015;117(2):178-191. doi:10.1161/CIRCRESAHA.117.305853
73. Cani PD, Amar J, Iglesias MA, *et al*. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*. Published online 2007. doi:10.2337/db06-1491
74. Raetz CRH, Whitfield C. Lipopolysaccharide Endotoxins. *Ann Rev Biochem*. 2002;71(1):635-700. doi:10.1146/annurev.biochem.71.110601.135414
75. Aderem A, Underhill DM. Mechanisms of Phagocytosis in Macrophages. *Ann Rev Immunol*. 1999;17(1):593-623. doi:10.1146/annurev.immunol.17.1.593
76. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. 2008;42(2):145-151.
77. Tobias PS, Soldau K, Gegner JA, Mintz D, Ulevitch RJ. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J Biol Chem*. 1995;270(18):10482-10488. doi:10.1074/jbc.270.18.10482
78. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*. 1990;249(4975):1431-1433. doi:10.1126/science.1698311
79. Maa MC, Chang MY, Hsieh MY, *et al*. Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. *J Nutr Biochem*. Published online 2010. doi:10.1016/j.jnutbio.2009.10.004
80. Masson GS, Nair AR, Dange RB, Silva-Soares PP, Michelini LC, Francis J. Toll-like receptor 4 promotes autonomic dysfunction, inflammation and microglia activation in the hypothalamic paraventricular nucleus: role of endoplasmic reticulum stress. *PloS ONE*. 2015;10(3):e0122850-e0122850. doi:10.1371/journal.pone.0122850
81. Sandiego CM, Gallezot JD, Pittman B, *et al*. Imaging robust microglial activation after lipopolysaccharide administration in humans with PET. *Proc Natl Acad Sci USA*. 2015;112(40):12468-12473. doi:10.1073/pnas.1511003112
82. Mancia G, Grassi G. The autonomic nervous system and hypertension. *Circ Res*. 2014;114(11):1804-1814. doi:10.1161/CIRCRESAHA.114.302524
83. Burnstock G, Loesch A. Sympathetic innervation of the kidney in health and disease: Emphasis on the role of purinergic cotransmission. *Auton Neurosci*. 2017;204:4-16. doi:10.1016/j.autneu.2016.05.007
84. Fisher JP, Young CN, Fadel PJ. Central sympathetic overactivity: Maladies and mechanisms. *Auton Neurosci*. 2009;148(1-2):5-15. doi:10.1016/j.autneu.2009.02.003

85. de Kloet AD, Liu M, Rodríguez V, Krause EG, Sumners C. Role of neurons and glia in the CNS actions of the renin-angiotensin system in cardiovascular control. *Am J Physiol Regul Integr Comp Physiol*. Published online 2015. doi:10.1152/ajpregu.00078.2015
86. Shi P, Grobe JL, Desland FA, *et al.* Direct pro-inflammatory effects of prorenin on microglia. PLoS ONE. Published online 2014. doi:10.1371/journal.pone.0092937
87. Berthoud HR, Blackshaw LA, Brookes SJH, Grundy D. Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. *Neurogastroenterol Motil*. 2004;16:28-33. doi:10.1111/j.1743-3150.2004.00471.x
88. Furness JB, Callaghan BP, Rivera LR, Cho HJ. The enteric nervous system and gastrointestinal innervation: Integrated local and central control. *Adv Exp Med Biol*. 2014;817:39-71. doi:10.1007/978-1-4939-0897-4_3
89. Gershon MD. The enteric nervous system: A second brain. *Hospital Practice*. Published online 1999. doi:10.3810/hp.1999.07.153
90. Yano JM, Yu K, Donaldson GP, *et al*. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*. 2015;161(2):264-276. doi:10.1016/j.cell.2015.02.047
91. Schäper J, Wagner A, Enigk F, *et al.* Regional sympathetic blockade attenuates activation of intestinal macrophages and reduces gut barrier failure. *Anesthesiology.* Published online 2013. doi:10.1097/ALN.0b013e3182784c93
92. Zubcevic J, Jun JY, Kim S, *et al*. Altered inflammatory response is associated with an impaired autonomic input to the bone marrow in the spontaneously hypertensive rat. *Hypertension*. 2014;63(3):542-550. doi:10.1161/HYPERTENSIONAHA.113.02722
93. Santisteban MM, Qi Y, Zubcevic J, *et al*. Hypertension-Linked Pathophysiological Alterations in the Gut. *Circ Res*. 2017;120(2):312-323. doi:10.1161/CIRCRESAHA.116.309006
94. Kim HL, Kim SH. Pulse wave velocity in atherosclerosis. *Front Cardiovasc Med*. 2019;6:41.
95. van Popele NM, Grobbee DE, Bots ML, *et al*. Association Between Arterial Stiffness and Atherosclerosis. *Stroke*. 2001;32(2):454-460. doi:10.1161/01.STR.32.2.454
96. Ridker PM, Cannon CP, Morrow D, *et al*. C-Reactive Protein Levels and Outcomes after Statin Therapy. *N Engl J Med*. 2005;352(1):20-28. doi:10.1056/NEJMoa042378
97. Ridker PM, Everett BM, Thuren T, *et al.* Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. Published online 2017. doi:10.1056/NEJMoa1707914
98. Ott SJ, El Mokhtari NE, Musfeldt M, *et al.* Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation*. 2006;113(7):929-937. doi:10.1161/CIRCULATIONAHA.105.579979
99. Koren O, Spor A, Felin J, *et al.* Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci USA*. 2011;108(Supplement_1):4592-4598. doi:10.1073/pnas.1011383107
100. Lanter BB, Sauer K, Davies DG. Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture. *mBio*. 2014;5(3). doi:10.1128/mBio.01206-14
101. Mitra S, Drautz-Moses DI, Alhede M, *et al.* In silico analyses of metagenomes from human atherosclerotic plaque samples. *Microbiome*. 2015;3(1):38. doi:10.1186/

s40168-015-0100-y
102. Lindskog Jonsson A, Hållenius FF, Akrami R, *et al.* Bacterial profile in human atherosclerotic plaques. *Atherosclerosis*. 2017;263:177-183. doi:10.1016/j.atherosclerosis.2017.06.016
103. Epstein SE, Zhu J, Burnett MS, Zhou YF, Vercellotti G, Hajjar D. Infection and Atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2000;20(6):1417-1420. doi:10.1161/01.ATV.20.6.1417
104. Rosenfeld ME, Campbell LA. Pathogens and atherosclerosis: Update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis. *Thromb Haemost*. Published online 2011. doi:10.1160/TH11-06-0392
105. Grayston JT. Antibiotic treatment of atherosclerotic cardiovascular disease. *Circulation*. 2003;107(9):1228-1230.
106. Song Z, Brassard P, Brophy JM. A meta-analysis of antibiotic use for the secondary prevention of cardiovascular diseases. *Can J Cardiol*. 2008;24(5):391-395. doi:10.1016/S0828-282X(08)70603-2
107. Jonsson AL, Bäckhed F. Role of gut microbiota in atherosclerosis. *Nat Rev Cardiol*. 2017;14(2):79-87. doi:10.1038/nrcardio.2016.183
108. Karlsson FH, Fåk F, Nookaew I, *et al.* Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat Comm*. 2012;3(1):1-8. doi:10.1038/ncomms2266
109. Jie Z, Xia H, Zhong SL, *et al.* The gut microbiome in atherosclerotic cardiovascular disease. *Nat Comm*. Published online 2017. doi:10.1038/s41467-017-00900-1
110. Liu H, Chen X, Hu X, *et al.* Alterations in the gut microbiome and metabolism with coronary artery disease severity. *Microbiome*. 2019;7(1):68. doi:10.1186/s40168-019-0683-9
111. Menni C, Lin C, Cecelja M, *et al.* Gut microbial diversity is associated with lower arterial stiffness in women. *Eur Heart J*. Published online 2018. doi:10.1093/eurheartj/ehy226
112. Brandsma E, Kloosterhuis NJ, Koster M, *et al.* A Proinflammatory Gut Microbiota Increases Systemic Inflammation and Accelerates Atherosclerosis. *Circ Res*. 2019;124(1):94-100. doi:10.1161/CIRCRESAHA.118.313234
113. Rath S, Heidrich B, Pieper DH, Vital M. Uncovering the trimethylamine-producing bacteria of the human gut microbiota. *Microbiome*. Published online 2017. doi:10.1186/S40168-017-0271-9
114. Bennett BJ, Vallim TQDA, Wang Z, *et al.* Trimethylamine-N-Oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. Published online 2013. doi:10.1016/j.cmet.2012.12.011
115. Papandreou C, Moré M, Bellamine A. Trimethylamine N-Oxide in Relation to Cardiometabolic Health—Cause or Effect? *Nutrients*. 2020;12(5):1330. doi:10.3390/nu12051330
116. Tang WHW, Wang Z, Kennedy DJ, *et al.* Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res*. 2014;116(3):448-455. doi:10.1161/CIRCRESAHA.116.305360
117. Chen K, Zheng X, Feng M, Li D, Zhang H. Gut microbiota-dependent metabolite Trimethylamine N-oxide contributes to cardiac dysfunction in western diet-

induced obese mice. *Front Physiol.* Published online 2017. doi:10.3389/fphys.2017.00139
118. Warrier M, Shih DM, Burrows AC, *et al.* The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. *Cell Rep*. Published online 2015. doi:10.1016/j.celrep.2014.12.036
119. Zhu W, Gregory JC, Org E, *et al.* Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell*. 2016;165(1):111-124. doi:10.1016/j.cell.2016.02.011
120. Wang Z, Klipfell E, Bennett BJ, *et al.* Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472(7341):57-65. doi:10.1038/nature09922
121. Koeth RA, Wang Z, Levison BS, *et al.* Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013;19(5):576-585. doi:10.1038/nm.3145
122. Heianza Y, Ma W, Manson JE, Rexrode KM, Qi L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. *J Am Heart Assoc*. 2017;6(7). doi:10.1161/JAHA.116.004947
123. Kaysen GA, Johansen KL, Chertow GM, *et al.* Associations of Trimethylamine N-Oxide With Nutritional and Inflammatory Biomarkers and Cardiovascular Outcomes in Patients New to Dialysis. *J Renal Nutr*. 2015;25(4):351-356. doi:10.1053/j.jrn.2015.02.006
124. Aldana-Hernández P, Leonard KA, Zhao YY, Curtis JM, Field CJ, Jacobs RL. Dietary choline or trimethylamine N-oxide supplementation does not influence atherosclerosis development in Ldlr−/− and Apoe−/− male mice. *J Nutr.* Published online 2020. doi:10.1093/jn/nxz214
125. Mueller DM, Allenspach M, Othman A, *et al.* Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis*. Published online 2015. doi:10.1016/j.atherosclerosis.2015.10.091
126. Tang WHW, Wang Z, Levison BS, *et al.* Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013;368(17):1575-1584. doi:10.1056/NEJMoa1109400
127. Lever M, George PM, Slow S, *et al.* Betaine and trimethylamine-N-oxide as predictors of cardiovascular outcomes show different patterns in diabetes mellitus: An observational study. *PLoS ONE*. 2014;9(12). doi:10.1371/journal.pone.0114969
128. Qi J, You T, Li J, *et al.* Circulating trimethylamine N-oxide and the risk of cardiovascular diseases: a systematic review and meta-analysis of 11 prospective cohort studies. *J Cell Mol Med*. 2018;22(1):185-194. doi:10.1111/jcmm.13307
129. Davey Smith G, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol.* 2003;32(1):1-22.
130. Jia J, Dou P, Gao M, *et al.* Assessment of causal direction between gut microbiota-dependent metabolites and cardiometabolic health: A bidirectional mendelian randomization analysis. *Diabetes*. 2019;68(9):1747-1755. doi:10.2337/db19-0153
131. Wang Z, Roberts AB, Buffa JA, *et al.* Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis. *Cell*. 2015;163(7):1585-1595. doi:10.1016/j.cell.2015.11.055

132. Vrieze A, Out C, Fuentes S, *et al.* Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *J Hepatol*. Published online 2014. doi:10.1016/j.jhep.2013.11.034
133. Baars A, Oosting A, Knol J, Garssen J, van Bergenhenegouwen J. The Gut Microbiota as a Therapeutic Target in IBD and Metabolic Disease: A Role for the Bile Acid Receptors FXR and TGR5. *Microorganisms*. Published online 2015. doi:10.3390/microorganisms3040641
134. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap—bile acids in metabolic control. *Nat Rev Endocrinol*. 2014;10(8):488-498.
135. Hagenbuch B, Dawson P. The sodium bile salt cotransport family SLC10. *Pflügers Archiv*. 2004;447:566-570.
136. Hofmann AF, Eckmann L. How bile acids confer gut mucosal protection against bacteria. *Proc Natl Acad Sci USA*. 2006;103(12):4333-4334.
137. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev.* 2005;29(4):625-651.
138. Brufau G, Groen AK, Kuipers F. Reverse cholesterol transport revisited: Contribution of biliary versus intestinal cholesterol excretion. *Arterioscler Thromb Vasc Biol*. Published online 2011. doi:10.1161/ATVBAHA.108.181206
139. Selwyn FP, Csanaky IL, Zhang Y, Klaassen CD. Importance of Large Intestine in Regulating Bile Acids and Glucagon-Like Peptide-1 in Germ-Free Mice. *Drug Metabolism and Disposition*. Published online 2015. doi:10.1124/dmd.115.065276
140. Sayin SI, Wahlström A, Felin J, *et al.* Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab*. Published online 2013. doi:10.1016/j.cmet.2013.01.003
141. Hodge RJ, Nunez DJ. Therapeutic potential of Takeda-G-protein-receptor-5 (TGR5) agonists. Hope or hype? *Diabetes Obes Metab*. Published online 2016. doi:10.1111/dom.12636
142. Yoneno K, Hisamatsu T, Shimamura K, *et al.* TGR5 signalling inhibits the production of pro-inflammatory cytokines by in vitro differentiated inflammatory and intestinal macrophages in Crohn's disease. *Immunology*. 2013;139(1):19-29. doi:10.1111/imm.12045
143. Pols TWH, Nomura M, Harach T, *et al.* TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. *Cell Metab*. 2011;14(6):747-757. doi:10.1016/j.cmet.2011.11.006
144. Hodge RJ, Lin J, Vasist Johnson LS, Gould EP, Bowers GD, Nunez DJ. Safety, pharmacokinetics, and pharmacodynamic effects of a selective TGR5 Agonist, SB-756050, in Type 2 Diabetes. *Clin Pharmacol Drug Dev*. Published online 2013. doi:10.1002/cpdd.34
145. Eggink HM, Soeters MR, Pols TW. TGR5 ligands as potential therapeutics in inflammatory diseases. *Interferon Cytokine Mediat Res*. Published online 2014:27-38.
146. Hanniman EA, Lambert G, McCarthy TC, Sinal CJ. Loss of functional farnesoid X receptor increases atherosclerotic lesions in apolipoprotein E-deficient mice. *J Lipid Res*. Published online 2005. doi:10.1194/jlr.M500390-JLR200
147. Zhang Y, Wang X, Vales C, *et al.* FXR deficiency causes reduced atherosclerosis in Ldlr-/- mice. *Arterioscler Thromb Vasc Biol*. Published online 2006. doi:10.1161/01.ATV.0000235697.35431.05

148. Guo GL, Santamarina-Fojo S, Akiyama TE, *et al.* Effects of FXR in foam-cell formation and atherosclerosis development. *Biochim Biophys Acta*. Published online 2006. doi:10.1016/j.bbalip.2006.09.018
149. Hartman HB, Gardell SJ, Petucci CJ, Wang S, Krueger JA, Evans MJ. Activation of farnesoid X receptor prevents atherosclerotic lesion formation in LDLR-/- and apoE -/- mice. *J Lipid Res*. Published online 2009. doi:10.1194/jlr.M800619-JLR200
150. Mencarelli A, Renga B, Distrutti E, Fiorucci S. Antiatherosclerotic effect of farnesoid X receptor. *Am J Physiol Heart Circ Physiol*. Published online 2009. doi:10.1152/ajpheart.01075.2008
151. Hambruch E, Miyazaki-Anzai S, Hahn U, *et al.* Synthetic farnesoid X receptor agonists induce high-density lipoprotein-mediated transhepatic cholesterol efflux in mice and monkeys and prevent atherosclerosis in cholesteryl ester transfer protein transgenic low-density lipoprotein receptor (-/-) mice. *J Pharmacol Exp Ther.* Published online 2012. doi:10.1124/jpet.112.196519
152. Siddiqui MS, Van Natta ML, Connelly MA, *et al.* Impact of obeticholic acid on the lipoprotein profile in patients with non-alcoholic steatohepatitis. *J Hepatology*. Published online 2019. doi:10.1016/j.jhep.2019.10.006
153. Miyazaki-Anzai S, Masuda M, Levi M, Keenan AL, Miyazaki M. Dual activation of the bile acid nuclear receptor FXR and G-Protein-Coupled receptor TGR5 protects mice against atherosclerosis. *PLoS ONE*. Published online 2014. doi:10.1371/journal.pone.0108270
154. Baghdasaryan A, Claudel T, Gumhold J, *et al.* Dual farnesoid X receptor/TGR5 agonist INT-767 reduces liver injury in the Mdr2 -/- (Abcb4 -/-) mouse cholangiopathy model by promoting biliary HCO3- output. *Hepatology*. 2011;54(4):1303-1312. doi:10.1002/hep.24537
155. Jadhav K, Xu Y, Xu Y, *et al.* Reversal of metabolic disorders by pharmacological activation of bile acid receptors TGR5 and FXR. *Mol Metabol*. 2018;9:131-140. doi:10.1016/j.molmet.2018.01.005
156. Iracheta-Vellve A, Calenda CD, Petrasek J, *et al.* FXR and TGR5 Agonists Ameliorate Liver Injury, Steatosis, and Inflammation After Binge or Prolonged Alcohol Feeding in Mice. *Hepatol Comm*. 2018;2(11):1379-1391. doi:10.1002/hep4.1256
157. Wang XX, Wang D, Luo Y, *et al.* FXR/TGR5 dual agonist prevents progression of nephropathy in diabetes and obesity. *J Am Soc Nephrol*. Published online 2018. doi:10.1681/ASN.2017020222
158. Hiippala K, Jouhten H, Ronkainen A, *et al.* The potential of gut commensals in reinforcing intestinal barrier function and alleviating inflammation. *Nutrients*. 2018;10(8):988.
159. Gilijamse PW, Hartstra AV, Levin E, *et al.* Treatment with Anaerobutyricum soehngenii: a pilot study of safety and dose–response effects on glucose metabolism in human subjects with metabolic syndrome. *npj Biofilms Microbiomes*. 2020;6(1). doi:10.1038/s41522-020-0127-0
160. Quigley EM. Prebiotics and probiotics in digestive health. *Clin Gastroenterol Hepatol.* 2019;17(2):333-344.
161. McFarland LV, Evans CT, Goldstein EJ. Strain-specificity and disease-specificity of probiotic efficacy: a systematic review and meta-analysis. *Front Med*. 2018;5:124.
162. Dailey FE, Turse EP, Daglilar E, Tahan V. The dirty aspects of fecal microbiota

transplantation: a review of its adverse effects and complications. *Curr Opin Pharmacol.* 2019;49:29-33.
163. Hecht GA, Blaser MJ, Gordon J, *et al.* What is the value of a food and drug administration investigational new drug application for fecal microbiota transplantation to treat Clostridium difficile Infection? *Clin Gastroenterol Hepatol.* 2014;12(2):289-291.
164. Lai CY, Sung J, Cheng F, *et al.* Systematic review with meta-analysis: review of donor features, procedures and outcomes in 168 clinical studies of faecal microbiota transplantation. *Aliment Pharmacol Ther.* 2019;49(4):354-363.
165. Draper LA, Ryan FJ, Smith MK, *et al.* Long-term colonisation with donor bacteriophages following successful faecal microbial transplantation. *Microbiome*. Published online 2018. doi:10.1186/s40168-018-0598-x
166. Smits LP, Kootte RS, Levin E, *et al.* Effect of vegan fecal microbiota transplantation on carnitine- and choline-derived trimethylamine-N-oxide production and vascular inflammation in patients with metabolic syndrome. *J Am Heart Assoc*. 2018;7(7). doi:10.1161/JAHA.117.008342
167. Kootte RS, Levin E, Salojärvi J, *et al.* Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. *Cell Metab*. 2017;26(4):611-619.e6. doi:10.1016/J.CMET.2017.09.008
168. Vrieze A, Van Nood E, Holleman F, *et al.* Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology*. 2012;143(4):913-916.e7. doi:10.1053/j.gastro.2012.06.031
169. Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes*. 2017;8(2):172-184. doi:10.1080/19490976.2017.1290756
170. Martínez-González MA, Gea A, Ruiz-Canela M. The Mediterranean diet and cardiovascular health: A critical review. *Circ Res*. 2019;124(5):779-798.
171. Siervo M, Lara J, Chowdhury S, Ashor A, Oggioni C, Mathers JC. Effects of the Dietary Approach to Stop Hypertension (DASH) diet on cardiovascular risk factors: a systematic review and meta-analysis. *Br J Nutr.* 2015;113(1):1-15.

4

Oral butyrate increases daytime systolic blood pressure in hypertensive patients: a randomized, placebo-controlled trial

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5

Plasma metabolite profiles and blood pressure in the HELIUS study: the role of formylmethionine

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In preparation

6

Sex differences in associations of plasma metabolites with blood pressure and heart rate variability: the HELIUS study

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Abstract

Background and aims: Since plasma metabolites can modulate blood pressure (BP) and vary between men and women, we examined sex differences in plasma metabolite profiles associated with BP and sympathicovagal balance. Our secondary aim was to investigate associations between gut microbiota composition and plasma metabolites predictive of BP and heart rate variability (HRV).

Methods: From the HELIUS cohort, we included 196 women and 173 men. Office systolic BP and diastolic BP were recorded, and heart rate variability and baroreceptor sensitivity (BRS) were calculated using finger photoplethysmography. Plasma metabolomics was measured using untargeted LC-MS/MS. Gut microbiota composition was determined using 16S sequencing. We used machine learning models to predict BP and HRV from metabolite profiles, and to predict metabolite levels from gut microbiota composition.

Results: In women, best predicting metabolites for systolic BP included dihomo-lineoylcarnitine, 4-hydroxyphenylacetateglutamine and vanillactate. In men, top predictors included sphingomyelins, N-formylmethionine and conjugated bile acids. Best predictors for HRV in men included phenylacetate and gentisate, which were associated with lower HRV in men but not in women. Several of these metabolites were associated with gut microbiota composition, including phenylacetate, multiple sphingomyelins and gentisate.

Conclusions: Plasma metabolite profiles are associated with BP in a sex-specific manner. Catecholamine derivatives were more important predictors for BP in women, while sphingomyelins were more important in men. Several metabolites were associated with gut microbiota composition, providing potential targets for intervention.

Introduction

Hypertension is the leading cause of cardiovascular disease and mortality worldwide for both men and women.[1,2] Although hypertension is generally more prevalent in men, hypertension prevalence at older age is higher in women due to a steeper increase in blood pressure (BP) than men from the third decade on.[3] Additionally, women have a higher risk of cardiovascular disease compared to men for every increment in BP and a higher prevalence of hypertension mediated organ damage.[4–6] Despite established sex differences in BP life trajectories and autonomic cardiovascular control,[3,7] sex differences in pathophysiology of hypertension remain incompletely understood.

The autonomic nervous system has a central role in BP regulation. Changes in BP lead to activation of the baroreceptors that regulate heart rate, myocardial contractility and vascular resistance.[8] As such, the sympathovagal tone mediates baroreceptor sensitivity, which is the interaction between changes in blood pressure and heart rate, and heart rate variability, defined by the variation in time intervals between heartbeats.[9,10] The complex interplay of cardiometabolic and neurohumoral systems that regulate BP is reflected by the plasma metabolome.[11] The plasma metabolome can be defined as the collection of products and intermediates of cellular metabolism smaller than 1.5 kDa.

Sex is an important determinant of metabolome profiles, since more than half of the metabolites have been shown to differ between men and women.[12] These differentially abundant metabolites not only include metabolites of sex hormones, but also other lipids, such as carnitines and sphingomyelins, and a range of amino acids. Other key determinants of the plasma metabolome are metabolic conditions, dietary intake, and medication use.[13–16] In addition, accumulating evidence shows that the gut microbiome can modulate the circulating pool of small-molecule metabolites.[17,18] The gut microbiota, and particularly short chain fatty acid producing microbes, have been associated with BP levels, and could modulate BP by production of metabolites.[19] Indeed, the gut microbiome has been shown to be able to modulate the plasma metabolome in intervention studies targeting the gut microbiota such as fecal microbiota transplantations.[20]

Previous studies that assessed the relation between plasma metabolite profiles and BP found that long chain fatty acids such as hexadecadienoate, ceramide, glycerolipids, and several amino acids levels were predictive of BP.[21–24] However, most studies did not look at sex-specific associations of metabolites with BP or only conducted sensitivity analyses in a subset of metabolites. We assessed sex-specific plasma metabolite profiles that are associated with blood pressure and auto-

nomic cardiovascular control, in order to better understand sex differences in hypertension. We performed machine learning analyses to predict BP, baroreceptor sensitivity (BRS) and heart rate variability (HRV) from plasma metabolite profiles for men and women separately. As a secondary analysis, we investigated the associations between gut microbiota composition and the plasma levels of the metabolites predicting BP and HRV, as these could associations could provide potential targets for intervention.

Highlights

- Sphingomyelins (men) and catecholamine metabolites (women) showed sex-specific associations with blood pressure (BP).
- Several metabolites were associated with gut microbiota composition, providing potential targets for intervention.
- Plasma metabolites could have sex-dependent effects on BP and are often only associated with BP in either men or women.

Patients and methods

Study population

We used baseline data and plasma samples collected between 2011 and 2015 from the HEalthy LIfe in an Urban Setting (HELIUS) cohort study, a large prospective multi-ethnic population-based study conducted in Amsterdam, the Netherlands.[25] Individuals aged between 18 and 70 years were randomly sampled from the municipality registry stratified for ethnicity (Dutch, Surinamese, Ghanaian, Turkish or Moroccan origin). All participants of HELIUS provided written informed consent and the HELIUS study was approved by the medical ethical review board of the Amsterdam UMC, location AMC. This study followed the principles of the Declaration of Helsinki.

Data were collected by questionnaire and during morning study visits at local research sites. A total of 24,788 participants could be included in HELIUS, of which 22,164 completed a visit at the research location. Of those, 6,048 handed in a stool sample. All participants were asked to refrain from using any vasoactive medication and smoking. Medication use was registered. Height and weight were measured and body mass index (BMI) was calculated. BP was measured after 5 minutes of rest in the supine position, using the average of two consecutive measurements of a validated semi-automatic oscillometric device (Microlife WatchBP

Home; Microlife AG, Switzerland). Following 10 minutes of supine rest, non-invasive continuous blood pressure measurements were taken for 5 minutes using finger photoplethysmography (Nexfin, Edwards Lifesciences, Irvine, California). Venous blood samples were drawn, from which fasting glucose and creatinine levels were measured. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI formula. Urinary albumin-to-creatinine ratio was determined from early morning spot urine samples. Albuminuria was categorized into different stages using the ACR KDIGO classification.[26] Diabetes was defined based on fasting glucose levels (≥7 mmol/L) or use of glucose-lowering medication. Hypertension was defined according to guidelines as an elevated systolic BP >140 mmHg or diastolic BP (DBP) >90 mmHg or use of antihypertensive medication.[27]

For the metabolomics substudy, 370 subjects were selected from 4 ethnic groups (South-Asian Surinamese, African Surinamese, Ghanaian and Dutch origin), and had preserved renal function (estimated glomerular filtration rate (eGFR) > 60 ml/min). Per ethnic group, we selected subjects from the cohort so that 50% had early-stage albuminuria (ACR KDIGO stage A2, 3-30 mg/mmol) in an otherwise random manner.

Plasma metabolomics

For untargeted metabolite profiling, fasting EDTA plasma samples were collected and shipped to Metabolon (Durham, North Carolina, USA). Plasma metabolites were measured by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) in 369 samples.

The plasma samples were kept at -80°C until further processing. Several recovery standards were added prior to the first step in the extraction process for QC purposes. Proteins were precipitated with methanol under vigorous shaking for 2 min followed by centrifugation. The extract was divided over different samples for the several UPLC methods, and put briefly on an evaporator to remove the solvent. Controls such as a sample with pooled plasma from all samples (technical replicate) and extracted water samples (blanks) were included in the same batch. A cocktail of QC standards was spiked into each analyzed sample for instrument performance monitoring and chromatographic alignment.

The UPLC methods used a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible with each of the four methods. The reverse phase meth-

ods used C18 columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) while the HILIC method used a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm). The MS analysis alternated between MS and data-dependent MSn scans (mass range 70-1000 m/z) using dynamic exclusion. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Biochemical identifications were based on a retention index within a narrow window, accurate mass match ±10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. For more details on the UPLC-MS/MS methods we refer to **Supplement 1** and a list of identified metabolites is provided in **Supplement 2**.

Missing values were attributed to sample measurement falling below detection limits and were imputed with the minimal observed value. Prior to the analyses, we excluded metabolites that were labeled by Metabolon as xenobiotic (super pathway), which included metabolites of medication, resulting in a dataset of a total of 722 metabolites for analysis. Lipid annotations that were too long to show in the data visualizations were abbreviated using the short lipid species level notation of the LIPID MAPS nomenclature [28]. For instance, sphingomyelin (d18:2/14:0, d18:1/14:1)* was annotated as SM 32:2.

Processing of continuous finger BP measurements

xBRS and HRV data calculated from continuous finger BP measurements was available for 139 subjects. Raw beat-to-beat data consisting of inter-beat intervals (IBI) and systolic BP values were exported from the Nexfin device and analyzed in Matlab (R2019a; The MathWorks, Inc.).[29] A moving average filter was applied to the beat-to-beat data to exclude measurement artefacts and ectopic beats.[30] A recording was excluded from further analysis if more than 20% of beats needed to be removed or if there was no continuous segment of 30 beats without internal calibration available. To quantify HRV, the standard deviation of normal-to- normal intervals (SDNN) was calculated.[31] BRS was determined with a cross-correlation method.[32] Each 10 second interval of IBI was cross-correlated with a 10 second interval of systolic BP measurements, with a time shift varying between 0 and 5 seconds, in which systolic BP preceded IBI. The time delay with maximum correlation was chosen, from which cross-correlation estimation of BRS (xBRS) was calculated by dividing the standard deviation (SD) of the IBI by the SD of the systolic BP for that segment. xBRS of the complete recording was defined as the geometric mean of all segments with significant positive correlation ($p<0.05$).

Gut microbiota composition

Participants were asked to bring a fresh faecal sample within 6 hours after collection, or, if not possible, to store the sample overnight in a freezer. Samples were stored at −20°C at the study visit location for a maximum of 1 day before transportation to the central freezer (-80°C). Samples obtained from participants who either had diarrhoea in the week prior to collection or used antibiotics within three weeks prior to collection were excluded. Samples were shipped to the Wallenberg Laboratory (Sahlgrenska Academy at University of Gothenburg, Sweden) for sequencing. DNA was extracted from 150 mg aliquot of faecal samples using a repeated bead-beating protocol.[33] Faecal microbiota composition was determined by sequencing the V4 region of the 16S rRNA gene on an Illumina MiSeq (llumina RTA v1.17.28; MCS v2.5, San Diego, CA, USA) using 515F and 806R primers designed for dual-indexingand the V2 Illumina kit (2x250 bp paired-end reads).[34] PCR was performed in duplicate reactions as previously described.[35] Preprocessing of the raw sequencing data is described in **Supplement 3**.

Machine learning models

We used machine learning analyses for three different aims: 1) to predict sex from metabolite profiles (as descriptive analysis); 2) to predict BP, HRV and xBRS from metabolite profiles; 3) to predict highest ranked metabolites resulting from the previous models from gut microbiota composition. The advantages of a tree-based machine learning model compared to univariate tests (t-tests, regressions) are the possibility to include a large number of variables in one model which improves power in this relatively small sample size, and the robustness to nonnormal distributions of variables. XGBoost is a commonly used algorithm that uses extreme gradient boosting to improve accuracy, and has been shown to provide accurate and efficient predictions across different omics analyses.[36–38]

All machine learning models used the XGBoost algorithm in a nested cross-validation design (**Supplement 4**).[39] In each iteration, the dataset was randomly split into a test set containing 20% of the subjects and a training set with the remaining 80%. Within the train set, 5-fold cross-validation was performed in order to optimize the model hyperparameters. Two random variables were added to the determinants in each iteration to serve as a benchmark. The resulting model was evaluated on the test set which yielded an area under the receiver-operator curve (AUC) for classification models, and explained variance (%) for continuous outcomes as main model quality metrics. In addition, each iteration resulted in a ranked list of metabolites with their relative importance to the prediction. These

were recorded for each iteration and were averaged across 200 iterations. Because of the definition of the explained variance score formula,[40] the explained variance score could also be a negative value, meaning that the prediction is worse than an intercept. To ensure that these models were not overfitted, we ran identical models in which the data was permuted prior to every iteration. Results of these permuted models can be found in **Supplement 5**.

First, we used the machine learning classification model described above to assess which of the 722 plasma metabolites (determinants) were most predictive of sex (as binary outcome). This resulted in a median AUC and a list of the 15 metabolites that had the highest feature importance for this prediction. The feature importance was set at 100% for the first metabolite, with the other metabolites' importance calculated relative to the first.

Thereafter, we ran machine learning models to predict systolic BP, diastolic BP, xBRS and HRV from the 722 plasma metabolites for men and women separately. Each of these eight models resulted in a median explained variance score and ranking of the most important metabolites for the prediction. In addition, since sex differences in BP change during aging, we performed subgroup analyses stratified for age (50 and >50 years) with an identical set-up. Since a proportion of 12.2% of participants had diabetes, and diabetes impacts both metabolite profiles and blood pressure substantially, we decided to also perform a sensitivity analysis in subjects without diabetes. With these models, we aimed to assess if the associations in the main models were driven by subjects with diabetes. The design of these models was identical to the models for systolic and diastolic BP described above.

To assess which of the highest ranked metabolites could be explained by gut microbiota composition, we used the same machine learning models, but with the ASVs (as gut microbiota composition) as determinants and the metabolite concentrations as outcome. We selected the top 10 metabolites from the BP, HRV and BRS models with an explained variance of more than 5% by the metabolite profiles. We selected ASVs with more than 5 counts in 30% of the subjects, resulting in a data set with 368 subjects and 146 ASVs. For each of the selected metabolites, we ran a separate machine learning regression model to predict the levels of this metabolite from the ASVs.

The machine learning models were implemented in Python (v.3.8.6) using the XGBoost (v.1.2.0), numpy (v.1.19.2), pandas (v.1.1.4), and scikit-learn (v.0.23.2) packages.[39,40] The conda environment set-up (yaml file), two parameter grids (one for metabolite models and one for microbiota models) and outputs of all models were shared in a public repository (doi:10.5281/zenodo.7684283).

Statistics

Differences in descriptive and outcome variables between men and women were tested using t-tests for continuous variables with normal distributions, Wilcoxon rank tests for continuous variables with non-normal distributions and chi-square tests for categorical variables using the tableone package (v.0.13.2). As a descriptive analysis to assess sex differences in the metabolite profiles, we first performed a principal component analysis after scaling and centering of the metabolite data set containing 722 metabolites using a variance-covariance matrix (*prcomp* function in R).

To obtain effect sizes for the associations of the top 10 highest ranked metabolites for each machine learning model (systolic BP, diastolic BP, HRV and xBRS), we used linear regression models that were adjusted for age, BMI, eGFR, diabetes and albuminuria, and stratified for sex. We only ran these linear regression models if the machine learning model had an explained variance higher than 1%. The effect sizes were plotted in a forest plot showing the beta (per SD increase in metabolite levels) with 95%-confidence intervals (95%-CI). HRV and xBRS were log10-transformed because of their skewed distributions. We used unstratified models with the same covariates to test the interaction of the associations with sex. For the each of 10 highest ranked ASVs per metabolite model, we used linear regression models to assess associations between these ASVs and the respective metabolite. For these models, the log10-transformed ASV counts were used as determinants and the metabolite levels (scaled with a mean of 0 and SD of 1) as outcomes.

All analyses and data visualizations were performed in RStudio (v.2022.7.2.576) using R (v.4.2.1). The scripts of all analyses and an renv lockfile were shared in a public repository (doi:10.5281/zenodo.7684283).

Data availability

The HELIUS clinical and metabolomics data are owned by the Amsterdam UMC, location AMC in Amsterdam, The Netherlands. As participants gave consent for re-use only within the aims of the HELIUS study, access to the HELIUS data needs to be granted by the HELIUS board. Any researcher can request the data by submitting a proposal to the HELIUS Executive Board (heliuscoordinator@amsterdamumc.nl) as outlined at http://www.heliusstudy.nl/en/researchers/collaboration. The HELIUS Executive Board will check proposals for compatibility with the general objectives, ethical approvals and informed consent forms of the HELIUS study. Access is granted to all researchers affiliated with an inter-

nationally recognized research institution who request to use the HELIUS data, after having signed the data transfer agreement. The 16S sequencing data are available in the European Genome-Phenome Archive (EGA), accession number EGAD00001004106 (https://ega-archive.org/datasets/EGAD00001004106).

Results

Population characteristics and top predicting metabolites

Population characteristics of this subset of the HELIUS cohort are shown in **Table 1**. The study population consisted of 173 (46.9%) men and 196 (53.1%) women. Men were on average older than women but had on average lower BMI, although these differences were not significant. More men than women were active smokers ($p<0.01$). Both systolic and diastolic BP were significantly higher in men than in women (**Figure 1A,B**), and accordingly, hypertension was more prevalent in men. However, use of antihypertensive medication was comparable between the groups. xBRS was higher in women than men ($p<0.01$; **Figure 1C**). In addition, HRV tended to be higher in women compared with men ($p=0.15$; **Figure 1D**). The characteristics of the subgroup of patients with available xBRS and HRV data are presented in **Supplement 6**.

A principal component analysis showed slightly different metabolome profiles between men and women, but also substantial overlap (**Figure 1E**). A classification model to predict sex from metabolite profiles had a median AUC of 0.96. Best predicting metabolites for sex included testosterone metabolites (5α-androstan-3α,17β-diol derivatives), a progesterone metabolite (pregnanediol disulfate), amino acids including creatinine, and a sphingomyelin (SM 32:2; **Figure 1F**). All of these metabolites had higher levels in men, except SM 32:2, which was higher in women (**Supplement 7).**

Different plasma metabolites predict BP in males and females

The machine learning analyses to predict BP from metabolite profiles showed an explained variance for systolic BP of 7.59% for men and 11.16% for women, while the models for diastolic BP had an explained variance of only 0.03% for men and 7.75% for women (**Supplement 8**). To further investigate the low explained variance for diastolic BP in male subjects, we repeated this analysis stratified for age group (<50 and ≥50 years; **Supplement 9**). This subgroup analysis showed an ex-

Table 1: Study population

	Overall	Women	Men	p
n	369	196	173	
Age (years)	51.7±11.3	50.7±11.6	52.9± 10.9	0.063
Age ≥50 years	154 (41.7)	91 (46.4)	63 (36.4)	0.066
BMI (kg/m²)	27.5±5.2	27.9±5.9	27.0±4.2	0.065
Ethnicity				0.263
Dutch	84 (22.8)	39 (19.9)	45 (26.0)	
South-Asian Surinamese	101 (27.4)	50 (25.5)	51 (29.5)	
African Surinamese	105 (28.5)	62 (31.6)	43 (24.9)	
Ghanaian	79 (21.4)	45 (23.0)	34 (19.7)	
Current smoking	67 (18.2)	24 (12.3)	43 (24.9)	0.003
Diabetes	45 (12.2)	19 (9.7)	26 (15.0)	0.160
Hypertension	215 (58.3)	98 (50.0)	117 (67.6)	0.001
Antihypertensive medication	107 (29.0)	53 (27.0)	54 (31.2)	0.443
Systolic BP (mmHg)	136.2±21.2	133.0±21.7	139.8±19.9	0.002
Diastolic BP (mmHg)	83.2±11.5	80.2±10.8	86.7±11.3	<0.001
xBRS (ms/mmHg)	11.8±6.6	10.3±5.2	13.6±7.5	0.003
HRV (SDNN)	0.05±0.02	0.05±0.02	0.05±0.02	0.358
eGFR (ml/min/1.73m²)	95.0±19.5	97.8±19.8	91.8±18.7	0.003
Albuminuria	169 (45.8)	87 (44.4)	82 (47.4)	0.635
LDL (mmol/L)	3.1±0.9	3.1±0.9	3.1±0.9	0.836
HDL (mmol/L)	1.5±0.4	1.6±0.4	1.3±0.4	<0.001
Triglycerides (mmol/L)	1.1±0.9	0.9±0.5	1.3±1.1	<0.001

Data is presented as mean±SD or n (%). Sex differences were tested with t-test for continuous variables and chi-square tests for categorical variables. BMI = body mass index, BP = blood pressure, eGFR = estimated glomerular filtration rate (CKD-EPI), HRV = heart rate variability, SDNN = standard deviation of NN intervals, xBRS = cross-correlation baroreceptor sensitivity.

plained variance of 9.1% for diastolic BP for the young age group in males, while there was no explained variance in older males. In systolic BP, there also was a clear difference in explained variance between young and older subjects for both men and women.

In women, best predictors for systolic BP included long chain acylcarnitines (dihomo-lineoylcarnitine), catecholamine degradation products (4-hydroxyphenylacetateglutamine and vanillactate) and metabolites that could potentially be related to S-adenosylmethionine formation (2,3-dihydroxy-5-methylthio-4-pentenoic acid (DMTPA) and 1-methyladenosine). The best predictor for diastolic BP in women was acetylcitrulline, a urea cycle metabolite. In addition, the list of best predictors included several steroids, including androgenic, progestin and corticosteroids, as well as catecholamine metabolites and serotonin. In men, best predicting metabolites for systolic BP included sphingomyelins, N-formylmethionine, conjugated bile acids, and N-acyl amino acids.

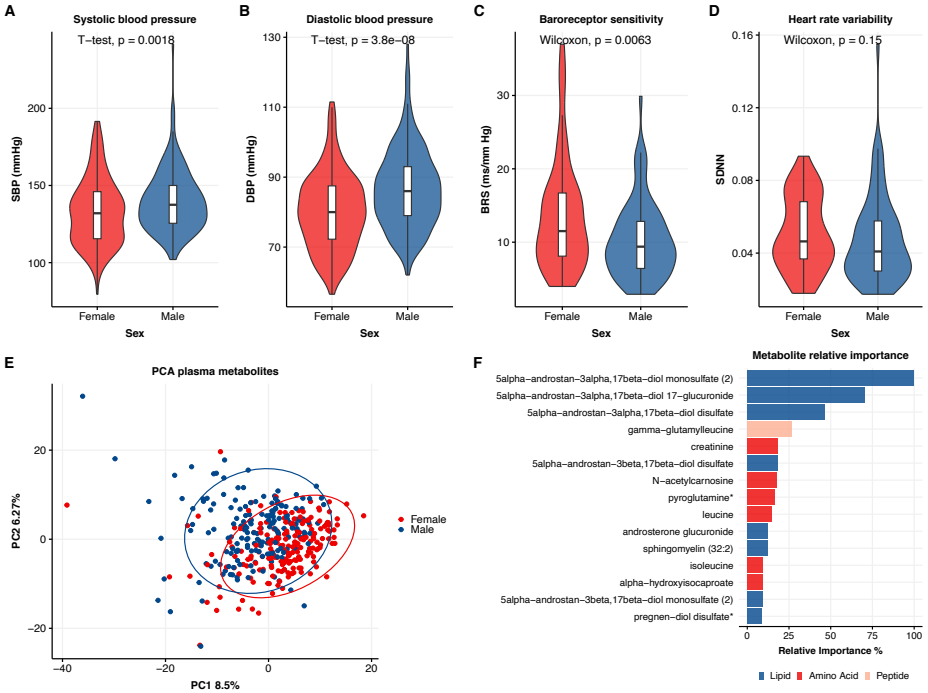
Next, we performed a sensitivity analysis excluding diabetic patients, to assess if the machine learning results are driven by the presence of diabetes (**Supplement 10**). The best predictors for BP in these analyses showed overlap with the findings in the total study population. In women, top predictors for systolic BP still included acylcarnitines, vanillactate, and DMTPA, and acetylcitrulline persisted as the highest ranked predictor for diastolic BP. However, uridine-related metabolites and C-glycosyltryptophan became higher ranked predictors for systolic BP than in the general model. In men, the highest ranked predictors for systolic BP were comparable to the total population.

BP-predicting metabolites have interactions with sex in adjusted models

We used linear regression models to obtain effect sizes of the associations between the top predicting metabolites and BP, while adjusting for relevant confounders such as age, sex, BMI, eGFR, diabetes and albuminuria (**Figure 2**). The three highest ranked metabolites for systolic BP in women, a long chain acylcarnitine and two catecholamine related metabolites, had significant interactions with sex and were only associated with higher systolic BP in women but not in men. Dihomo-lineoylcarnitine, 4-hydroxyphenylacetylglutamine and vanillactate showed similar effect sizes and were associated with a 4.54 mmHg (95%-CI 1.81, 7.28), 3.74 mmHg (95%-CI 0.54, 6.94) and 4.36 mmHg (95%-CI 1.33, 7.39) mmHg higher systolic BP per SD increase in women.

Sphingomyelins (SM 38:3, SM 42:4 and SM 40:3) and conjugated bile acids

Figure 1: Descriptive characteristics

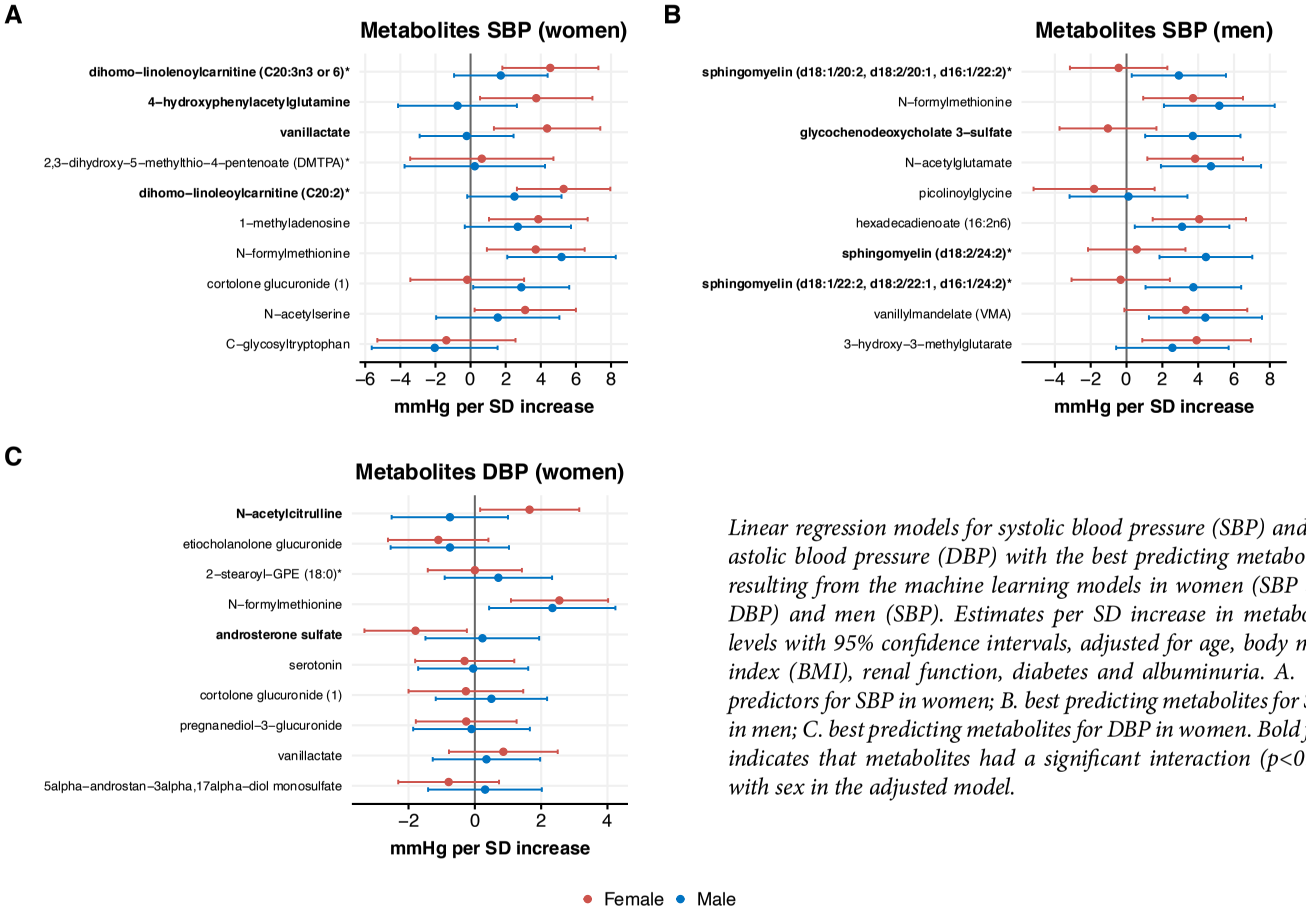


Descriptive characteristics of the study population. Differences in A. systolic BP, B. diastolic BP, C. baroreceptor sensitivity, and D. heart rate variability between female and male subjects are shown in the upper row. E. Principal component plot showing two principal components (PC1 and PC2) and the differences in metabolite profiles between female and male subjects. The ellipses were drawn around 95% of the subjects for each group. F. Relative importance of metabolites resulting from the machine learning model that predicted sex from metabolite profiles (mean area under the receiver-operator curve (AUC): 0.96).

had significant interactions with sex and were only associated with systolic BP in men, while N-acetylglutamate showed a stronger association in men than women, but had no significant interactions. SM 38:3 (+2.92 mmHg (95%-CI 0.29, 5.55) per SD increase), N-formylmethionine (+5.18 mmHg (95%-CI 2.09, 8.27)), and glycochenodeoxycholate (+3.70 mmHg (95%-CI 1.04, 6.36)) had the largest effect sizes and were associated with higher systolic BP in the adjusted linear regression model.

We only performed linear regression analyses for the best predicting metabolites associated with diastolic BP in women (**Figure 2C**), since the machine learning model for men had very low explained variance. N-acetylcitrulline (+1.65 mmHg (95%-CI 0.16, 3.15)), a nitric oxide pathway metabolite, and N-formylmethionine (+2.55mmHg (95%-CI 1.09, 4.02) were associated with higher diastolic

Figure 2: Linear regression models plasma metabolites and BP



Linear regression models for systolic blood pressure (SBP) and diastolic blood pressure (DBP) with the best predicting metabolites resulting from the machine learning models in women (SBP and DBP) and men (SBP). Estimates per SD increase in metabolite levels with 95% confidence intervals, adjusted for age, body mass index (BMI), renal function, diabetes and albuminuria. A. best predictors for SBP in women; B. best predicting metabolites for SBP in men; C. best predicting metabolites for DBP in women. Bold font indicates that metabolites had a significant interaction ($p<0.05$) with sex in the adjusted model.

BP in women, while androsterone sulfate (-1.79mmHg (95%-CI -3.33, -0.24) was associated with lower diastolic BP.

Sex specific differences in predicting xBRS and HRV from plasma metabolites

The machine learning models for xBRS and HRV performed better in men than in women. The explained variance of xBRS was 3.5% in men compared to 0.8% in women, while the explained variance of HRV was 15.6% in men and none in women. Stratification for age of the HRV models in female participants did not improve model performance substantially (data not shown). Of the top 10 predictors for xBRS and HRV in men, 6 predictors were overlapping. These included sphingomyelin, N–acetylneuraminate (also known as sialic acid), isobutyrylcarnitine, phenylacetate and 3beta–hydroxy–5–cholestenoate. For women, the top predictors for xBRS included several pregnane steroids.

For the linear regression analyses, we focused on the best predictors for men, since the models in women had essentially no explained variance. Of the best predictors for HRV in male participants, five metabolites were associated with HRV in the adjusted regression models (**Supplement 11**). Four metabolites were associated with a higher HRV (N-acetylneuraminate, isobutyrylcarnitine, gentisate, phenylacetate) and one with lower HRV (3beta-hydroxy-5-cholestenoate). In addition, two metabolites were associated with higher xBRS (isobutyrylcarnitine and gentisate) and two with lower xBRS (1,5-anhydroglucitol and 1-oleoyl-GPC). Several metabolites, including N-acetylneuraminate, phenylacetate, gentisate, and 3beta-hydroxy-5-cholestenoate, had an interaction with sex and were only associated with HRV or xBRS in men but not in women, or even showed opposite associations, such as for isobutyrylcarnitine.

Gut microbiota composition is associated with plasma metabolite levels

Since gut microbiota composition is a key determinant of the plasma metabolite profile, we wanted to assess whether the top 10 metabolites for systolic BP (men and women), diastolic BP (women) and HRV (men) models could be explained by gut microbiota composition. Using machine learning prediction models, we found ten metabolites that were associated with gut microbiota composition (**Figure 3**).

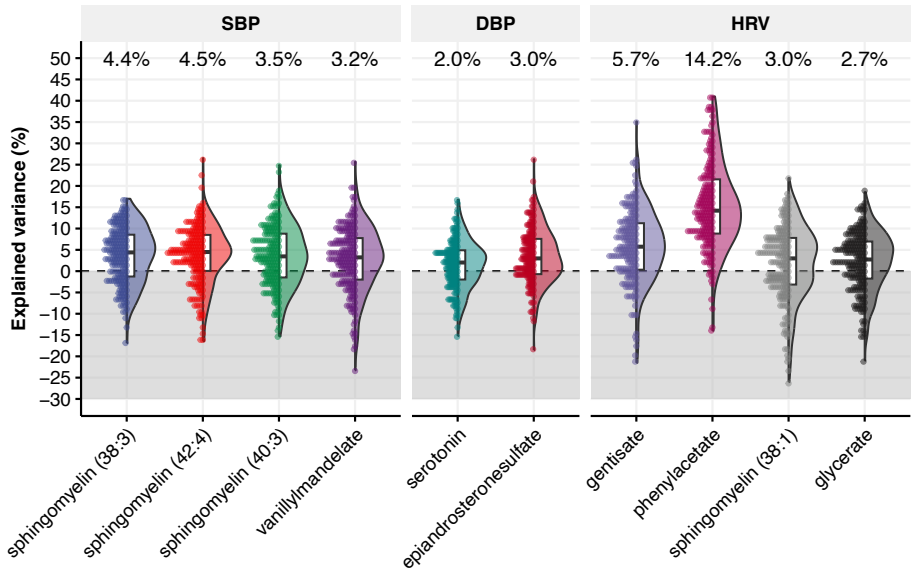
Phenylacetate had the highest explained variance (14.2%) by gut microbiota composition. The top microbial predictors for phenylacetate levels predominantly consisted of microbes from the *Ruminococcaceae* family, including several

ASVs from UCG-002 and UCG-005 genera, and *Intestinimonas* spp, from the *Oscillospiraceae* family in the same order (**Figure 4G**). These were associated with higher phenylacetate levels in an adjusted regression model. In contrast, higher abundance of *Blautia* spp., from the *Lachnospiraceae* family, was associated with lower phenylacetate levels. The associations with *Blautia* spp. and *Ruminococcaceae* spp. had interactions with sex and were stronger in men than women in the stratified models (**Supplement 12**).

Gut microbiota composition explained 5.7% of gentisate variance. The top predictors were associated with higher gentisate levels in the adjusted model, and mostly included microbes from the Clostridiales order, and more specifically from *Ruminococcaceae* (*Ruminococcaceae UCG-005, Faecalibacterium prausnitzii, Subdoligranulum* spp.), *Lachnospiraceae* (*Marvinbryantia* spp., *Roseburia* spp.), and *Christensenellaceae* (*Christensenellaceae R-7 group* spp.) families (**Figure 4H**).

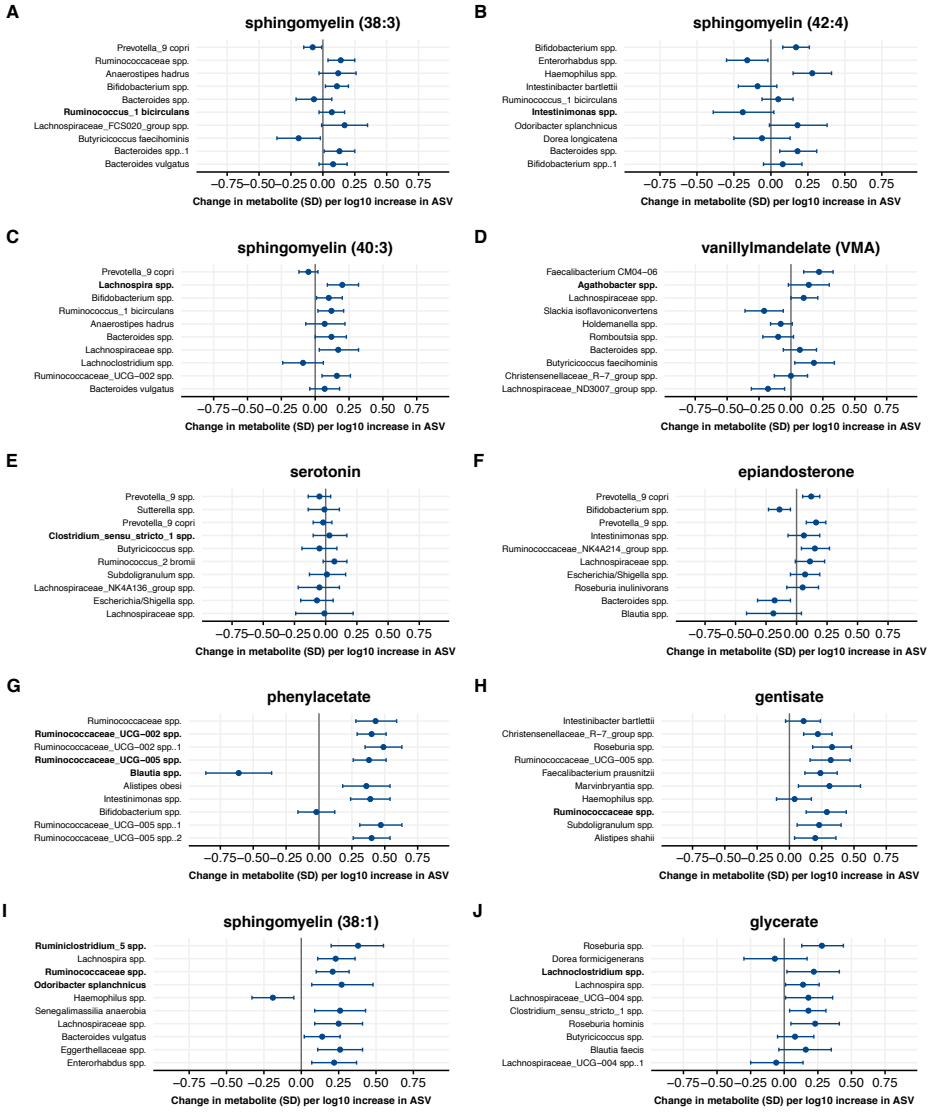
Four sphingomyelins could be explained by gut microbiota composition by 3.5-4.4%. Microbes from the *Ruminococcaceae* family were associated with higher sphingomyelin levels across the different sphingomyelins (**Figure 4A,B,C,I**). Vanillylmandelate (**Figure 4D**), serotonin (**Figure 4E**), epiandosterone (**Figure 4F**) and glycerate (**Figure 4J**) had explained variances ranging from 2.0 to 3.2%.

Figure 3: Explained variance of plasma metabolites by microbiota composition



Explained variance in % per iteration is shown. Each metabolite is annotated with the median explained variance of the machine learning model (XGBoost algorithm).

Figure 4: Linear regression models microbes and metabolites



Linear regression models for associations between plasma metabolites and best predicting microbes: estimates per log10 increase in abundance of microbe with 95% confidence intervals, adjusted for age, sex, body mass index (BMI), renal function, diabetes and albuminuria. Bold font indicates that metabolites had a significant interaction ($p<0.05$) with sex in the adjusted model. The estimates show how metabolite concentration changes (in standard deviations) for a log10 increase in the amplicon sequence variant (ASV) counts; e.g. in Figure 4G a 10-fold (log10) increase in Blautia spp. counts is associated with a 0.61 SD decrease in phenylacetate.

Discussion

We found that plasma metabolite profiles are associated with blood pressure and autonomic cardiovascular control in a sex-specific manner. Sphingomyelins and conjugated bile acids were more predictive of BP in men, while metabolites from acylcarnitine and catecholamine pathways were better predictors in women. In addition, we could predict HRV from metabolite profiles in men, but not in women, further underscoring potential sex differences in BP physiology. Several of the best predicting metabolites were associated with gut microbiota composition. This could indicate that inventions targeted at the gut microbiota might have sex-specific effects, since plasma metabolites derived from the microbiota are differently associated with blood pressure for men and women.

Acylcarnitines were among the top predictors for systolic BP in women, including the metabolite with the highest feature importance, dihomo-linolenoylcarnitine. Acylcarnitines are byproducts of incomplete beta-oxidation, which takes place in the mitochondria to generate acetyl-CoA from fatty acids. Accumulation of acylcarnitines could lead to inflammation through induction of nuclear factor kappa beta activity and phosphorylation of *JNK* and *ERK*.[41,42] Other metabolomics studies also found associations between acylcarnitines and BP, including one cohort with only female subjects, but these studies did not investigate sex differences.[22]

Interestingly, there were several metabolites belonging to the tyrosine pathway among the top predictors. The tyrosine pathway results in the synthesis of catecholamines, including dihydroxyphenylalanine (DOPA), dopamine, norepinephrine and epinephrine. The second-best predictor for systolic BP in women was 4-hydroxyphenylacetateglutamine, a glutamine conjugate of 4-hydroxyphenylacetate, which is a derivative of tyrosine, an essential component of dopamine, norepinephrine and epinephrine. Vanillactate, the third ranked predictor, is a degradation product of DOPA. The high ranking of 4-hydroxyphenylacetylglutamine and vanillactate in the prediction of systolic BP in women could indicate that different catecholamine profiles have sex-specific effects. This is in line with a study on sex differences in sympathetic activation in reaction to norephinephrine, that showed that there are sex differences in physiological mechanisms that regulate sympathetic cardiovascular control.[7]

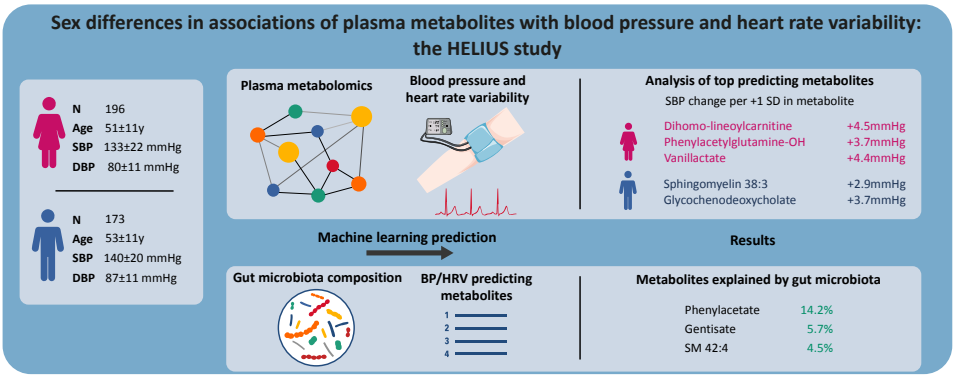
Not all machine learning models in our analyses performed well. The prediction model for diastolic BP in men had essentially no explained variance, which could have been caused by the changing relation between plasma metabolites and BP with age, considering the improved model performance after age stratifica-

tion. In contrast to systolic BP, diastolic BP shows an inverted U-shape trend with increasing age,[43] and this nonlinear relation with age might have complicated the diastolic BP prediction from the metabolite profiles. The xBRS models had lower model metrics than the HRV models, possibly due to more noise in the xBRS data than in the HRV data, since xBRS is calculated using a combination of parameters (heart rate, blood pressure). Both the xBRS and HRV models had lower explained variance in women compared to men, which is in line with studies that showed that there are sex differences in the sympathetic neuro-humoral balance. For instance, while there is a close association between sympathetic activity and vascular resistance in men, this association is absent in premenopausal women.[7,44,45]

The HRV model in male subjects showed that N-acetylneuraminate, the most abundant sialic acid in human cells, was the highest ranked predictor for HRV. Sialylation (e.g. binding of sialic acid) of IgG can convert IgG from a pro-inflammatory to an anti-inflammatory state.[46] In mice, supplementation with a sialic acid precursor could prevent obese mice from developing hypertension, and in humans, sialylated IgG was inversely associated with systolic BP.[47] Another metabolite associated with higher HRV was phenylacetate. Gut microbiota can produce phenylacetate from phenylalanine, and phenylacetate can be conjugated into phenylacetylglutamine by the liver.[48] In our analyses, we could predict phenylacetate levels from gut microbiota composition, but not 4-hydroxyphenylacetylglutamine, in contrast to other studies.[49,50] Higher *Blautia* spp. abundance was associated with higher phenylacetate levels and *Ruminococcaceae* spp. abundance was associated with lower phenylacetate levels. Altogether, this indicates that reducing phenylacetate levels through dietary or gut microbiome interventions could be a strategy to increase HRV.

Sphingomyelins (SM 38:3, SM 42:4, SM 40:3 and SM 38:1) were among the best predictors for both systolic BP and HRV in males. These are membrane sphingolipids that can be used in the synthesis of ceramide. Ceramide is central to sphingolipid metabolism, and has been shown to suppress phosphorylation of Akt and eNOS, thereby possibly lowering nitric oxide production and as a result, inducing vasoconstriction.[51] In addition, sphingolipids have been associated with hypertension and coronary artery disease.[52,53] There are established sex differences in sphingolipid levels, as has also been shown in the HELIUS cohort,[54] which could be caused by indirect effects of estrogen on adipose tissue or direct effects on the expression of sphingomyelinase.[55] Gut microbiota including *Bacteroides* spp. have been previously shown to be able to produce sphingolipids and affect host ceramide metabolism in mice.[56] In addition, we found that other prevalent microbes

Graphical abstract



from the *Bifidobacterium* genus, *Ruminococcaceae* family, *Lachnospiraceae families* were associated with sphingomyelin levels.

Glycochenodeoxcyholate-3-sulfate, a glycine-conjugated bile acid, was also associated with systolic BP in men, but not in women. Men have a larger total bile acids pool than women,[57] and accordingly, the levels of this metabolite were higher in male subjects in our cohort. Bile acids could affect BP by affecting water and electrolyte homeostasis.[58] Bile acids could stimulate sodium retention through the epithelial sodium channel in the distal tubules, and water retention through upregulation of aquaporin 2 by the receptor TGR5 and nuclear receptor FXR.[58] Although other studies have found associations between gut microbiota and deoxycholate, another secondary bile acid, we could not predict levels of glycochenodeoxcyholate-3-sulfate from gut microbiota composition.[50]

Our study has several limitations. Our metabolomics data was semi-quantitative and as such does not provide concentrations of the detected metabolites, only relative abundances that enable inter-sample comparisons. Moreover, our analyses were cross-sectional. Therefore, we cannot draw conclusions about causality and cannot assess whether the best predicting metabolites reflect pathological or protective mechanisms. Future studies are needed focusing on specific metabolites to further validate our findings and to determine exact plasma concentrations and underlying mechanisms. For this study, we did not include menopausal status, and therefore, we could not compare the differences between premenopausal and postmenopausal women, whereas hormonal changes over lifetime are likely to affect metabolomic profiles. A relatively high number of participants in our study sample had mild albuminuria, which might have affected the metabolomic

profiles, despite the preserved glomerular filtration rate of the included participants. However, we adjusted for presence of albuminuria in our linear regression analyses. Since diabetes diagnoses could have influenced our results, we ran a separate sensitivity analysis without subjects with diabetes, that yielded comparable results. Strengths of this study include the ethnic diversity of the cohort and the extensive phenotyping of this population including clinical data, metabolomics and gut microbiome data. Although plasma metabolites have been associated with BP in other studies, we are the first to report that significant sex differences exist in the association with BP using untargeted metabolomics and a machine learning approach.

While hypertension guidelines do not distinguish between men and women, there is accumulating evidence showing that there are sex differences in inflammatory patterns underlying hypertension and physiology of blood pressure regulation. Our analyses underline that plasma metabolites could have sex-dependent effects on blood pressure and are often only associated with blood pressure in either men or women. Future studies on the role of plasma metabolites in hypertension should therefore consider sex stratification, whether that is in fundamental studies, animal studies or clinical trials.

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Author contributions

B.J.H.B., M.N., D.H.R. and H.G. contributed to the design of the study. C.M.M. was responsible for the handling of plasma samples and D.C. for the processing of Nexfin data. B.J.H.V. performed the statistical analyses. B.J.H.B., M.N., D.H.R., E.R. and M.M. contrib-

uted to the interpretation of the results. B.J.H.V. wrote the first draft of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

Conflict of interest

M.N. is in the scientific board of Caelus Pharmaceuticals, the Netherlands. However, this position is not directly relevant for the current paper. All other authors have no competing interest.

References

1. *Global health risks: mortality and burden of disease attributable to selected major risks.* World Health Organization, 2009.
2. Abramson, B. L. & Melvin, R. G. Cardiovascular Risk in Women: Focus on Hypertension. *Can J Cardiol* 30, 553–559 (2014).
3. Ji, H. *et al.* Sex Differences in Blood Pressure Trajectories Over the Life Course. *JAMA Cardiol* 5, 255–262 (2020).
4. Palatini, P. *et al.* Premenopausal Women Have Increased Risk of Hypertensive Target Organ Damage Compared with Men of Similar Age. *J Womens Health* 20, 1175–1181 (2011).
5. Connelly, P. J., Currie, G. & Delles, C. Sex Differences in the Prevalence, Outcomes and Management of Hypertension. *Curr Hypertens Rep* 24, (2022).
6. Wei, Y. C., George, N. I., Chang, C. W. & Hicks, K. A. Assessing Sex Differences in the Risk of Cardiovascular Disease and Mortality per Increment in Systolic Blood Pressure: A Systematic Review and Meta-Analysis of Follow-Up Studies in the United States. *PLoS ONE* 12, e0170218 (2017).
7. Hart, E. C. *et al.* Sex differences in sympathetic neural-hemodynamic balance implications for human blood pressure regulation. *Hypertension* 53, 571–576 (2009).
8. Spyer, K. M. Neural organisation and control of the baroreceptor reflex. in *Rev Physiol Biochem Pharmacol, Volume 88:* 23–124 (Springer, 1981). doi:10.1007/BFb0034536.
9. La Rovere, M. T., Pinna, G. D. & Raczak, G. Baroreflex sensitivity: measurement and clinical implications. *Ann Noninv Electrocardiol* 13, 191–207 (2008).
10. van Ravenswaaij-Arts, C. M. A., Kollee, L. A. A., Hopman, J. C. W., Stoelinga, G. B. A. & van Geijn, H. P. Heart Rate Variability. *Ann Intern Med* 118, 436–447 (1993).
11. Tzoulaki, I., Iliou, A., Mikros, E. & Elliott, P. An Overview of Metabolic Phenotyping in Blood Pressure Research. *Curr Hypertens Rep* vol. 20 1–8 Preprint at https://doi.org/10.1007/s11906-018-0877-8 (2018).
12. Darst, B. F., Koscik, R. L., Hogan, K. J., Johnson, S. C. & Engelman, C. D. Longitudinal plasma metabolomics of aging and sex. *Aging* 11, 1262–1282 (2019).
13. Li, J. *et al.* The Mediterranean diet, plasma metabolome, and cardiovascular disease risk. *Eur Heart J* 41, 2645–2656 (2020).
14. Wilmanski, T. *et al.* Blood metabolome predicts gut microbiome α-diversity in humans. *Nat Biotechnol* 37, 1217–1228 (2019).
15. Liu, J. *et al.* Integration of epidemiologic, pharmacologic, genetic and gut microbiome data in a drug–metabolite atlas. *Nat Med* 26, 110–117 (2020).

16. Bell, J. A. *et al.* Sex differences in systemic metabolites at four life stages: cohort study with repeated metabolomics. *BMC Medicine* 19, 1–13 (2021).
17. Fromentin, S. *et al.* Microbiome and metabolome features of the cardiometabolic disease spectrum. *Nat Med* 28, 303–314 (2022).
18. Ma, Y., Liu, X. & Wang, J. Small molecules in the big picture of gut microbiome-host cross-talk. *eBioMedicine* vol. 81 Preprint at https://doi.org/10.1016/j.ebiom.2022.104085 (2022).
19. Verhaar, B. J. H. *et al.* Associations between gut microbiota, faecal short-chain fatty acids, and blood pressure across ethnic groups: the HELIUS study. *Eur Heart J* (2020) doi:10.1093/eurheartj/ehaa704.
20. van der Vossen, E. W. J. *et al.* Effects of fecal microbiota transplant on DNA methylation in subjects with metabolic syndrome. *Gut Microbes* 13, 1993513 (2021).
21. Zheng, Y. *et al.* Metabolomics and incident hypertension among blacks: The atherosclerosis risk in communities study. *Hypertension* (2013) doi:10.1161/HYPERTENSIONAHA.113.01166.
22. Menni, C. *et al.* Metabolomic Identification of a Novel Pathway of Blood Pressure Regulation Involving Hexadecanedioate. *Hypertension* (2015) doi:10.1161/HYPERTENSIONAHA.115.05544.
23. Lin, Y.-T. *et al.* Global Plasma Metabolomics to Identify Potential Biomarkers of Blood Pressure Progression. *Arterioscler Thromb Vasc Biol* 40, E227–E237 (2020).
24. Louca, P. *et al.* Cross-Sectional Blood Metabolite Markers of Hypertension: A Multicohort Analysis of 44,306 Individuals from the COnsortium of METabolomics Studies. *Metabolites* 12, 601 (2022).
25. Snijder, M. B. *et al.* Cohort profile: The Healthy Life in an Urban Setting (HELIUS) study in Amsterdam, the Netherlands. *BMJ Open* 7, 1–11 (2017).
26. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Workgroup. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney Int Suppl* 3, 4–4 (2013).
27. The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J* 39, 3021–3104 (2018).
28. Liebisch, G. *et al.* Shorthand notation for lipid structures derived from mass spectrometry. *J Lipid Res* 54, 1523–1530 (2013).
29. MATLAB, R. & Release, B. D. B. The MathWorks, Inc., Natick, Massachusetts. Preprint at (2019).
30. Bakema, M. J. *et al.* Associations Between Child Maltreatment, Autonomic Regulation, and Adverse Cardiovascular Outcome in an Urban Population: The HELIUS Study. *Front Psychiatry* 11, (2020).
31. Task Force of the European Society of Cardiology. Heart rate variability: standards of measurement, physiological interpretation and clinical use. *Circulation* 93, 1043–1065 (1996).
32. Wesseling, K. H. *et al.* Validity and variability of xBRS: instantaneous cardiac baroreflex sensitivity. *Physiol Rep* 5, e13509 (2017).
33. Mobini, R. *et al.* Metabolic effects of Lactobacillus reuteri DSM 17938 in people with type 2 diabetes: A randomized controlled trial. *Diabetes Obes Metab* (2017) doi:10.1111/dom.12861.

34. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl Environ Microbiol* (2013) doi:10.1128/AEM.01043-13.
35. Deschasaux, M. *et al.* Depicting the composition of gut microbiota in a population with varied ethnic origins but shared geography. *Nat Med* 24, 1526-1531 (2018) doi:10.1038/s41591-018-0160-1.
36. Liebal, U. W., Phan, A. N. T., Sudhakar, M., Raman, K. & Blank, L. M. Machine Learning Applications for Mass Spectrometry-Based Metabolomics. *Metabolites* 10, 243 (2020).
37. Wang, X.-W. & Liu, Y.-Y. Comparative study of classifiers for human microbiome data. *Medicine in Microecology* 4, 100013 (2020).
38. Hou, X.-W. *et al.* Machine Learning-Based Integration of Metabolomics Characterisation Predicts Progression of Myopic Retinopathy in Children and Adolescents. *Metabolites* 13, 301 (2023).
39. Chen, T. & Guestrin, C. XGBoost: A scalable tree boosting system. in *Proceedings of the ACM SIGKDD International Conference on Knowledge Discovery and Data Mining* 785–794 (2016). doi:10.1145/2939672.2939785.
40. Pedregosa, F. *et al.* Scikit-learn: Machine learning in Python. *J Mach Learn Res* 12, 2825–2830 (2011).
41. Rutkowsky, J. M. *et al.* Acylcarnitines activate proinflammatory signaling pathways. *American Journal of Physiology - Endocrinology and Metabolism* 306, (2014).
42. Adams, S. H. *et al.* Plasma Acylcarnitine Profiles Suggest Incomplete Long-Chain Fatty Acid β-Oxidation and Altered Tricarboxylic Acid Cycle Activity in Type 2 Diabetic African-American Women. *J Nutr* 139, 1073–1081 (2009).
43. Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants - The Lancet. https://www.thelancet.com/journals/lancet/article/piiS0140-6736(21)01330-1/fulltext.
44. Christou, D. D. *et al.* Women have lower tonic autonomic support of arterial blood pressure and less effective baroreflex buffering than men. *Circulation* 111, 494–498 (2005).
45. Schmitt, J. A. M., Joyner, M. J., Charkoudian, N., Wallin, B. G. & Hart, E. C. Sex differences in α-adrenergic support of blood pressure. *Clin Autonom Res* 20, 271–275 (2010).
46. Liu, Y. *et al.* Sialylation of IgG inhibits the formation of galactose-deficient IgA1-containing immune complexes and protects mesangial cells from injury in IgA nephropathy. *BMC Nephrology* 23, 25 (2022).
47. Peng, J. *et al.* Supplementation with the Sialic Acid Precursor N-Acetyl-D-Mannosamine Breaks the Link between Obesity and Hypertension. *Circulation* 140, 2005–2018 (2019).
48. Nemet, I. *et al.* A Cardiovascular Disease-Linked Gut Microbial Metabolite Acts via Adrenergic Receptors. *Cell* 180, 862-877.e22 (2020).
49. Bar, N. *et al.* A reference map of potential determinants for the human serum metabolome. *Nature* 588, 135–140 (2020).
50. Dekkers, K. F. *et al.* An online atlas of human plasma metabolite signatures of gut microbiome composition. *Nat Commun* 13, 5370 (2022).

51. Cantalupo, A. *et al.* Endothelial Sphingolipid De Novo Synthesis Controls Blood Pressure by Regulating Signal Transduction and NO via Ceramide. *Hypertension* 75, 1279–1288 (2020).
52. Poss, A. M. *et al.* Machine learning reveals serum sphingolipids as cholesterol-independent biomarkers of coronary artery disease. *J Clin Invest* 130, 1363–1376 (2020).
53. Spijkers, L. J. A. *et al.* Hypertension Is Associated with Marked Alterations in Sphingolipid Biology: A Potential Role for Ceramide. *PLoS ONE* 6, e21817 (2011).
54. Muilwijk, M., Callender, N., Goorden, S., Vaz, F. M. & van Valkengoed, I. G. M. Sex differences in the association of sphingolipids with age in Dutch and South-Asian Surinamese living in Amsterdam, the Netherlands. *Biol Sex Differ* 12, 1–14 (2021).
55. Ishii, T. & Warabi, E. Mechanism of Rapid Nuclear Factor-E2-Related Factor 2 (Nrf2) Activation via Membrane-Associated Estrogen Receptors: Roles of NADPH Oxidase 1, Neutral Sphingomyelinase 2 and Epidermal Growth Factor Receptor (EGFR). *Antioxidants* 8, 69 (2019).
56. Johnson, E. L. *et al.* Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. *Nat Commun 2020 11:1* 11, 1–11 (2020).
57. Bennion, L. J. *et al.* Sex differences in the size of bile acid pools. *Metabolism* 27, 961–969 (1978).
58. Ishimwe, J. A., Dola, T., Ertuglu, L. A. & Kirabo, A. Bile acids and salt-sensitive hypertension: a role of the gut-liver axis. *Am J Physiol Heart Circ Physiol* 322, H636–H646 (2022).

Supplements

Supplement 1: Untargeted metabolomics methods

Methods as provided by Metabolon, Morrisville, NC, USA

Sample shipment and processing

All samples were kept at -80°C until processed. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company). Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a Zymark TurboVap® evaporator to remove the solvent. The sample extracts were stored in nitrogen overnight before preparation for analysis.

QA/QC

Several types of controls were analyzed together with the study plasma samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. **Table 1** and **Table 2** describe these QC samples and standards.

Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections, as outlined in **Figure S1**.

Table 1: Description of Metabolon QC Samples

Type	Description	Purpose
MTRX	Large pool of human plasma maintained by Metabolon that has been characterized extensively.	Assure that all aspects of the Metabolon process are operating within specifications.
CMTRX	Pool created by taking a small aliquot from every customer sample.	Assess the effect of a non-plasma matrix on the Metabolon process and distinguish biological variability from process variability.
PRCS	Aliquot of ultra-pure water	Process Blank used to assess the contribution to compound signals from the process.
SOLV	Aliquot of solvents used in extraction.	Solvent Blank used to segregate contamination sources in the extraction.

Table 2: Metabolon QC Standards

Type	Description	Purpose
RS	Recovery Standard	Assess variability and verify performance of extraction and instrumentation.
IS	Internal Standard	Assess variability and performance of instrument.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

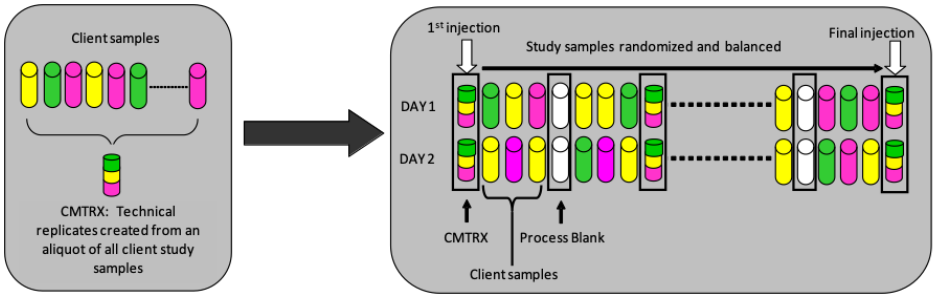
All UPLC-MS/MS methods used a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the

extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Bioinformatics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

Figure S1: Preparation of client-specific technical replicates



A small aliquot of each client sample (colored cylinders) is pooled to create a CMTRX technical replicate sample (multi-colored cylinder), which is then injected periodically throughout the platform run. Variability among consistently detected biochemicals can be used to calculate an estimate of overall process and platform variability.

Data Extraction and Compound Identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ±10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

Curation

A variety of curation procedures were carried out to ensure that a high-quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary. Peaks were quantified using area-under-the-curve.

Metabolite Quantification and Data Normalization

Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (termed the "block correction").

Supplement 2: Infofile metabolites

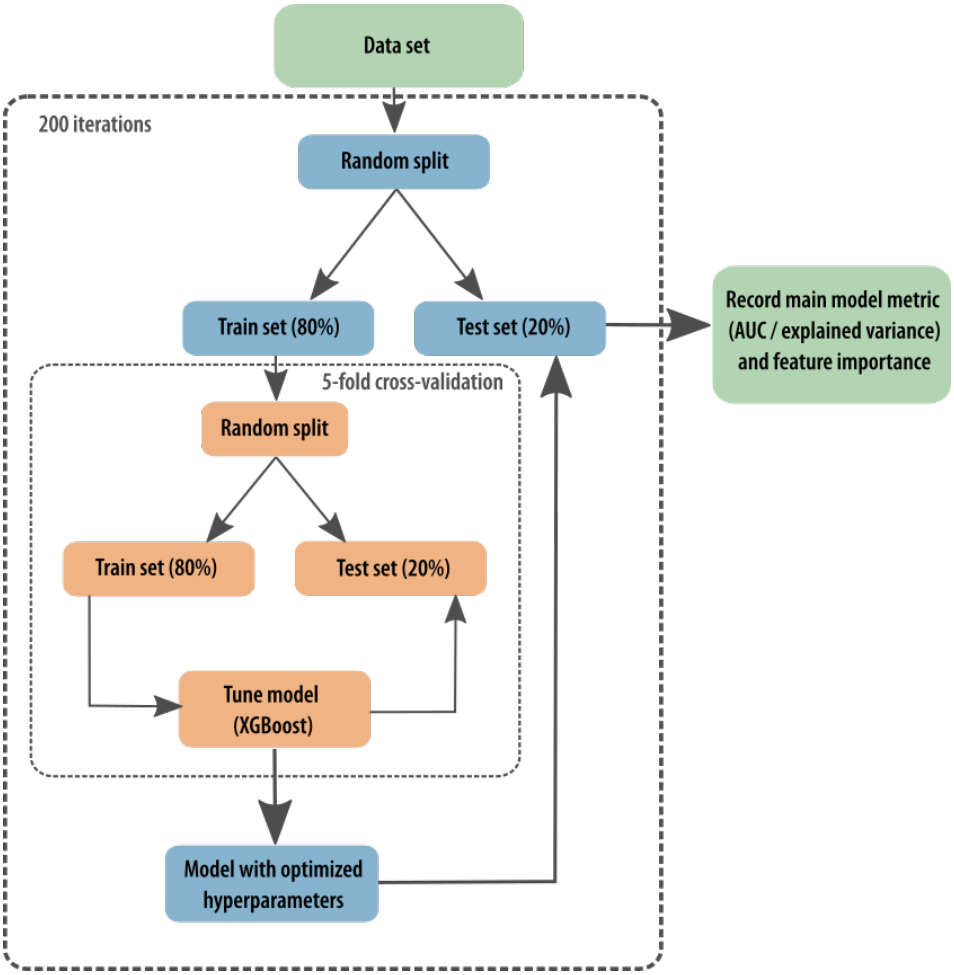
This excel file containing a list of metabolites is too large to be printed. The table can be found digitally, through the online publication of the paper: https://doi.org/10.1016/j.atherosclerosis.2023.05.016.

Supplement 3: Preprocessing of raw sequencing data

Raw sequencing reads were processed using USEARCH (v11.0.667).[1] Paired-end reads were merged allowing a maximum of 30 differences in the overlapping region and a maximum of 1 expected error in the merged contig. Expected error-based read quality filtering was performed as described in Edgar et al.[2] Remaining contigs were dereplicated and unique sequences were denoised using the UNOISE3 algorithm to infer Amplicon Sequence Variants (ASVs).[2] All merged reads were subsequently mapped against the resulting ASVs to produce a count table. ASVs not matching expected amplicon length were removed (i.e. ASV sequences longer than 260 bp or shorter than 250 bp). Taxonomy was assigned with the 'assignTaxonomy' function from the 'DADA2' R package (v 1.12.1) using the SILVA (v. 132) reference database.[3,4] ASVs sequences were then aligned using MAFFT (v.7.427) using the auto settings.[5] A phylogenetic tree was constructed from the resulting multiple sequence alignment with FastTree (v.2.1.11 Double Precision) using a generalized time-reversible model.[6] The ASV table, taxonomy, and tree were integrated using the 'phyloseq' R package (v.1.28.0). The ASV table was rarefied to 14932 counts per sample.[7] Of 6056 sequenced samples, 24 had insufficient counts (<5000 counts per sample) and were excluded at the rarefying stage.

1. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* (2010) doi:10.1093/bioinformatics/btq461
2. Edgar RC. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv* (2016). doi:10.1101/081257
3. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* (2016) doi:10.1038/nmeth.3869
4. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res* (2013) doi:10.1093/nar/gks1219
5. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* (2013) doi:10.1093/molbev/mst010
6. Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS ONE* (2010) doi:10.1371/journal.pone.0009490
7. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* (2013) doi:10.1371/journal.pone.0061217

Supplement 4: Machine learning design

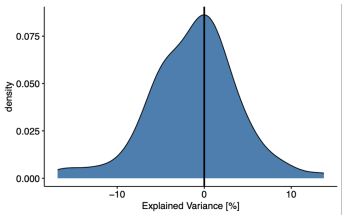


Supplement 5: Permuted models

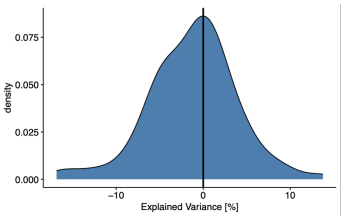
The results of the permuted machine learning models are presented below. These have an identical design as the models that used actual data, except that the data was permuted prior to each iteration. These are expected to have a mean explained variance around 0%, that is, if they do not overfit.

Systolic blood pressure

Women

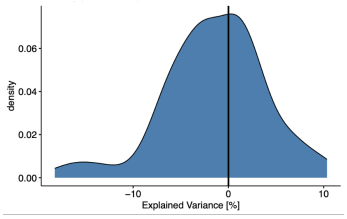


Men

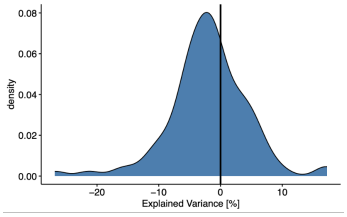


Diastolic blood pressure

Women

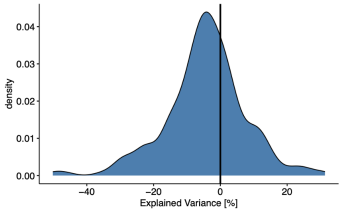


Men

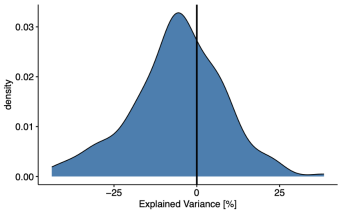


Heart rate varability (SDNN)

Women

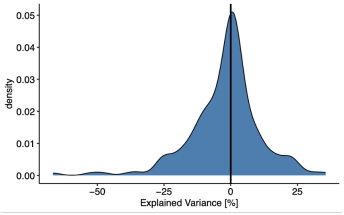


Men

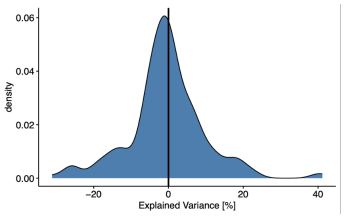


Baroreceptor sensitivity (xBRS)

Women



Men

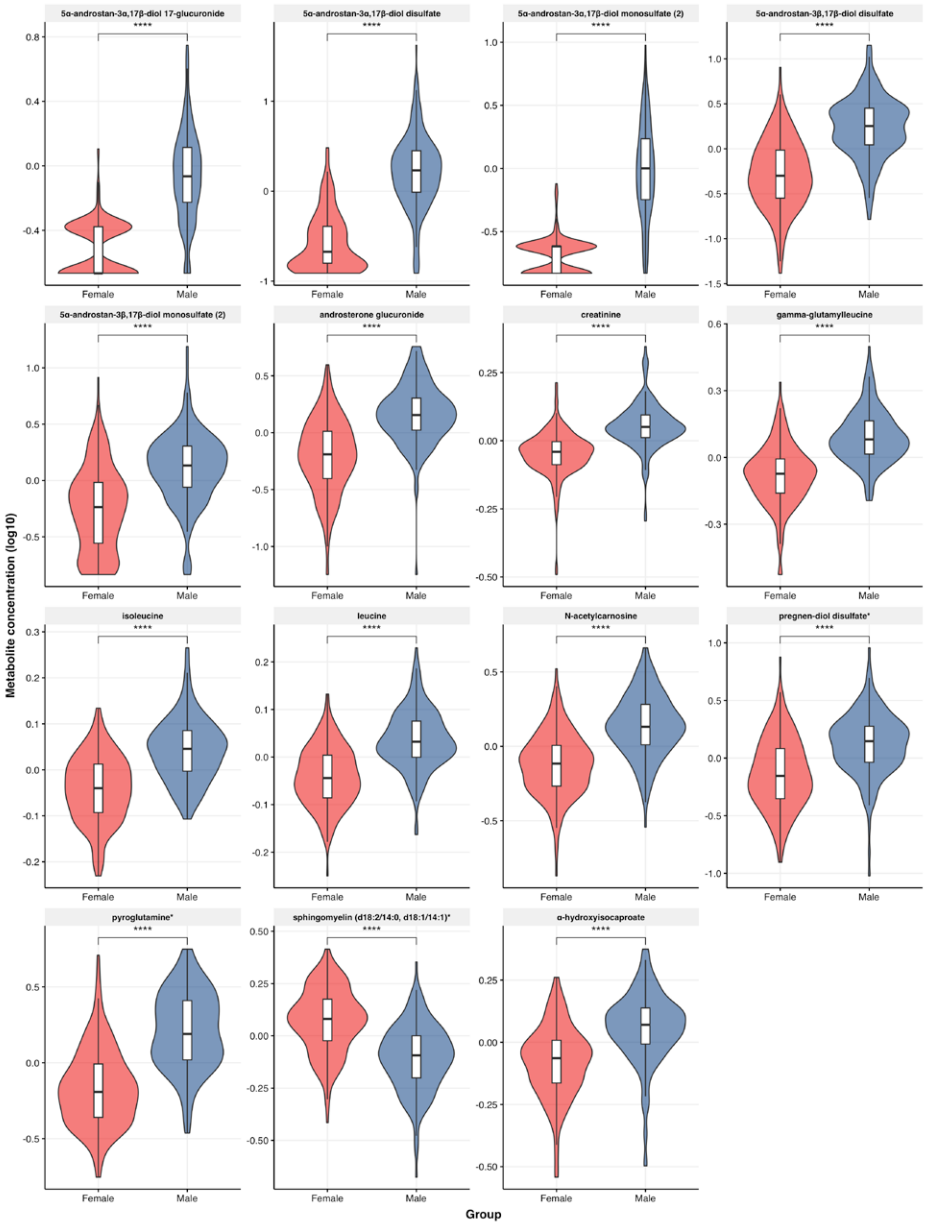


Supplement 6: Population characteristics of subset with available xBRS and HRV data

	Overall	Women	Men	p
N	139	64	75	
Age, years	52.46±10.34	50.77 ±11.04	53.91±9.54	0.074
Age ≤50 years	58 (41.7)	35 (54.7)	23 (30.7)	0.007
Ethnicity				0.007
Dutch	25 (18.0)	7 (10.9)	18 (24.0)	
South-Asian Surinamese	39 (28.1)	13 (20.3)	26 (34.7)	
African Surinamese	40 (28.8)	26 (40.6)	14 (18.7)	
Ghanaian	35 (25.2)	18 (28.1)	17 (22.7)	
BMI, kg/m²	28.08±4.94	29.06±6.06	27.23±3.56	0.029
Current smoking	26 (18.7)	8 (12.5)	18 (24.0)	0.130
Diabetes	17 (12.2)	5 (7.8)	12 (16.0)	0.227
Hypertension	85 (61.2)	33 (51.6)	52 (69.3)	0.049
Antihypertensive medication	43 (30.9)	16 (25.0)	27 (36.0)	0.225
Systolic BP, mmHg	137.26±20.95	132.99±20.67	140.89±20.64	0.026
Diastolic BP, mmHg	83.26±11.37	79.24±10.44	86.69±11.06	<0.001
xBRS, ms/mmHg	11.80±6.57	13.55±7.53	10.30±5.23	0.003
HRV, SDNN	0.05±0.02	0.05±0.02	0.05±0.02	0.358
eGFR, ml/ min/1.73m²	93.81±19.90	99.37±18.82	89.06±19.68	0.002
Albuminuria (stage A2 KDIGO)	69 (49.6)	31 (48.4)	38 (50.7)	0.927

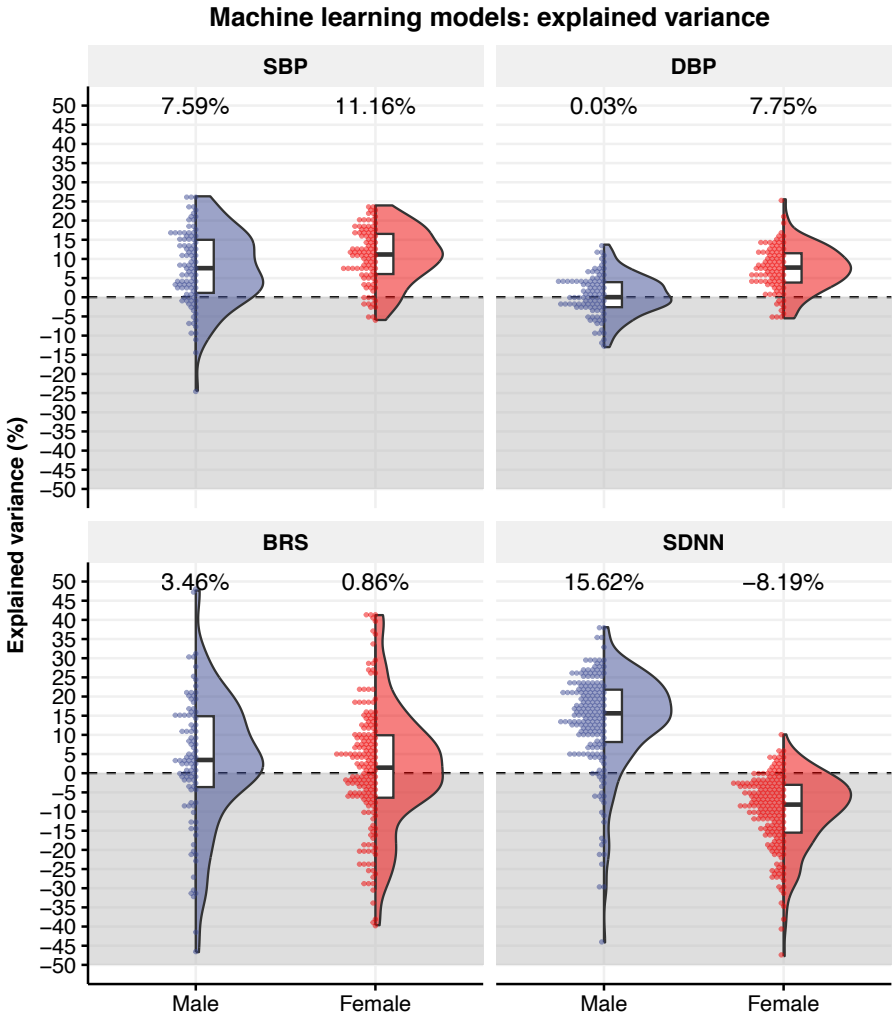
Data is presented as mean±SD or n (%). BMI = body mass index, BP = blood pressure, eGFR = estimated glomerular filtration rate (CKD-EPI), HRV = heart rate variability, xBRS = cross-correlation baroreceptor sensitivity.

Supplement 7: Differences between men and women in highest ranked metabolites in the prediction for sex



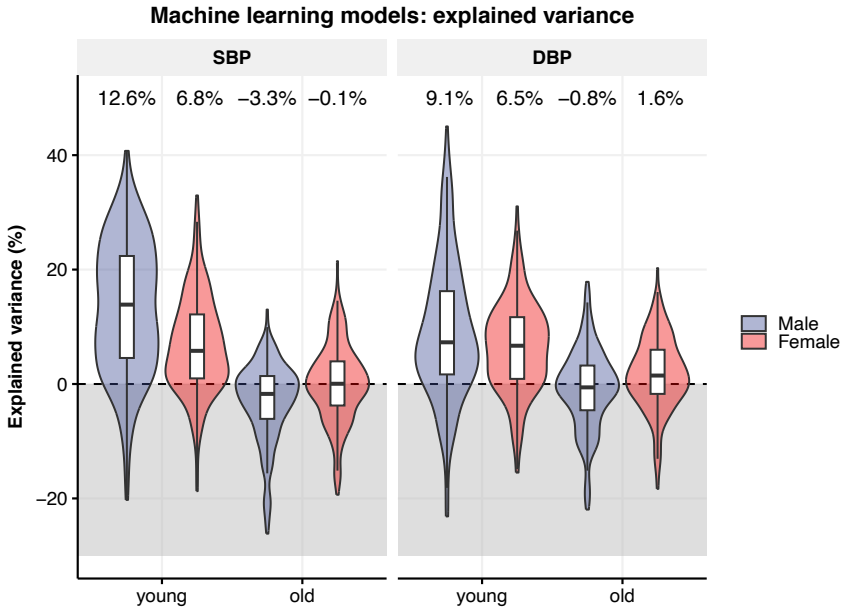
*Differences in metabolite concentrations (log10 scale) between men and women. Metabolite levels were log10-transformed since some of these metabolites had a nonnormal distribution. All differences between men and women were very significant, **** = p-value<1*10^{-15} using Mann-Whitney U tests.*

Supplement 8: Explained variance of hemodynamic parameters by metabolites



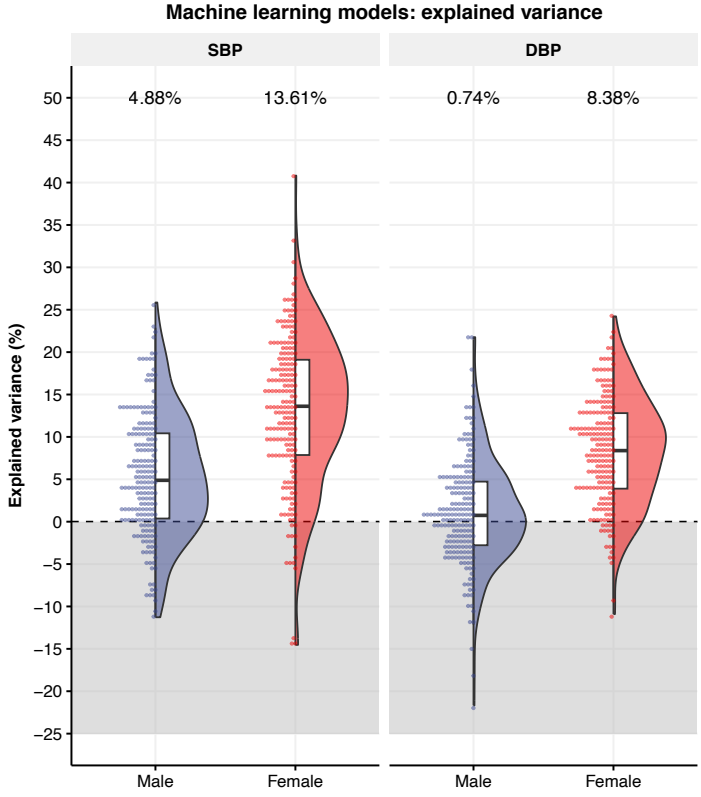
Explained variance in % per iteration is shown, with a total of 200 iterations per model. Each model is annotated with the median explained variance of the machine learning model (XGBoost algorithm).

Supplement 9: Machine learning models for old and young subjects, in men and women



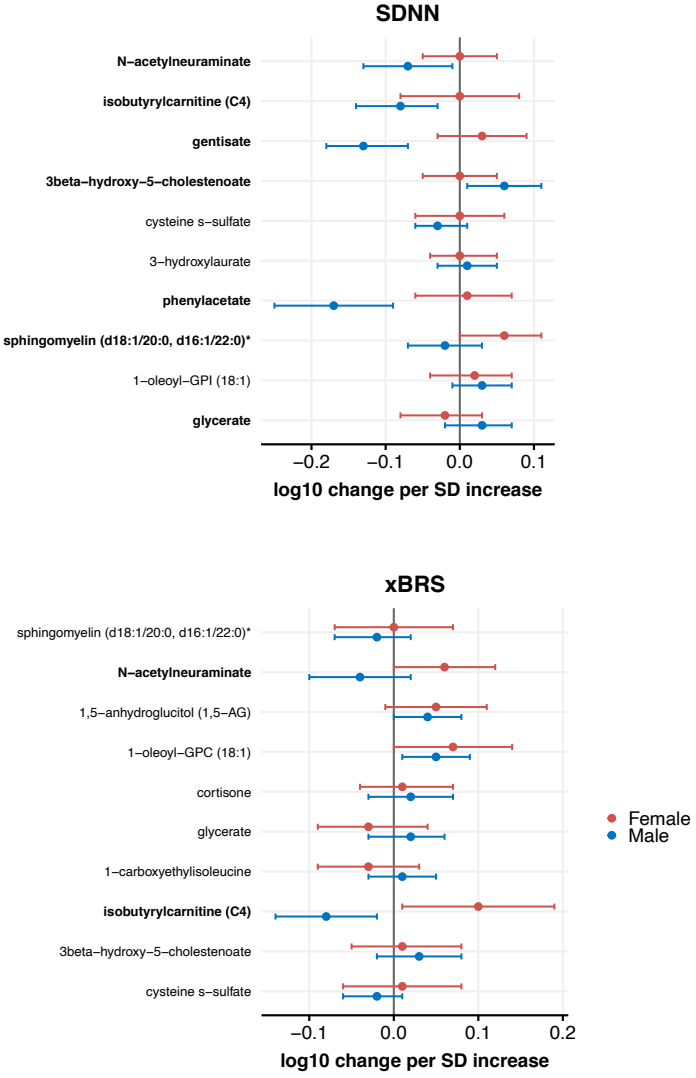
Explained variance in % per iteration is shown, with a total of 200 iterations per model. Each model is annotated with the median explained variance of the machine learning model (XGBoost algorithm).

Supplement 10: Sensitivity analysis in subjects without diabetes



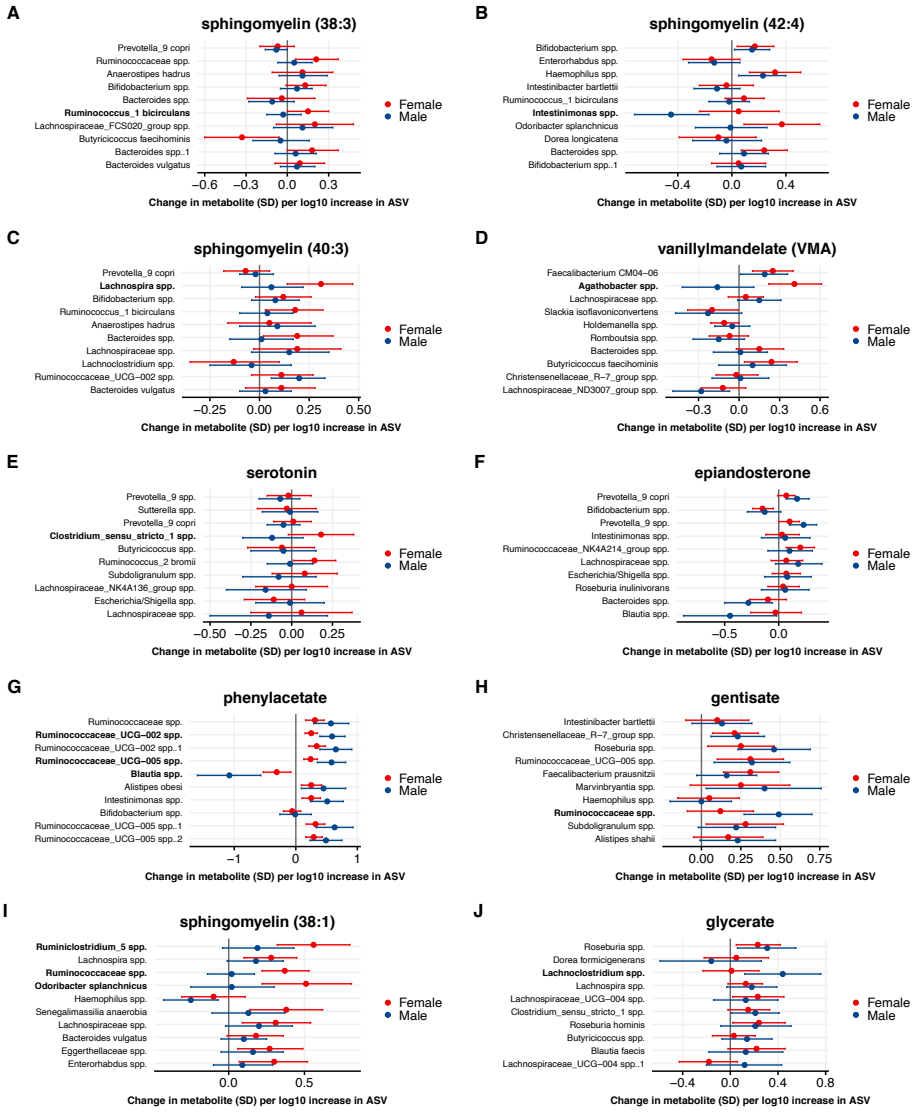
Explained variance in % per iteration is shown, with a total of 200 iterations per model. Each model is annotated with the median explained variance of the machine learning model (XGBoost algorithm).

Supplement 11: Linear regression models HRV (SDNN) and xBRS

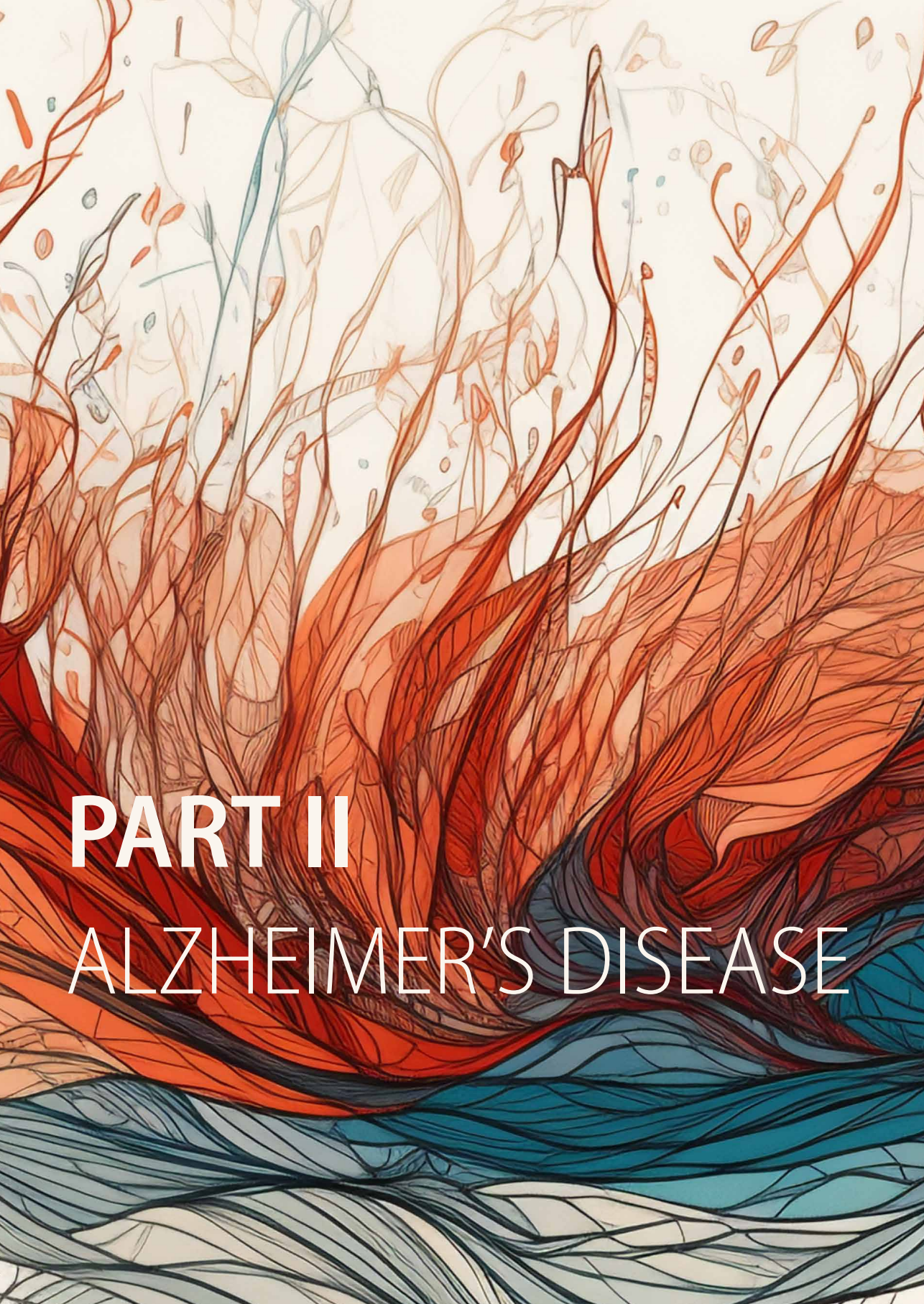


Linear regression models for SDNN and xBRS: log change in outcome per SD increase in metabolite levels with 95% confidence intervals, adjusted for age, body mass index (BMI), renal function, diabetes and albuminuria. Left plot shows the best predictors for SDNN (as measure of heart rate variability) in men; right plot shows the best predicting metabolites for xBRS in men. Bold font indicates that metabolites had a significant interaction ($p<0.1$) with sex in the adjusted model. SDNN and xBRS were log10-transformed for this analysis.

Supplement 12: Associations between microbiota and metabolite levels stratified by sex



Linear regression models for associations between plasma metabolites and best predicting microbes stratified for sex: estimates per log10 increase in abundance of microbe with 95% confidence intervals, adjusted for age, body mass index (BMI), renal function, diabetes, albuminuria and smoking. Bold font indicates that metabolites had a significant interaction ($p<0.05$) with sex in the unstratified model.



PART II
ALZHEIMER'S DISEASE

7

Nutritional status and structural brain changes in Alzheimer's disease: the NUDAD project

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Abstract

Introduction: Weight loss is associated with higher mortality and progression of cognitive decline, but its associations with MRI changes related to AD are unknown.

Methods: We included 412 patients from the NUDAD project, comprising 129 with AD dementia, 107 with mild cognitive impairment (MCI) and 176 controls. Associations between nutritional status and MRI measures were analyzed using linear regression, adjusted for age, sex, education, cognitive functioning, and cardiovascular risk factors.

Results: Lower body mass index, fat mass and fat free mass index were associated with higher medial temporal atrophy (MTA) scores. Lower body mass index, fat mass and waist circumference were associated with more microbleeds. Stratification by diagnosis showed that the observed associations with microbleeds were only significant in MCI.

Discussion: Lower indicators of nutritional status were associated with more MTA and microbleeds, with largest effect sizes in MCI.

Background

Changing nutritional status, including weight loss, is already prevalent in early (pre-dementia) stages of Alzheimer's disease (AD).[1,2] A suboptimal nutritional status has been associated with higher mortality and progression of cognitive decline.[3-6] However, it is not clear what the relation is between nutritional status and the neurodegenerative process implicated in AD.

On MRI, AD is characterized by cerebral atrophy, including global cortical atrophy (GCA) and medial temporal atrophy (MTA).[7] Particularly MTA is an early marker for AD pathology.[7] Cerebrovascular damage in AD is characterized by white matter hyperintensities (WMH) and microbleeds on MRI.[8] The observation of the latter has been linked to underlying cerebral amyloid angiopathy and AD patients with microbleeds have been shown to have more abnormal concentrations of amyloid-beta in their cerebrospinal fluid.[8,9]

In clinical populations of AD and mild cognitive impairment (MCI), studies have shown conflicting results regarding the association between body mass index (BMI) and cerebral atrophy.[10-14] These conflicting findings could be due to differences in study populations, since in some populations cardiovascular risk factors were more prevalent than in others. Alternatively, they could be the consequence of the complex relationship between nutritional status and atrophy, as most of these former studies only evaluated BMI, but nutritional status refers to a broader concept, including parameters such as body composition (i.e. fat mass (FM), fat free mass index (FFMI)) and malnutrition as assessed using mini nutritional assessment (MNA). In this study, we aimed to investigate associations between BMI, FM, FFMI, waist circumference and MNA, as indicators of nutritional status and structural brain changes, including measures of brain atrophy and cerebrovascular pathology, in a memory clinic population with AD dementia, MCI and controls.

Research in context

1. **Systematic review:** A PubMed search yielded several articles on the relation between body mass index (BMI) and cerebral atrophy.[10-14] These articles showed conflicting results, and only a few of these studies took more in-depth parameters of nutritional status into account.
2. **Interpretation:** In our cohort, lower nutritional parameters were associated with more medial temporal atrophy and microbleeds, with largest effect sizes in patients with mild cognitive impairment. Our results extend previous reports by simultaneously evaluating multiple nutritional status parameters in relation to different MRI measures of neurodegenerative and vascular pathology in a clinical AD sample covering the entire cognitive spectrum from cognitively normal to dementia.
3. **Future directions:** Our results indicate that worse nutritional status might have a role in the development of AD, either as early consequence of underlying pathology or as an aggravating factor. This should be further studied in intervention studies that focus on optimizing nutritional status in AD.

Methods

Study population

NUDAD (Nutrition, the Unrecognized Determinant in Alzheimer's Disease) is a prospective cohort study that aims to investigate nutritional determinants in AD dementia and pre-dementia stages, with a clinical follow-up of three years.[15] The NUDAD cohort is nested within the Amsterdam Dementia Cohort and consists of patients that visited the Alzheimer Center of the Amsterdam UMC between September 2015 and August 2017, were diagnosed with AD dementia, MCI or subjective cognitive decline (SCD) and had a mini-mental state examination (MMSE) score >16.[16] Here, we present cross-sectional baseline data of the 412 NUDAD participants with available MRI scans, including 129 patients with AD, 107 patients with MCI and 176 individuals with SCD, who served as controls. Patients underwent standardized dementia screening, including extensive neuropsychological assessment, neurological examination, MRI, lumbar puncture and laboratory tests.[17] MCI and AD diagnoses were established by consensus in a multidisciplinary meeting according to the National Institute on Aging-Alzheimer's Association criteria.[18,19] As controls, we used subjects with SCD who presented with memory complaints but performed normal on all clinical and cognitive exa-

minations, i.e. did not fulfill criteria for MCI, dementia or psychiatric diagnoses.[17] Informed consent was obtained from all participants and the protocol was approved by the Ethics Committee of the Amsterdam UMC.

Descriptive characteristics included age, sex, educational levels according to the Verhage score (low: 1-3, medium: 4-5, high: 6-7),[20] living situation (independent alone, independent together or institutionalized), medical history (history of diabetes mellitus, hypertension, hypercholesterolemia, myocardial infarction or peripheral artery disease – either self-reported or as described in referral letter), smoking status (current, former, never) and alcohol use (in number of consumptions per day). In addition, global cognitive functioning was assessed using the MMSE (scale 0-30).[21] Cardiovascular risk was defined as a cumulative score that increased with one point for the presence of one of the following variables: a self-reported history of diabetes mellitus, hypertension, hypercholesterolemia, or self-reported medication use for either of these conditions, self-reported history of peripheral artery disease or myocardial infarction, or self-reported positive smoking status (current or former smoking).

Indicators of nutritional status

From measured body height and body weight, body mass index (BMI, kg/m^2) was calculated for all patients. Waist circumference, available in 400 patients, was measured in standing position with a measuring tape at the smallest part between the lowest rib and hip. After multifrequency bio-electrical impedance analysis (50 kHz, Bodystat Quadscan 4000), FFM (kg) was calculated using the Kyle formula, and FM (kg) was calculated by subtracting FFM from total body weight.[22] Subsequently, FFM was divided by squared body height to calculate FFM index (FFMI, kg/m^2). Data on FM and FFMI were available for 346 patients. Nutritional status was evaluated in 267 patients using the validated MNA that has a maximum score of 30 points with higher scores indicating a better nutritional status.[23,24] If necessary, study partners assisted patients in completing this questionnaire. Patients scoring lower than 23.5 points are generally regarded as being at risk of malnutrition and lower than 17 points as malnourished. For the analyses, a modified MNA score was used with a maximum score of 28, in which the question on neuropsychological functioning was omitted to avoid that putative group differences in MNA were driven by diagnosis.[25]

MRI visual scores

MRI scans were performed on a 3.0T scanner. The MRI protocol included T1-weighted, T2-weighted, fluid-attenuated inversion recovery (FLAIR) and gradient echo T2*-weighted images. A trained neuroradiologist evaluated all scans using visual rating scales. MTA was rated on coronal reconstructions of T1-weighted images on a 5-point rating scale (scores 0-4) that has been previously described by Scheltens et al.[26] MTA was rated on both sides, perpendicular to the long axis of the hippocampus. For the analyses, for each patient an average MTA score was calculated from left and right scores. GCA was quantified on transverse FLAIR images using a 4-point rating scale (scores 0-3) that has been previously described by Pasquier et al.[27] WMHs were assessed on the same sequences using the 4-point Fazekas scale (scores 0-3).[28] Microbleeds were defined as small (up to 10 mm) round hypointense lesions on T2*-weighted MRI.[29] Microbleeds counts were categorized as follows: no microbleeds, 1 microbleed, 2-4 microbleeds and ≥5 microbleeds.

Amyloid status

Amyloid status determined by either positive emission tomography (PET) or cerebrospinal fluid (CSF) was available for 356 patients (PET n=198, CSF n=158). Amyloid PET-scans were made after injection of a tracer dose of either approximately 250 MBq ± 20% [^{18F}]florbetaben (Neuraceq) or approximately 370 MBq

Table 1: Population characteristics

Characteristics	Categories	N	Total N=412	Controls N=176	MCI N=107	AD dementia N=129	p-value
General							
Sex	Female	412	188 (54.4)	95 (54.0)	67 (62.6)	62 (48.1)	0.082
Age		412	64.6 ± 8.3	60.8 ± 7.6	66.9 ± 7.5‡	68.0 ± 7.8‡	<0.001
Education level	Low	412	27 (6.6)	9 (5.1)	9 (8.4)	9 (7.0)	0.012
	Medium		174 (42.2)	59 (33.5)	52 (48.6)	63 (48.8)	
	High		211 (51.2)	108 (61.4)	46 (43.0)	57 (44.2)	
Living situation	Independent, with partner	412	310 (75.2)	129 (72.9)	85 (79.4)	96 (74.4)	0.514
	Independent, alone		100 (24.3)	47 (27.1)	21 (19.6)	32 (24.8)	
	Nursing home		2 (0.5)	0 (0.0)	1 (0.9)	1 (0.8)	

Characteristics	Categories	N	Total N=412	Controls N=176	MCI N=107	AD dementia N=129	p-value
MMSE		412	27 [24-29]	29 [27-29]	27 [25-28]‡	24 [21-26]‡§	<0.001†
Amyloid status	Positive	356	187 (52.5)	34 (23.4)	50 (51.0)	103 (91.2)	<0.001
Cardiovascular risk factors							
Smoking status	Smoker	412	55 (13.3)	22 (12.5)	16 (15.0)	17 (13.2)	0.938
	Former smoker		154 (37.4)	65 (36.9)	42 (39.3)	47 (36.4)	
	Never		203 (49.3)	89 (50.6)	49 (45.8)	65 (50.4)	
Alcohol use per day		412	1.0 ± 1.3	1.0 ± 1.3	1.1 ± 1.3	0.9 ± 1.2	0.553†
Hypertension		412	103 (25.0)	40 (22.7)	31 (29.0)	32 (24.8)	0.500
Hypercholesterolemia		412	52 (12.6)	17 (9.7)	14 (13.1)	21 (16.3)	0.225
Diabetes mellitus		412	38 (9.2)	13 (7.4)	17 (15.9)	8 (6.2)	0.020
Myocardial infarction		412	12 (2.9)	3 (1.7)	5 (4.7)	4 (3.1)	0.350
Peripheral artery disease		412	2 (0.5)	2 (1.1)	0 (0.0)	0 (0.0)	0.260
Indicators of nutritional status							
BMI		412	25.8 ± 4.1	26.6 ± 4.7	25.3 ± 3.5‡	25.0 ± 3.7‡	0.001
Waist circumference		400	91.3 ± 12.5	92.6 ± 13.5	91.7 ± 11.7	89.1 ± 11.5‡	0.054
Fat mass		346	25.8 ± 8.2	27.0 ± 8.3	25.3 ± 7.8	24.3 ± 8.1‡	0.026
Fat free mass		346	52.9 ± 10.5	53.6 ± 11.5	53.4 ± 9.5	51.5 ± 9.8	0.256
Fat free mass index		346	17.3 ± 2.4	17.5 ± 2.6	17.2 ± 2.2	17.1 ± 2.1	0.282
MNA-modified score		267	25.0 [23.5-25.5]	25.0 [23.5-25.5]	25.0 [23.0-26.0]	25.0 [23.0-26.0]	0.052†
MRI markers							
MTA		412	0.90 ± 0.89	0.36 ± 0.51	1.07 ± 0.89‡	1.48 ± 0.86‡§	<0.001†
GCA		412	0.66 ± 0.68	0.31 ± 0.50	0.83 ± 0.69‡	0.98 ± 0.67‡	<0.001†
WMH		411	0.96 ± 0.81	0.69 ± 0.70	1.23 ± 0.89‡	1.12 ± 0.76‡	<0.001†
Microbleeds (≥1)		403	79 (19.6)	24 (13.9)	30 (28.3)	25 (20.2)	0.013

*Table on the left: Data is presented as mean ± SD, n (%), or median [interquartile range]. Differences were tested with one-way ANOVA or Kruskal Wallis tests for continuous variables and chi-square tests for categorical variables. *=p-value < 0.05; †=Kruskal-Wallis test; ‡=significantly different from controls upon post-hoc; §=significantly different from MCI upon post-hoc. GDS = Geriatric Depression Scale, MMSE = Mini Mental State Examination, BMI = body mass index, MNA = Mini Nutritional Assessment, MTA = medial temporal atrophy, GCA = global cortical atrophy, WMH = white matter hyperintensities.*

[18F]florbetapir (Amyvid). Images were assessed for amyloid positivity by an experienced nuclear medicine physician.[30,31] CSF was obtained by lumbar puncture using a 25-gauge needle and collected in 10 ml polypropylene tubes (Sarstedt). Amyloid-$\beta_{1\text{-}42}$ ($A\beta_{42}$) concentrations were determined with sandwich ELISAs (Fujirebio).[32] Patients were classified as having a positive amyloid status, indicative for AD pathology, if they had a either positive amyloid PET scan,[30] or abnormal cerebrospinal fluid (CSF) biomarkers, defined as $A\beta_{42}$ drift corrected values lower than 813 pg/ml.[33] In total, 187 (52%) patients were classified as amyloid positive.

Statistical analysis

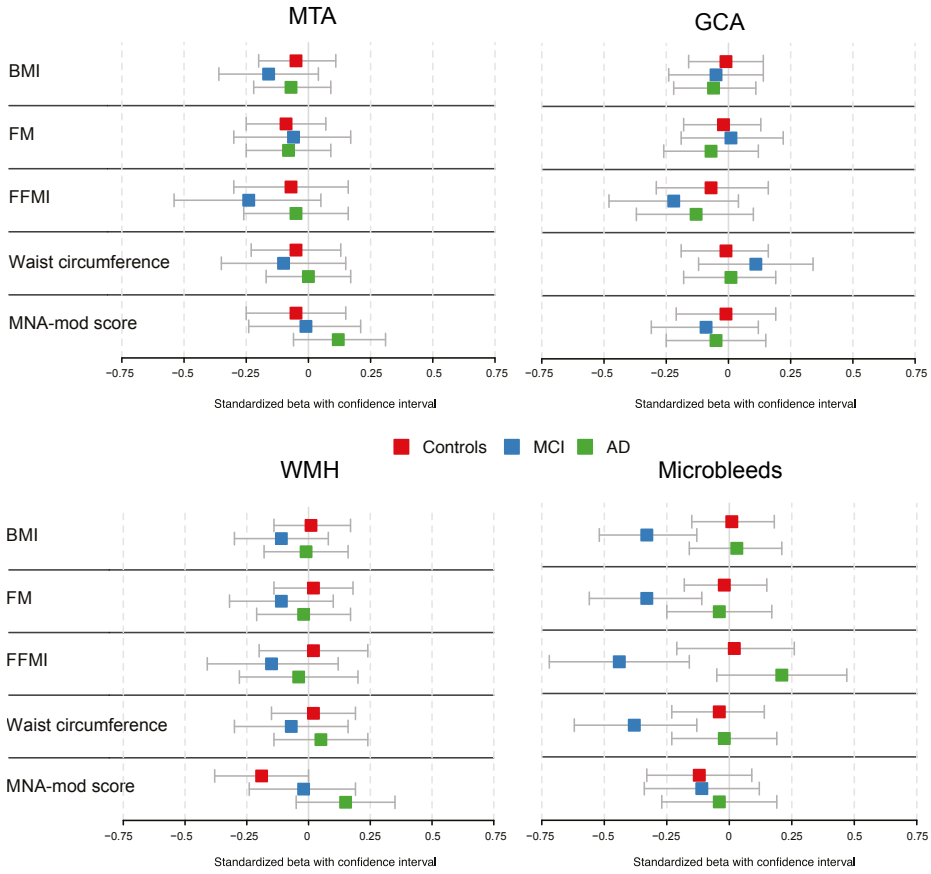
Differences in descriptive variables, nutritional status parameters and MRI scores between diagnosis groups were tested using analysis of variance or Kruskal-Wallis tests for continuous variables and chi-square tests for categorical variables. For ease of comparison, nutritional status parameters were transformed into Z-scores. Linear regression analysis in the total sample was used to evaluate associations between nutritional status parameters and MRI measures in two models: model 1 was adjusted for age, sex and education (continuous Verhage score); model 2 was adjusted for age, sex, education, MMSE and cardiovascular risk composite score. Subsequently, we repeated model 2 stratified for diagnosis. Lastly, we performed a sensitivity analysis for the stratified model 2 including amyloid positive patients only. Significance level was set at $p<0.05$ for all analyses. All statistical analyses were performed with SPSS version 22.0 for Windows and plots were created with RStudio 3.4.2 for Windows using the forestplot package.[34]

Table 2: Associations between nutritional parameters and MRI markers

Determinant	Model 1	Model 2
MTA		
BMI	-0.12 (-0.20;-0.03)*	-0.12 (-0.21;-0.03)*
FM	-0.11 (-0.20;-0.01)*	-0.11 (-0.20;-0.02)*
FFMI	-0.14 (-0.27;-0.02)*	-0.18 (-0.30;-0.06)*
Waist circumference	-0.10 (-0.20; 0.00)	-0.09 (-0.19; 0.01)
MNA-mod score	-0.04 (-0.15; 0.07)	0.00 (-0.11; 0.10)
GCA		
BMI	-0.06 (-0.14; 0.03)	-0.06 (-0.15; 0.02)
FM	-0.04 (-0.13; 0.06)	-0.04 (-0.14; 0.05)
FFMI	-0.12 (-0.25; 0.00)	-0.15 (-0.27;-0.03)*
Waist circumference	-0.01 (-0.11; 0.08)	-0.01 (-0.11; 0.09)
MNA-mod score	-0.10 (-0.20; 0.01)	-0.06 (-0.17; 0.05)
WMH		
BMI	-0.02 (-0.11; 0.07)	-0.05 (-0.15; 0.04)
FM	0.00 (-0.09; 0.10)	-0.04 (-0.14; 0.06)
FFMI	-0.05 (-0.18; 0.08)	-0.08 (-0.21; 0.05)
Waist circumference	0.02 (-0.08; 0.12)	-0.02 (-0.12; 0.09)
MNA-mod score	-0.05 (-0.16; 0.06)	-0.02 (-0.13; 0.09)
Microbleeds		
BMI	-0.09 (-0.19; 0.01)	-0.11 (-0.21; 0.00)*
FM	-0.12 (-0.22;-0.01)*	-0.14 (-0.25;-0.03)*
FFMI	-0.10 (-0.24; 0.04)	-0.11 (-0.25; 0.04)
Waist circumference	-0.14 (-0.25;-0.03)*	-0.16 (-0.27;-0.04)*
MNA-mod score	-0.09 (-0.21; 0.03)	-0.10 (-0.22; 0.03)

*Associations between nutritional parameters and MRI markers are presented as standardized betas with confidence intervals. Model 1 is adjusted for age, sex and education; model 2 is adjusted for age, sex, education, MMSE and cardiovascular risk composite score. *= $p<0.05$.*

Figure 1: Associations stratified for diagnosis



Forest plots with associations between nutritional parameters and MRI markers stratified for diagnosis, presented as standardized betas with confidence intervals. MTA, medial temporal atrophy; GCA, global cortical atrophy; WMH, white matter hyperintensities; BMI, body mass index; FM, fat mass; FFMI, fat free mass index; MNA, mini nutritional assessment.

Results

Patients with MCI and AD dementia were older, had received less education and had lower MMSE scores than controls (**Table 1**). There were no differences in sex and living situation. Regarding the nutritional parameters, MCI and AD dementia patients had lower BMI and lower FM than controls. MTA and GCA were most severe in patients with AD dementia, followed by patients with MCI and controls. WMH and microbleed load were most severe in MCI patients compared to controls with AD dementia in between.

Linear regression analyses (**Table 2**) showed that lower BMI (β -0.12 (-0.21, -0.02), p<0.01, model 2), lower FM (β -0.11 (-0.20, 0.02), p<0.05, model 2) and lower FFMI (β -0.18 (-0.30, -0.06), p<0.01, model 2) were associated with higher MTA scores in both models. In addition, lower FFMI was associated with more GCA (β -0.15 (-0.27, 0.03), p<0.05, model 2). Lower FM (β -0.14 (-0.25, -0.03), p<0.05, model 2) and lower waist circumference (β -0.16 (-0.27, -0.04), p<0.01, model 2) were associated with more microbleeds in both models. Lower BMI was only associated with more microbleeds in model 2 (β -0.11 (-0.21, 0.00), p<0.05). There were no associations between nutritional parameters and WMH.

Subsequently, we stratified model 2 for diagnosis. Although statistical significance of most associations was lost due to smaller group sizes, effect sizes remained similar. Moreover, associations between nutritional parameters, including lower BMI (β -0.33 (-0.52, -0.13), p<0.01), FM (β -0.33 (-0.56, -0.11), p<0.01), FFMI (β -0.44 (-0.72, -0.16), p<0.01) and waist circumference (β -0.38 (-0.62, -0.13), p<0.01) and having more microbleeds were significant in MCI patients. There were no significant associations in AD dementia and controls. There was an association between MNA and WMH in controls (β -0.19 (-0.38, 0.00), p<0.05), but not in MCI or AD. Associations between lower BMI, FM, FFMI and waist circumference and higher MTA were largely similar in direction and effect size across diagnosis groups, with somewhat larger effect sizes in patients with MCI.

Finally, we performed a sensitivity analysis for model 2 in the subgroup of 187 patients with positive amyloid status, with a mean age of 66.5±7.6 years, 102 (51.3%) females, 54 (27%) patients with MCI, 104 (52%) patients with AD dementia and 41 (21%) controls (**Table 3**). Associations with MTA and GCA became stronger than in the total group. There were no associations between nutritional parameters and WMH or microbleeds. After stratification for diagnosis in amyloid positive patients (**Figure 2**), effect sizes of MTA with nutritional parameters were largest in controls on visual inspection, while effect sizes of WMH and microbleeds with these parameters were largest in MCI.

Table 3: Sensitivity analysis in amyloid positive patients

Determinant	Model 1	Model 2
MTA		
BMI	-0.18 (-0.31, -0.05)*	-0.18 (-0.31, -0.05)*
FM	-0.15 (-0.30, -0.01)*	-0.16 (-0.31, -0.02)*
FFMI	-0.18 (-0.36, 0.01)	-0.23 (-0.41, -0.06)*
Waist circumference	-0.15 (-0.31, 0.00)*	-0.15 (-0.30, 0.00)
MNA-mod score	0.02 (-0.14, 0.19)	0.06 (-0.10, 0.21)
GCA		
BMI	-0.11 (-0.25, 0.03)	-0.12 (-0.26, 0.02)
FM	-0.06 (-0.22, 0.09)	-0.08 (-0.24, 0.08)
FFMI	-0.23 (-0.42, -0.03)*	-0.27 (-0.46, -0.08)*
Waist circumference	-0.06 (-0.22, 0.10)	-0.07 (-0.23, 0.09)
MNA-mod score	-0.11 (-0.28, 0.06)	-0.09 (-0.26, 0.08)
WMH		
BMI	-0.06 (-0.20, 0.08)	-0.09 (-0.23, 0.06)
FM	-0.03 (-0.19, 0.12)	-0.08 (-0.24, 0.08)
FFMI	-0.10 (-0.30, 0.09)	-0.12 (-0.32, 0.07)
Waist circumference	0.01 (-0.15, 0.17)	-0.02 (-0.18, 0.14)
MNA-mod score	0.13 (-0.03, 0.30)	0.14 (-0.03, 0.30)
Microbleeds		
BMI	0.02 (-0.13, 0.17)	0.03 (-0.12, 0.19)
FM	-0.04 (-0.21, 0.12)	-0.03 (-0.20, 0.14)
FFMI	0.09 (-0.12, 0.29)	0.12 (-0.08, 0.33)
Waist circumference	-0.09 (-0.26, 0.08)	-0.08 (-0.26, 0.09)
MNA-mod score	-0.01 (-0.19, 0.17)	-0.02 (-0.20, 0.16)

*Associations between nutritional parameters and MRI markers in amyloid positive patients are presented as standardized betas with confidence intervals. Model 1 was adjusted for age, sex and education; model 2 was adjusted for age, sex, education, MMSE and cardiovascular risk composite score. *= $p<0.05$.*

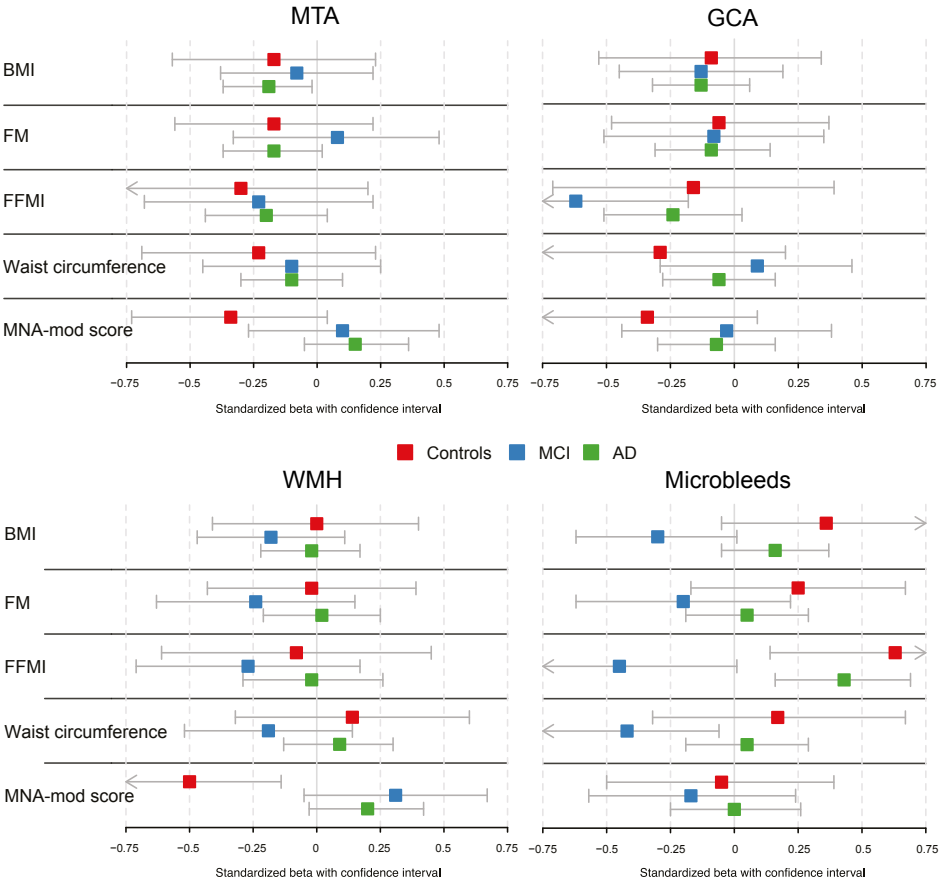
Discussion

The main finding of this study is that lower parameters of nutritional status, including lower BMI, FM and FFMI, were associated with more severe MTA and more microbleeds. Effect sizes were largest in patients with MCI, although for the associations with MTA significance was lost. Our results extend previous reports by simultaneously evaluating multiple parameters of nutritional status in relation to different MRI measures of neurodegenerative and vascular pathology in a clinical AD sample covering the entire cognitive spectrum of cognitively normal to dementia.

Our findings are in line with two former studies in patients with AD dementia and controls that described associations between lower BMI and more severe MTA,[10] and between lower FFM and higher GCA.[11] By contrast, in two other studies comprising AD and MCI patients, higher BMI was associated with lower total brain or hippocampal volumes.[12,14] However, these studies used a clinical AD diagnosis, while in our study AD diagnosis was confirmed with CSF amyloid in the majority of patients. As such, our cohort probably contains more patients with AD pathology than other studies, which provides the possibility to evaluate the association between nutritional status and Alzheimer-related disease processes. In line with this notion, of the four MRI markers in our analyses, MTA, the most AD specific MRI marker, showed strongest associations in the amyloid positive subgroup. A former study in a geriatric outpatient population described associations between malnutrition, as assessed with MNA, and WMH, but not with MTA.[25] This discrepancy could be due to difference in population, since the former study evaluated a more heterogeneous geriatric population, while our study focused on the clinical spectrum of AD. In line with this notion, we found an association between MNA and WMH in controls only.

In addition, we observed that lower FM and waist circumference were associated with more microbleeds, especially in MCI. Microbleeds are more prevalent in MCI and AD dementia patients and have been related to Alzheimer pathology.[8,9] In the sensitivity analysis with amyloid positive MCI patients, the association with waist circumference remained intact, providing further support for the notion that the relationship between nutritional status and microbleeds is AD specific.

Figure 2: Associations stratified for diagnosis in amyloid positive patients



Forest plots with associations between nutritional parameters and MRI markers in amyloid positive patients (N=187), stratified for diagnosis, presented as standardized betas with confidence intervals. MTA, medial temporal atrophy; GCA, global cortical atrophy; WMH, white matter hyperintensities; BMI, body mass index; FM, fat mass; FFMI, fat free mass index; MNA, mini nutritional assessment.

This study has several limitations. Firstly, the cross-sectional nature hampers causal interpretation of our findings. Longitudinal studies with repeated imaging and data on for instance body weight history are needed to assess if patients with worse nutritional status indeed develop more AD-specific structural brain changes. Secondly, we used visual MRI scores to quantify brain atrophy and white matter hyperintensities rather than volumetric measurements. Although perhaps somewhat less precise, visual MRI ratings for cerebral atrophy and WMH have nonetheless been shown to be as valid and reliable as volumetric measurements.[35,36] Moreover, these measures have clinical applicability, as they are fairly easy to implement in clinical practice. Strengths of this study include the relatively large clinical cohort that underwent standardized work-up, availability of AD biomarkers including PET scans and CSF. Diagnoses were made carefully, and although we can never rule out misdiagnosis, widely accepted diagnostic criteria were used. In addition, we used several parameters of nutritional status, including BMI, FM, FFMI, waist circumference and MNA. Of note, average BMI of the study population could be considered as overweight. Nonetheless, within this sample of patients in the earliest stages of AD, we find that lower nutritional parameters were associated with more MTA and microbleeds. This is in line with the notion that the process of changing nutritional status in AD is a continuous, longer trajectory and that in fact many patients may come from obesity in midlife.[37]

The altered nutritional status in AD could be caused by elevated energy expenditure, lower intake or malabsorption of nutrients.[38] A mechanism that could explain the associations between lower indicators of nutritional status and MRI measures of AD pathology is a lower availability of important nutrients for maintenance and repair of brain tissue, such as proteins and fat. In addition, lower levels of specific nutrients required for phospholipid synthesis could result in more synapse loss, ultimately leading to more atrophy. In line with this hypothesis, a recent meta-analysis showed that patients with AD have lower CSF levels of these phospholipid precursors and cofactors such as docosahexaenoic acid (DHA), choline-containing lipid, folate, vitamin B12, vitamin C and vitamin E.[39]

Alternatively, we cannot rule out reverse causality, in which cerebral atrophy and resulting cognitive decline could have led to lower energy intake, weight loss and deteriorating nutritional status. However, the observed associations were already present in amyloid positive controls and in patients with MCI. This suggests that the observed relations between nutritional status and structural brain changes are not a mere consequence of cognitive decline but rather a prodrome. To further address the issues of underlying mechanisms and causal directionality

regarding lower intake versus change in energy expenditure, future studies should take dietary intake into account.

The associations observed in MCI and controls suggest that an impaired nutritional status has a role in the development of disease, either as early consequence of the underlying pathology or as an aggravating factor. This provides further evidence for the notion that nutrition could also be a target for secondary prevention. This should be further studied in intervention studies that focus on optimizing nutritional status. A recent intervention study, LipiDiDiet, with supplementation that includes precursors and cofactors for phospholipid synthesis, has shown a favorable effect on hippocampal atrophy and functional decline in patients with prodromal AD.[40,41] This underlines the potential benefit of intervening early in the disease process, within the time window where it can still make a difference in terms of neurodegeneration. Whether positive results can also be obtained by intervening on the level of macronutrient intake needs to be elucidated.

Concluding, in our memory clinic cohort, worse nutritional status, indicated by BMI, FM and FFMI, was associated with more MTA and microbleeds. Our findings indicate that lower nutritional parameters might have a role in the development of AD, either as early consequence of the underlying pathology or as an aggravating factor.

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Conflicts of interest

C.T. received grants from the European Commission, the Dutch Research Council (ZonMW), Association of Frontotemporal Dementia/Alzheimer's Drug Discovery Foundation, The Weston Brain Institute, Alzheimer Netherlands. C.T. has a collaboration contract with ADx Neurosciences, performed contract research or received grants from Probiodrug, Biogen, Esai, Toyama, Janssen prevention center, Boehringer, AxonNeurosciences, Fujirebio, EIP farma, PeopleBio, and Roche. B.v.B. received research support from ZonMW, AVID radiopharmaceuticals, CTMM and Janssen Pharmaceuticals. He is a trainer for Piramal and GE. He receives no personal honoraria. F.B. is a consultant for Biogen-Idec, Janssen Alzheimer Immunotherapy, Bayer-Schering, Merck-Serono, Roche, Novartis, Genzyme, and Sanofi-Aventis; has received sponsorship from European Commission–Horizon 2020, National Institute for Health Research–University College London Hospitals Biomedical Research Centre, Scottish Multiple Sclerosis Register, TEVA, Novartis, and Toshiba; and serves on the editorial boards of Radiology, Brain, Neuroradiology, Multiple Sclerosis Journal, and Neurology®. P.S. has received consultancy/speaker fees from Lilly, GE Healthcare, Novartis, Sanofi, Nutricia, Probiodrug, Biogen, Roche, Avraham, and EIP Pharma. P.S. has acquired grant support from GE Healthcare, Danone Research, Piramal, and MERCK. All funding was paid to the institution. W.v.d.F. received grants from ZonMW, the Food cognition and behavior program of The Dutch Research Council (NWO-FCB), The EU Joint Programme – Neurodegenerative Disease Research (EU-JPND), Alzheimer Nederland, Health-Holland, Topsector Life Sciences & Health, Biogen MA Inc, Boehringer Ingelheim, Life Molecular Imaging, AVID Radiopharmaceuticals, Roche BV, Combinostics, Janssen Stellar, Gieskes Strijbis fonds, and Stichting Equilibrio. W.v.d.F. has performed contract research for Biogen MA Inc and Boehringer Ingelheim and has been an invited speaker at Boehringer Ingelheim and Biogen MA Inc. All funding was paid to the institution. BV, FdL, AD, JF, OvdR, MV, MdvdS, MK and MM report no disclosures.

References

1. Knopman, D. S., Edland, S. D., Cha, R. H., Petersen, R. C. & Rocca, W. A. Incident dementia in women is preceded by weight loss by at least a decade. *Neurology* 69, 739–746 (2007).
2. Johnson, D. K., Wilkins, C. H. & Morris, J. C. Accelerated weight loss may precede diagnosis in Alzheimer disease. *Arch. Neurol.* 63, 1312–1317 (2006).
3. Cronk, B. B., Johnson, D. K., Burns, J. M. & Initiative, A. D. N. Body mass index and cognitive decline in mild cognitive impairment. *Alzheimer Dis Assoc Disord* 24, 126 (2010).
4. Guerin, O. *et al*. Nutritional status assessment during Alzheimer's disease: results after one year (the REAL French Study Group). *J Nutr Health Aging* 9, 81–84 (2005).
5. Andrieu, S. et al. Nutritional risk factors for institutional placement in Alzheimer's disease after one year follow-up. *J Nutr Health Aging* 5, 113–117 (2001).
6. Vellas, B. *et al*. Impact of nutritional status on the evolution of Alzheimer's disease and on response to acetylcholinesterase inhibitor treatment. *J Nutr Health Aging* 9, 75–80 (2005).
7. Frisoni, G. B., Fox, N. C., Jack Jr, C. R., Scheltens, P. & Thompson, P. M. The clinical use of structural MRI in Alzheimer disease. *Nat Rev Neurol* 6, 67 (2010).

8. Benedictus, M. R. *et al*. Specific risk factors for microbleeds and white matter hyperintensities in Alzheimer's disease. *Neurobiol. Aging* 34, 2488–2494 (2013).
9. Goos, J. D. *et al*. Patients with Alzheimer disease with multiple microbleeds: relation with cerebrospinal fluid biomarkers and cognition. *Stroke* 40, 3455–3460 (2009).
10. Grundman, M., Corey-Bloom, J., Jernigan, T., Archibald, S. & Thal, L. J. Low body weight in Alzheimer's disease is associated with mesial temporal cortex atrophy. *Neurology* 46, 1585–1591 (1996).
11. Burns, J. M., Johnson, D. K., Watts, A., Swerdlow, R. H. & Brooks, W. M. Reduced lean mass in early Alzheimer disease and its association with brain atrophy. *Arch. Neurol.* 67, 428–433 (2010).
12. Boyle, C. P. *et al*. Physical activity, body mass index, and brain atrophy in Alzheimer's disease. *Neurobiol. Aging* 36, S194–S202 (2015).
13. Ho, A. J. *et al*. Obesity is linked with lower brain volume in 700 AD and MCI patients. *Neurobiol. Aging* 31, 1326–1339 (2010).
14. Ho, A. J. *et al*. Hippocampal volume is related to body mass index in Alzheimer's disease. *Neuroreport* 22, 10 (2011).
15. Doorduijn, A. S. *et al*. Associations of AD biomarkers and cognitive performance with nutritional status: The NUDAD project. *Nutrients* 11, 1161 (2019).
16. Folstein, M. F., Folstein, S. E. & McHugh, P. R. "Mini-mental state": a practical method for grading the cognitive state of patients for the clinician. *J. Psychiatr. Res.* 12, 189–198 (1975).
17. van der Flier, W. M. *et al*. Optimizing Patient Care and Research: The Amsterdam Dementia Cohort. *Journal of Alzheimer's Disease* 41, 313–327 (2014).
18. Albert, M. S. et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's Dement.* 7, 270–279 (2011).
19. McKhann, G. *et al*. Clinical diagnosis of Alzheimer's disease. *Neurology* 34, 939–939 (1984).
20. Verhage, F. Intelligence and age in a Dutch sample. *Hum. Dev*. 8, 238–245 (1965).
21. Tombaugh, T. N. & McIntyre, N. J. The Mini-Mental State Examination: A Comprehensive Review. *J. Am. Geriatr. Soc.* 40, 922–935 (1992).
22. Kyle, U. G. *et al*. Bioelectrical impedance analysis—part II: utilization in clinical practice. *Clin Nutrition* 23, 1430–1453 (2004).
23. Bauer, J. M., Kaiser, M. J., Anthony, P., Guigoz, Y. & Sieber, C. C. The Mini Nutritional Assessment®—its history, today's practice, and future perspectives. *Nutrition in clinical practice* 23, 388–396 (2008).
24. Meijers, J. M., Schols, J. M., Dassen, T., Janssen, M. A. & Halfens, R. J. Malnutrition prevalence in The Netherlands: results of the annual Dutch national prevalence measurement of care problems. *Br. J. Nutr.* 101, 417–423 (2008).
25. de van der Schueren, M. A. *et al*. Malnutrition and risk of structural brain changes seen on magnetic resonance imaging in older adults. *J. Am. Geriatr. Soc.* 64, 2457–2463 (2016).
26. Scheltens, P. *et al*. Atrophy of medial temporal lobes on MRI in "probable" Alzheimer's disease and normal ageing: diagnostic value and neuropsychological correlates. *J. Neurol. Neurosurg. Psychiatry* 55, 967–972 (1992).
27. Pasquier, F. *et al*. Inter-and intraobserver reproducibility of cerebral atrophy

assessment on mri scans with hemispheric infarcts. *Eur Neurology* 36, 268–272 (1996).
28. Fazekas, F., Chawluk, J. B., Alavi, A., Hurtig, H. I. & Zimmerman, R. A. MR signal abnormalities at 1.5 T in Alzheimer's dementia and normal aging. *Am. J. Roentgenology* 8, 421–426 (1987).
29. Wardlaw, J. M. *et al*. Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. *Lancet Neurol.* 12, 822–838 (2013).
30. de Wilde, A. *et al*. Alzheimer's biomarkers in daily practice (ABIDE) project: Rationale and design. *Alzheimer's Dement.: Diagn. Assess. Dis. Monit* 6, 143 (2017).
31. Slot, R. E. R. *et al*. Subjective Cognitive Impairment Cohort (SCIENCe): study design and first results. *Alzheimer's Res. Ther.* 2018 10:1 10, 1–13 (2018).
32. Mulder, C. *et al*. Amyloid-β(1–42), Total Tau, and Phosphorylated Tau as Cerebrospinal Fluid Biomarkers for the Diagnosis of Alzheimer Disease. *Clinical Chemistry* 56, 248–253 (2010).
33. Tijms, B. M. *et al*. Unbiased Approach to Counteract Upward Drift in Cerebrospinal Fluid Amyloid-β 1–42 Analysis Results. *Clinical Chemistry* 64, 576–585 (2018).
34. Gordon, M. & Lumley, T. forestplot: Advanced Forest Plot Using 'grid' Graphics. R package version 1, (2015).
35. Gouw, A. A. *et al*. Simple versus complex assessment of white matter hyperintensities in relation to physical performance and cognition: the LADIS study. *J. Neurol.* 253, 1189 (2006).
36. Wahlund, L.-O., Julin, P., Johansson, S.-E. & Scheltens, P. Visual rating and volumetry of the medial temporal lobe on magnetic resonance imaging in dementia: a comparative study. *J. Neurol. Neurosurg. Psychiatry* 69, 630–635 (2000).
37. Xu, W. L. *et al*. Midlife overweight and obesity increase late-life dementia risk: a population-based twin study. *Neurology* 76, 1568–1574 (2011).
38. Poehlman, E. T. & Dvorak, R. V. Energy expenditure, energy intake, and weight loss in Alzheimer disease. *Am. J. Clin. Nutr.* 71, 650S-655S (2000).
39. de Wilde, M. C., Vellas, B., Girault, E., Yavuz, A. C. & Sijben, J. W. Lower brain and blood nutrient status in Alzheimer's disease: Results from meta-analyses. *Alzheimer's Dement: Transl. Res. Clin. Intervent.* 3, 416–431 (2017).
40. Soininen, H. *et al*. 24-month intervention with a specific multinutrient in people with prodromal Alzheimer's disease (LipiDiDiet): a randomised, double-blind, controlled trial. *Lancet Neurol.* 16, 965–975 (2017).
41. Hendrix, S. B. *et al*. Alzheimer's Disease Composite Score: a Post-Hoc Analysis Using Data from the LipiDiDiet Trial in Prodromal Alzheimer's Disease. *J Prev Alzheimers Dis* 1–5 (2019).

8

Gut microbiota composition is related to AD pathology

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Abstract

Introduction: Several studies have reported alterations in gut microbiota composition of Alzheimer's disease (AD) patients. However, the observed differences are not consistent across studies. We aimed to investigate associations between gut microbiota composition and AD biomarkers using machine learning models in patients with AD dementia, mild cognitive impairment (MCI) and subjective cognitive decline (SCD).

Materials and Methods: We included 170 patients from the Amsterdam Dementia Cohort, comprising 33 with AD dementia (66±8 years, 46%F, mini-mental state examination (MMSE) 21[19-24]), 21 with MCI (64±8 years, 43%F, MMSE 27[25-29]) and 116 with SCD (62±8 years, 44%F, MMSE 29[28-30]). Fecal samples were collected and gut microbiome composition was determined using 16S rRNA sequencing. Biomarkers of AD included cerebrospinal fluid (CSF) amyloid-beta 1-42 (amyloid) and phosphorylated tau (p-tau), and MRI visual scores (medial temporal atrophy, global cortical atrophy, white matter hyperintensities). Associations between gut microbiota composition and dichotomized AD biomarkers were assessed with machine learning classification models. The two models with the highest area under the curve (AUC) were selected for logistic regression, to assess associations between the 20 best predicting microbes and the outcome measures from these machine learning models while adjusting for age, sex, BMI, diabetes, medication use, and MMSE.

Results: The machine learning prediction for amyloid and p-tau from microbiota composition performed best with AUCs of 0.64 and 0.63. Highest ranked microbes included several short chain fatty acid (SCFA)-producing species. Higher abundance of *[Clostridium] leptum* and lower abundance of *[Eubacterium] ventriosum* group spp., *Lachnospiraceae* spp., *Marvinbryantia* spp., *Monoglobus* spp., [*Ruminococcus*] *torques group* spp., *Roseburia hominis*, and *Christensenellaceae R-7* spp., was associated with higher odds of amyloid positivity. We found associations between lower abundance of *Lachnospiraceae* spp., *Lachnoclostridium* spp., *Roseburia hominis* and *Bilophila wadsworthia* and higher odds of positive p-tau status.

Conclusions: Gut microbiota composition was associated with amyloid and p-tau status. We extend on recent studies that observed associations between SCFA levels and AD CSF biomarkers by showing that lower abundances of SCFA-producing microbes were associated with higher odds of positive amyloid and p-tau status.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, and is characterized by the accumulation of amyloid beta in plaques and the formation of neurofibrillary tangles including hyperphosphorylated tau (p-tau). Another hallmark is chronic neuroinflammation, which is reflected by activation of microglia and increased cytokine production.[1] The gut microbiome has been shown to interact with the innate and adaptive immune system, by release of bacterial toxins and production of metabolites.[2,3] As has been shown in other neurological conditions such as multiple sclerosis,[4,5] gut microbiota could affect neuroinflammation.

The gut is populated with trillions of microbiota, including bacteria, viruses, fungi, archaea and protozoa.[6] Collectively, the genomes of these cells are referred to as the gut microbiome. The microbiota composition is affected by dietary factors, age, sex, body mass index (BMI) and medication use, including antibiotics, metformin, proton pump inhibitors and statins.[7] Gut microbiota live in symbiosis with the host and are needed for the degradation of macronutrients and production of metabolites.[8,9] Short chain fatty acids (SCFAs) are key metabolites of the gut microbiota, which are produced by fermentation of indigestible dietary fibers.[10]

Animal studies have reported differences in gut microbiota composition between AD and wild-type mice, including a decrease in SCFA-producing microbes.[11,12] Fecal microbiota transplantation from wild type mice to AD-like animal models such as APP/PS1 and ADLP^{APT} mice resulted in a reduction of amyloid, suggesting a causal relation between gut microbes and AD.[12,13] Colonization of Tg2576 mice with *Bacteroides* exacerbates amyloid depositions, suggesting a mechanism for the impact of gut microbiota on AD pathology.[14] In addition, an intervention with sodium butyrate, an SCFA, in an AD mice model resulted in a reduction of AD pathology.[15]

In line with these animal studies, five human studies observed alterations in microbiota composition in patients with AD or mild cognitive impairment (MCI) compared to controls, with a lower abundance of SCFA-producing species in patients with AD.[16–20] However, the nature of the specific microbiota alterations was conflicting across studies, with for instance lower[16,19,20] and higher[17] abundance of *Ruminococcaceae* spp., and lower[17] and higher[16,18,19] abundance of the *Bacteroidetes* phylum of MCI or AD patients compared to controls. In addition, former studies did not take into account AD pathology as measured with AD biomarkers,[17–20] while studies that did focused on a limited set of microbes for these analyses.[16,21]

Hence, we aimed to assess the relation between gut microbiota composi-

tion, as measured with 16S rRNA sequencing, and biomarkers of AD pathology, including CSF biomarkers and MRI measures of vascular burden and neurodegeneration, in a memory clinic population with AD dementia, mild cognitive impairment (MCI) and subjective cognitive decline (SCD).

Methods

Study population

We invited 223 study participants from the Amsterdam Dementia Cohort and SCIENCe project, for fecal sample collection. All invited participants were diagnosed with AD dementia, MCI or SCD and had mini-mental state examination (MMSE) scores higher than 16. Of the invited participants, 175 subjects collected samples, and 170 subjects could be included in our analyses (**Figure 1**), comprising 33 patients with AD, 22 patients with MCI and 120 subjects with SCD.[22–24] All patients underwent comprehensive neuropsychological assessment, neurological examination, lumbar puncture and MRI as part of a standard dementia screening.[22] MCI and AD diagnoses were established by consensus in a multidisciplinary meeting according to the National Institute on Aging-Alzheimer's Association criteria.[25,26] Subjects with SCD presented with memory complaints but performed normal on cognitive examinations and did not fulfill criteria for MCI, dementia, psychiatric diagnoses or other neurological diagnoses.[22] Patients were seen annually for follow-up visits, during which cognitive assessments and medical examinations were repeated. Prior to these follow-up visits, patients were asked to collect fecal samples. The study protocol was approved by the Ethics Committee of the Amsterdam UMC, and all study participants provided written informed consent.

Descriptive characteristics included age, sex, medical history (history of hypertension, hypercholesterolemia and diabetes; self-reported or described in a referral letter), medication use (antihypertensive medication, glucose lowering medication, cholesterol lowering medication, proton pump inhibitors (PPI)), smoking status (current smoking yes/no) and alcohol use (in units per day). Global cognitive functioning was assessed using the MMSE (scale 0-30).[27]

Gut microbiota composition

Patients were sent a fecal collection kit prior to their memory clinic follow-up visit. Seven patients who used antibiotics within three months prior to collec-

tion were not included. Other exclusion criteria were diarrhea in the past week or severe gastro-intestinal conditions, including inflammatory bowel disease. A flowchart with the screening and recruiting procedure and reasons for exclusion at each stage is presented in **Figure 1**. The included patients were asked to store the sample in a freezer and to transport the samples to the hospital in a cooling bag. The 175 samples were shipped to Erasmus Medical Center, Rotterdam, the Netherlands, for sequencing. Aliquots of ~300 mg feces were homogenized and DNA was isolated using bead-beating and the InviMag Stool DNA kit (Invitek Molecular GmbH, Berlin, Germany) on a KingFisher Flex robot (Thermo Fisher Scientific, Breda, Netherlands). Fecal microbiota composition was determined by sequencing the V3 and V4 hypervariable regions of the 16S rRNA gene on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) primers and dual-indexing.[28] The processing of the raw sequencing data is described in **Supplement 1**, which after rarefying to 20.000 counts per sample resulted in a dataset with 170 samples and 7894 amplicon sequence variants (ASVs). Prior to the machine learning analyses, we filtered for ASVs that had at least 5 counts in 30% of the subjects, which resulted in a dataset with 181 ASVs. Of these ASVs, taxonomy was available up to species level for 32%, up to genus level for 88% and up to family level for 99%.

AD biomarkers

CSF was obtained by lumbar puncture using a 25-gauge needle and collected in 10 ml polypropylene tubes (Sarstedt). Amyloid-β1-42 (Aβ42) and p-tau concentrations were determined with sandwich ELISAs, using Innotest (Fujirebio) and Elecsys immunoassays. Patients were classified as having a positive amyloid status, indicative for AD pathology, if they had amyloid values lower than the platform-dependent cut-off (Innotest <813 pg/ml;[29,30] Elecsys <1000 pg/ml). A positive p-tau status was defined as having p-tau values higher than the platform-dependent cut-off (Innotest >52 pg/ml; Elecsys >19pg/ml). Because of the high correlations between these platforms, Elecsys values were converted to Innotest values,[31] CSF biomarkers were available for 116 patients at a median of 2.4 [IQR 2.2, 3.2] years before the time of fecal sampling.

MRI scans were performed on a 3.0T scanner and the protocol included T1-weighted, T2-weighted, fluid-attenuated inversion recovery (FLAIR) and gradient echo T2*-weighted images. A trained neuroradiologist evaluated all scans using visual rating scales. Medial temporal atrophy (MTA) was rated on coro-

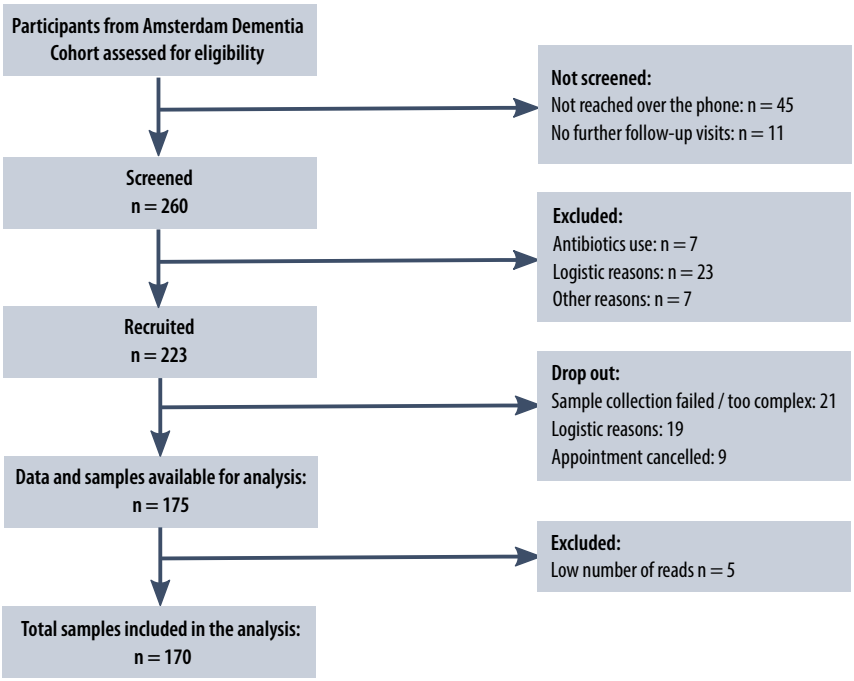
nal reconstructions of T1-weighted images of both sides, perpendicular to the long axis of the hippocampus (0-4 scale). MTA was averaged across left and right scores, and was dichotomized with a cut-off of ≥1.[32,33] Global cortical atrophy (GCA) was assessed on transverse FLAIR images and rated using a 4-point scale (0-3) and dichotomized (cut-off ≥1).[33,34] White matter hyperintensities (WMH) were assessed on the same sequences using the Fazekas scale for white matter hyperintensities (0-3) and dichotomized with a cut-off of ≥2.[35] Microbleeds were defined as oval or round hypointense lesions up to 10 mm on a T2*-weighted MRI. Microbleeds counts were dichotomized into present or absent.[36] MRI results were available for 136 patients at a median of 2.1 [IQR 0.5, 2.4] years before the time of fecal sampling.

Statistical analysis

Differences in descriptive and outcome variables between diagnosis groups were tested using analysis of variance for continuous variables with normal distributions, Kruskal-Wallis tests for continuous variables with non-normal distributions and chi-square tests for categorical variables. To compare microbiota composition between groups, we calculated alpha diversity indices, including Shannon index, richness and Faith's phylogenetic diversity.[37,38] In addition, we compared beta diversity between groups by testing differences in Bray-Curtis distance with a PERMANOVA test. We used the rarefied microbiota data to calculate alpha and beta diversity.

We used machine learning models to predict dichotomized AD biomarkers, including amyloid and p-tau status, MTA, GCA, WMH and microbleeds, from gut microbiota composition (i.e. the relative abundance of ASVs). Subjects were excluded for a particular model if data on that outcome variable were missing. Microbiota abundance data is compositional data, with skewed, zero-inflated and overdispersed distributions. We used gradient-boosted tree models (XGBoost algorithm),[39] which is a state-of-the art algorithm that has shown good accuracy in comparative microbiota studies.[40] To prevent overfitting, we used a nested cross-validation design in performing these models (**Supplement 2**). In each of the 200 iterations, the dataset was randomly split into a test set containing 20% of the subjects and a training set with the remaining 80%. Within the train set, 5-fold cross-validation was performed in order to optimize the model hyperparameters. Two random variables were added to the microbiota data in each iteration as a benchmark. The resulting model was evaluated on the test set which yielded an area under the receiver-operator curve (AUC) as main model quality metric, and

Figure 1: Study flowchart



Flowchart of the number of patients from the Amsterdam Dementia Cohort screened, recruited and included in the analysis, including reasons for exclusion at different stages. The flowchart was designed following the 'Strengthening The Organization and Reporting of Microbiome Studies' (STORMS) checklist.[41]

a ranked list of microbial predictors with their relative importance to the model. These were recorded for each iteration and were averaged across 200 iterations.

We selected the two machine learning models with the highest AUCs for logistic regression, to obtain effect sizes for the associations between the 20 highest ranked (i.e. highest feature importance) microbes and the dichotomous outcome of these machine learning models. We ran three models: model 1 adjusted for age, sex and BMI, model 2 with additional adjustment for diabetes, statin and proton pump inhibitor (PPI) use and model 3 with additional adjustment for MMSE. The effect sizes, reported as odds ratios (OR) per log2-increase in counts with 95%-confidence intervals (95%-CI) were visualized in a forest plot. Spearman rank correlation coefficients were calculated between the top 10 best predicting ASVs found by the two best performing machine learning models and the AD biomarkers and were visualized with a correlation heatmap. We used hierarchical clustering (Ward's method) to order the ASVs in this plot and to draw a dendrogram. The correlations with amyloid levels and MMSE scores were inversed for

interpretability, since lower levels are indicative for AD pathology in contrast to other biomarkers.

Machine learning was implemented in Python (v.3.7.4) using the XGBoost (v.0.90), numpy (v.1.16.4), pandas (v.0.25.1), and scikit-learn (v.0.21.2) packages. Statistical analyses and visualizations were performed using R (v.3.6.2). All R code was made publicly available (https://github.com/barbarahelena/ADC_microbiota).

Data availability

The sequencing data presented in this study can be found in an online repository, European Nucleotide Archive (ENA) accession number PRJEB49329 (https://www.ebi.ac.uk/ena/browser/view/PRJEB49329). Clinical data are available upon reasonable request at Alzheimer Center Amsterdam, Amsterdam UMC, location VUmc in Amsterdam, The Netherlands.

Results

Population characteristics

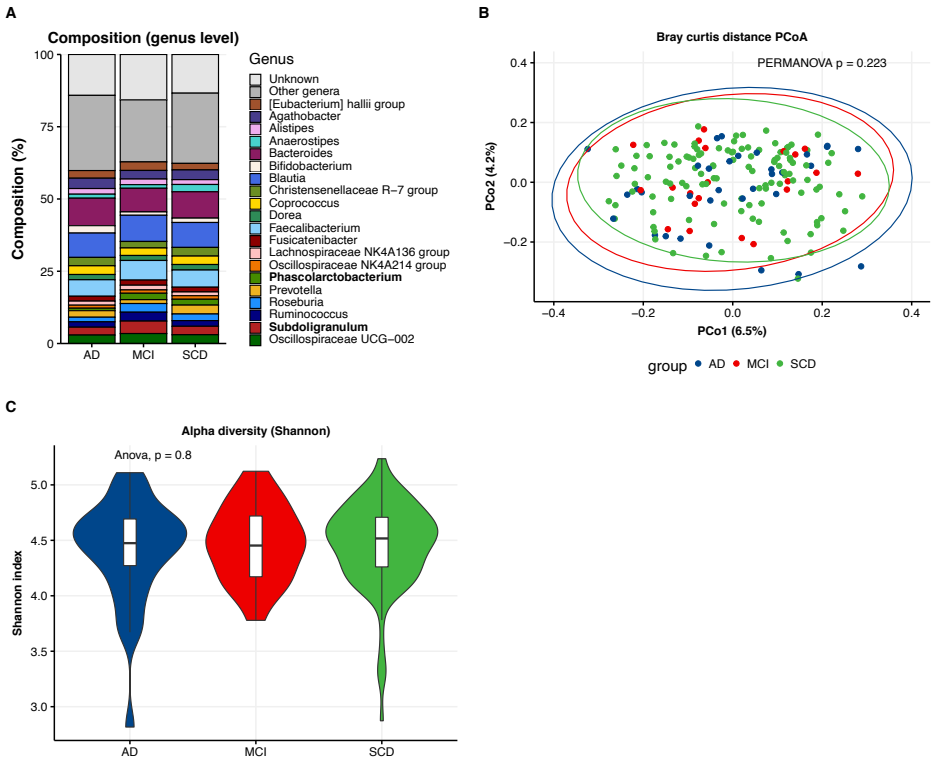
The mean age of the overall study population was 63 years (**Table 1**), with the AD dementia group (66.0±8.0) older than the SCD group (62.0±7.5; $p<0.05$). Patients with AD dementia, MCI and SCD were comparable in terms of sex, BMI, smoking status and alcohol use, as well as most cardiovascular risk factors. However, diabetes was more prevalent among patients with AD dementia and MCI compared to SCD ($p<0.05$). AD dementia and MCI patients more often had abnormal AD biomarkers than controls, such as positive amyloid and p-tau status ($p<0.001$), and MTA ($p<0.01$) and GCA scores $^{3}1$ ($p<0.05$). Distributions of amyloid and p-tau CSF levels are presented in Supplement 3. Prevalence of WMH $^{3}2$ and microbleeds tended to be higher in patients with MCI, but this difference was not significant. The gut microbiota composition on genus level of the three diagnosis groups is shown in Figure 2. When comparing the 20 most abundant genera between diagnosis groups, only two genera, Subdoligranulum ($p<0.05$) and Phascolarctobacterium ($p<0.05$), had different abundances between groups. There were no differences in beta diversity (PERMANOVA $p=0.223$), nor in alpha diversity, as measured with Shannon index, richness and Faith's phylogenetic diversity.

Table 1: Patient characteristics

	N	Overall	AD dementia	MCI	SCD	p
		170	33	21	116	
Age	170	63.1±7.8	66.0±8.0a	64.1±7.9	62.0±7.5	0.028
Female sex	170	75 (44.1)	15 (45.5)	9 (42.9)	51 (44.0)	0.981
BMI	144	25.3±4.0	25.2±3.7	24.0±3.3	25.6±4.1	0.289
Current smoking	129	12 (9.3)	0 (0.0)	2 (11.8)	10 (10.6)	0.338
Alcohol units/day	130	1.3±1.5	1.2±1.4	1.3±1.3	1.3±1.5	0.908
Hypertension	170	42 (24.7)	12 (36.4)	4 (19.0)	26 (22.4)	0.212
Diabetes	170	15 (8.8)	5 (15.2)	4 (19.0)	6 (5.2)	0.043
Hypercholesterolemia	170	29 (17.1)	5 (15.2)	5 (23.8)	19 (16.4)	0.671
Antihypertensive drugs	170	55 (32.4)	13 (39.4)	5 (23.8)	37 (31.9)	0.482
Cholesterol lowering drugs	170	48 (28.2)	11 (33.3)	6 (28.6)	31 (26.7)	0.758
Glucose lowering drugs	170	12 (7.1)	4 (12.1)	3 (14.3)	5 (4.3)	0.117
Proton pump inhibitors	170	29 (17.1)	6 (18.2)	2 (9.5)	21 (18.1)	0.618
MMSE	161	29 [26, 30]	21 [19, 24][a,b]	27 [25, 29][a]	29 [28, 30]	<0.001
ApoE4 allele	166	74 (44.6)	24 (75.0)[a]	12 (57.1)	38 (33.6)	<0.001
amyloid positive status	115	49 (42.6)	24 (96.0)[a,b]	8 (47.1)	17 (23.3)	<0.001
amyloid CSF levels	115	884 [646-1100]	589 [526-663][a,b]	875 [643-943][a]	1034 [828-1188]	<0.001
p-tau positive status	116	71 (61.2)	26 (100.0)[a]	14 (82.4)[a]	31 (42.5)	<0.001
p-tau CSF levels	116	56 [45-88]	100 [80-140][a,b]	78 [54-107][a]	49 [34-58]	<0.001
MTA≥1	137	41 (29.9)	12 (54.5)[a]	7 (41.2)	22 (22.4)	0.007
GCA≥1	137	49 (35.8)	11 (50.0)	10 (58.8)[a]	28 (28.6)	0.018
WMH≥2	137	15 (10.9)	2 (9.1)	3 (17.6)	10 (10.2)	0.633
Microbleeds present	137	24 (17.5)	4 (18.2)	6 (35.3)	14 (14.3)	0.109

Patient characteristics are presented as mean±SD, median [interquartile range] or n (%). Differences were tested with one-way ANOVA for continuous variables with normal distribution, and Kruskal-Wallis test for continuous variables with non-normal distribution, or chi-square tests for categorical variables. [a] = significantly different from SCD upon post-hoc testing, [b] = significantly different from MCI upon post-hoc testing. CSF=cerebrospinal fluid, MTA=medial temporal atrophy, GCA=global cortical atrophy, WMH=white matter hyperintensities. Significant p-values (p<0.05) are marked in bold.

Figure 2: Descriptive plots gut microbiota



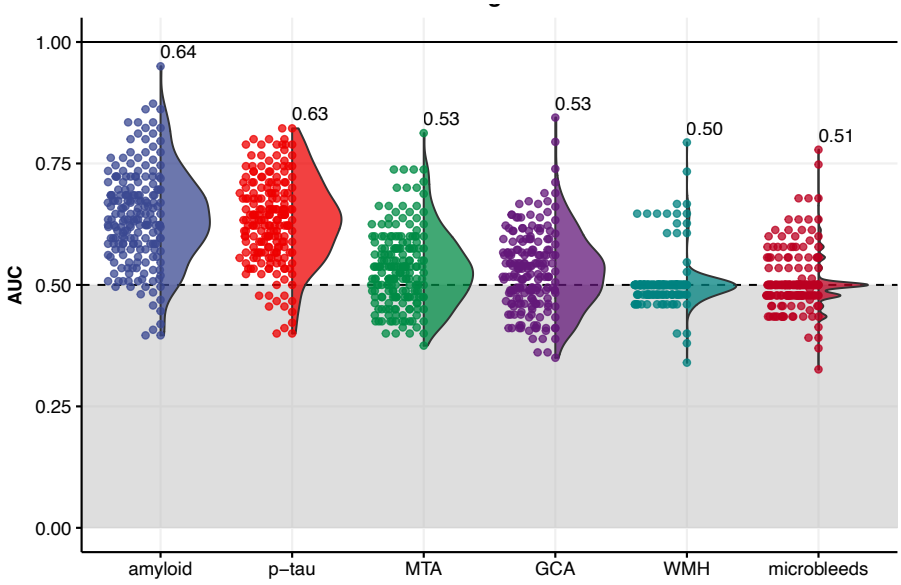
Descriptive characteristics of microbiota composition, differences between diagnosis groups. A. Compositional plot of top 20 genera with bars representing diagnosis groups: Alzheimer's disease dementia (AD), mild cognitive impairment (MCI) and subjective cognitive decline (SCD). "Unknown" refers to ASVs of which taxonomy was not known up to genus level. Genera with different abundances across groups (Kruskal-Wallis test, p <0.05) are marked in bold. B. Principal coordinate analysis (PCoA) plot of Bray-Curtis distances per diagnosis group with PERMANOVA test for group differences. C. Alpha diversity (Shannon index) of gut microbiota composition per diagnosis group.

Associations gut microbiota composition and AD biomarkers

The machine learning model for the prediction of amyloid status from gut microbiota composition performed best with an AUC of 0.64±0.10 (**Figure 3**). This model was closely followed by the p-tau model with an AUC of 0.63±0.09, while AUCs of the MRI visual scores ranged between 0.50 and 0.53. Highest ranked predictors of the amyloid (CSF) predicting model with all subjects included *[Eubacterium] ventriosum group* spp., *Subdoligranulum* spp., and *Anaerostipes* spp. In the model predicting p-tau, highest ranked microbes included *Lachnospiraceae* spp., *Lachnoclostridium edouardii* and *Blautia faecis*. These microbes are all anaerobic bacteria from the Firmicutes phylum and *Eubacterieae*, *Ruminococcaceae* and *Lachnospiraceae* families that are known for production of SCFAs. Some ASVs, including *Subdoligranulum* spp., *Roseburia hominis* and *Butyricoccus* spp., could be found in the top 20 predictors of both the amyloid and p-tau model. The receiver-operating curves (ROCs) of the amyloid and p-tau models with the relative importance of the highest ranked predictors can be found in **Supplement 4**.

Logistic regression models showed significant associations with amyloid status for 10 of the 20 highest ranked microbial predictors from the amyloid status machine learning model (**Figure 4A**) in model 1 and 2. Two ASVs, Coprococcus catus (OR 0.78 (0.63-0.97), p<0.05; model 2) and Oscillospiraceae UCG-005 spp. (OR 0.76 (0.59-0.93), p<0.05; model 2), were only associated with amyloid status in model 1 and 2. Eight associations remained significant in model 3, adjusting for age, sex, BMI, diabetes, proton pump inhibitor and statin use, and MMSE, including [Eubacterium] ventriosum group spp. (OR 0.76 (0.62-0.91) per log2-increase in counts, p<0.01), *Lachnospiraceae* spp. (OR 0.69 (0.49-0.97), p<0.05), *Marvinbryantia* spp. (OR 0.72 (0.53-0.96), p<0.05), *Monoglobus* spp. (OR 0.75 (0.57-0.98)), *[Ruminococcus] torques group* spp. (OR 0.84 (0.71-0.99), p<0.05), *Roseburia hominis* (OR 0.78 (0.63-0.95), p<0.05), and *Christensenellaceae R-7* spp. (OR 0.82 (0.68-0.96), p<0.05), and *[Clostridium] leptum* spp. (OR 1.55 (1.18-2.12), p<0.01). Six of the top 20 highest ranked microbial predictors from the p-tau status model were associated with p-tau status in the fully adjusted model 3 (**Figure 4B**). These included two *Lachnospiraceae* spp. ASVs (OR 0.49 (0.33-0.67), p<0.001, and OR 0.72 (0.54-0.94), p<0.05), *Lachnospiraceae edouardii* (OR 0.62 (0.41-0.85), p<0.01) and *Lachnoclostridium* spp. (OR 0.72 (0.54-0.94), p<0.01), which all belong to the *Lachnospiraceae* family. In addition, *Roseburia hominis* (OR 0.81 (0.64-0.99), p<0.05) and Bilophila wadsworthia (OR 0.72 (0.52-0.97), p<0.05) were lower abundant in patients with a positive p-tau status.

Figure 3: Machine learning models: AUCs

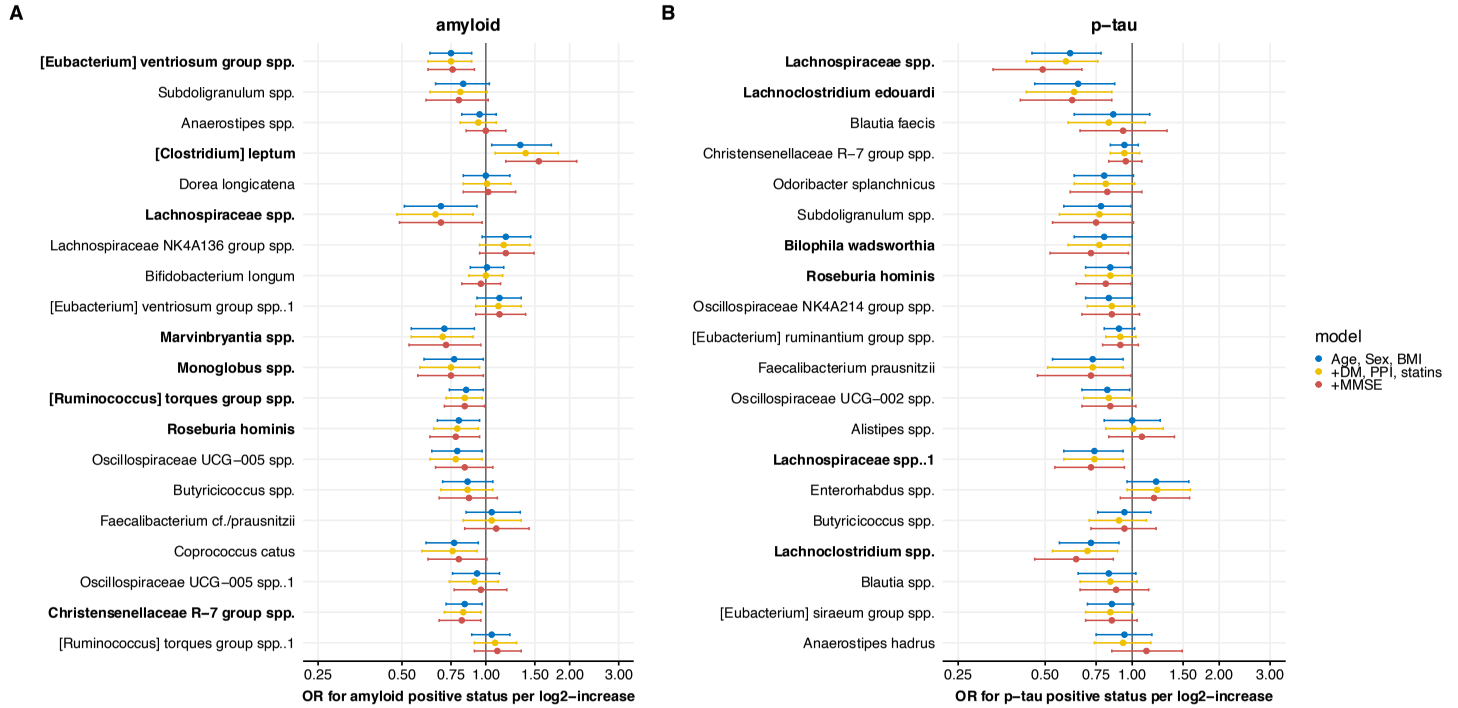


Distribution of area under the receiver-operating curves (AUCs) resulting from 200 iterations of the machine learning classification models (XGBoost algorithm) for each outcome. The labels indicate the mean AUC over 200 iterations. MTA=medial temporal atrophy, GCA=global cortical atrophy, WMH=white matter hyperintensities.

Associations of top predicting microbes with other biomarkers

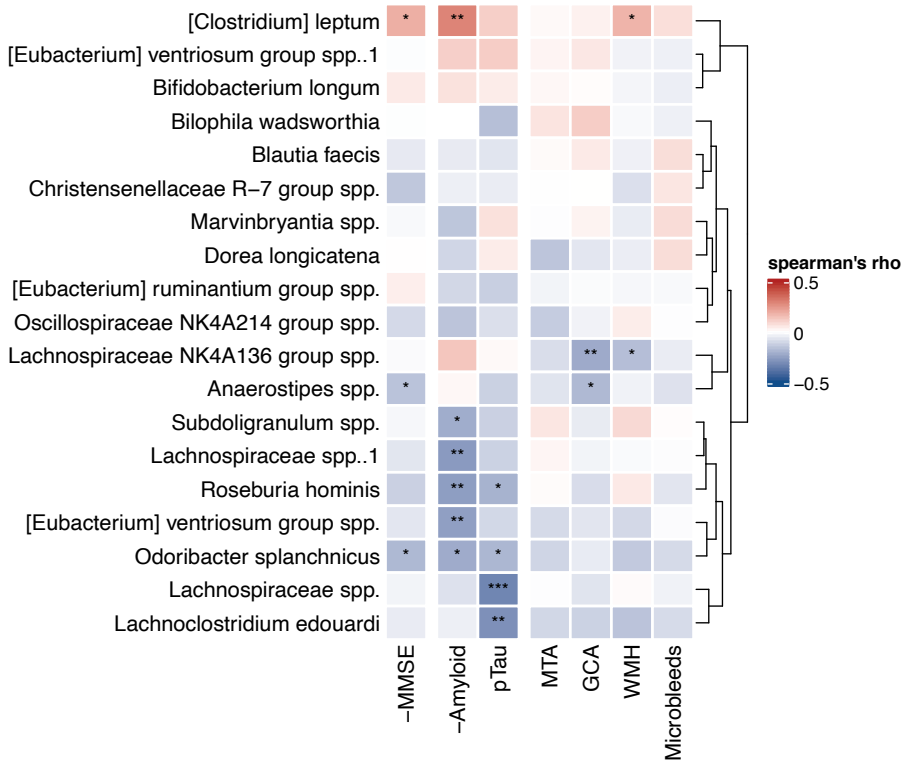
We also calculated Spearman's correlations between the 10 highest ranked microbes from the amyloid and p-tau models (19 microbes in total, because of an overlap of one ASV) and all AD biomarkers, including amyloid and p-tau levels (**Figure 5**). Five ASVs correlated with higher amyloid levels ($0.27<\rho<0.22$), while one ASV, *[Clostridium] leptum*, correlated with lower amyloid levels (ρ 0.29, $p<0.01$). Four ASVs correlated with lower p-tau levels ($-0.33<\rho<-0.19$). *Roseburia hominis* and *Odoribacter splanchicus* correlated with both higher amyloid and lower p-tau levels. *Lachnospiraceae NK4A136 group* spp. and *Anaerostipes* spp. correlated with lower GCA visual scores on MRI. In addition, *Anaerostipes* spp. and *Odoribacter splanchicus* correlated with higher MMSE scores, while *[Clostridium] leptum* correlated with lower MMSE scores.

Figure 4: Logistic regression models



Forest plots with results from the logistic regression models with associations between the 20 highest ranked microbial predictors from the machine learning model, ordered by ranking, and A. amyloid and B. p-tau positive status. Three models are shown: 1) adjusted for age, sex and body mass index (BMI), 2) additionally adjusted for diabetes mellitus (DM), use of proton pump inhibitors (PPI) and statins and 3) additionally adjusted for mini-mental state examination (MMSE) score. Results are presented as odds ratios (OR) with 95% confidence intervals. Microbes that were significantly associated with amyloid status in model 3 are marked in bold.

Figure 5: Heatmap of correlations with highest ranked predictors



*Spearman's correlations between 10 highest ranked microbial predictors from the amyloid and p-tau machine learning models and continuous AD biomarkers. Hierarchical clustering (Ward's method) was used to order the microbes and draw the dendrogram on the right. Correlations with MMSE and amyloid CSF levels are reversed for interpretability (-MMSE and -Amyloid), as lower values of these variables are indicative for pathology, in contrast to the other biomarkers. Negative (blue) correlations in this heatmap reflect correlations with less biomarkers indicative for AD pathology. Row (microbe) order was determined by hierarchical clustering. * p<0.05, ** p<0.01, *** p<0.001. MMSE=mini-mental state examination, P-tau=phosphorylated tau, MTA=medial temporal atrophy, GCA=global cortical atrophy, WMH=white matter hyperintensities.*

Discussion

Our main findings are the associations between gut microbiota composition and CSF amyloid and p-tau status. Discriminative value of the models predicting amyloid and p-tau status from gut microbiota composition was modest, but nonetheless we provide evidence that several SCFA-producing microbes are altered in patients with abnormal CSF amyloid and/or p-tau. We extend on animal studies reporting associations between SCFAs and amyloid pathology by showing that lower abundance SCFA-producing microbes was associated with lower odds of amyloid and p-tau positive status.[15,42]

Five cross-sectional studies of differences in gut microbiota between patients with AD and controls found that several microbes were less abundant in AD, including *Faecalibacterium prausnitzii, Eubacterium*, *Anaerostipes, Ruminococcus,* and *Roseburia* spp, while other microbes, such as *Odoribacter splanchicus*, *Bacteroides, Prevotella,* and *Alistipes* spp., were more abundant.[16–20] In line with these studies, we found that many of the highest ranked predictors for amyloid and p-tau status belonged to the *Lachnospiraceae* family, including *Roseburia hominis*, *[Ruminococcus] torques, Lachnoclostridium, Monoglobus* and *Marvinbryantia* spp. In contrast to earlier findings, higher abundance of *Odoribacter splanchicus* and *Alistipes* spp. correlated with more normal levels of AD biomarkers (higher amyloid and lower p-tau CSF levels) in our analyses, albeit these associations were lost after adjustment for covariates.

Two previous studies investigated associations between AD biomarkers and a specific subset of gut microbes.[16,21] One cross-sectional study correlated 13 microbial genera, that were differently abundant between AD patients and controls including a few that are SCFA-producing, with amyloid and p-tau levels in 40 patients. *Blautia* and *Bacteroides* spp. were associated with higher levels of biomarkers indicative of AD pathology, while *SMB53* and *cc115* spp. were associated with lower AD biomarkers. Of these genera, only *Blautia faecis* was also among the best predictors for p-tau status in our analyses, although this association was not significant in the adjusted analyses. These different findings could be explained by the older study population or by their inclusion of very low abundance taxa in the statistical analyses. A study that assessed differences between amyloid positive and negative patients in six microbes measured using qPCR found that *Escherichia/Shigella* spp. were more abundant while *Eubacterium rectale* was less abundant in amyloid positive patients[21] Indeed, several *Eubacterium* species were among the highest ranked predictors for amyloid status in our analyses. We did, howev-

er, not confirm the *Escherichia/Shigella* association, most likely because qPCR is more sensitive in finding changes in low abundant pathogens than 16S rRNA gene amplicon sequencing. *[Clostridium] leptum,* a microbe from the *Oscillospiraceae* family, was the only ASV associated with higher odds of amyloid positive status, and also correlated with lower continuous amyloid CSF levels. To our knowledge, we are the first to report an association between this microbe and AD biomarkers.

Our analyses allowed us to differentiate between predictors for amyloid and p-tau status. Microbial predictors for amyloid and p-tau status showed some overlap, such as *Roseburia hominis* and *Lachnospiraceae* spp. We also found differences in highest ranked predictors for amyloid compared to p-tau status; microbial strains from the *Eubacterium* and *Ruminococcus* genera were the highest ranked predictors for amyloid status, while several *Lachnoclostridium* spp. were among the highest predictors for p-tau status.

In contrast to our findings in CSF amyloid and p-tau, we did not find associations between microbiota composition and MRI measures including vascular markers such as WMH and microbleeds in our machine learning model (AUC 0.50), perhaps due to the low prevalence of cerebrovascular damage in this young study population. The low prevalence of cerebrovascular damage also makes it unlikely that the observed associations with amyloid and p-tau were mediated by vascular pathology.

There are several hypotheses regarding the mechanisms by which gut microbiota could affect AD pathology which involve several metabolites and toxins. Lipopolysaccharide (LPS) can be found in the outer membrane of gram-negative bacteria and has been shown to elicit peripheral inflammatory responses, affect the permeability of the blood-brain barrier and induce neuroinflammation.[43,44] In contrast, capsular polysaccharide A (PSA) of *Bacteroides fragilis* species has been shown to have anti-inflammatory effects on the peripheral immune system,[45] and to suppress central neuroinflammation by induction of T-regulatory cells in mice.[46] However, *Bacteroides fragilis* was not among the highest ranked predictors for amyloid nor p-tau status in our analyses, nor were other species from the gram-negative *Bacteroides* genus.

The highest ranked predictors were mostly species from the predominantly gram-positive Firmicutes phylum known for SCFA production. SCFAs, including acetate, propionate and butyrate, are produced by gut bacteria in fermentation processes of otherwise undigestible dietary fibers and have immunomodulatory potential.[10,47] SCFAs could have indirect effects on AD pathology by induction of peripheral inflammation or by altering the integrity of the blood-brain barrier, as

shown by a butyrate intervention study in germ-free mice.[42] Alternatively, SCFA could have direct anti-inflammatory effects on microglia as was shown in an in vitro study.[48] In that regard, future studies could focus on associations between fecal and plasma SCFA levels and inflammatory brain markers such as glial fibrillary acidic protein (GFAP).[49]

There are several limitations of our study including the cross-sectional design which warrants caution that observed associations should not be interpreted as causal relationships. Moreover, time lags between the biomarker measurements and the fecal sampling might have confounded some associations. Although we adjusted for relevant confounders such as age, sex, BMI, diabetes and medication use, we cannot rule out residual confounding. Dietary factors in particular have been shown to affect microbiota composition.[50] Since AD patients tend to lose weight over the course of the disease, it has been suggested that cognitive decline could lead to lower energy intake which might also affect microbiota composition.[51] However, we have found previously that macronutrient intake was not different between diagnosis groups in this cohort.[52] Moreover, associations between gut microbiota composition and AD biomarkers remained significant when adjusting for cognitive function (MMSE). Of note, higher abundance of SCFA-producing microbes is indicative for, but does not necessarily reflect higher gut or plasma SCFA levels. To assess microbial production of SCFAs, metagenomic sequencing would be needed, which was not within the scope of the current study.

Strengths of this study include the availability of several AD biomarkers, including CSF and MRI data, and the inclusion of patients in different stages of the AD disease continuum. Fecal samples were obtained using a standardized protocol, participants taking antibiotics were excluded, and microbiota composition was determined with 16S gene amplicon sequencing, which is a widely used sequencing method. Machine learning prediction models enabled us to simultaneously include all ASVs as features in order to find the best predicting microbes. Nested cross-validation ensured robustness of the models and prevented overfitting.

The putative relation between gut microbiota composition and AD pathology, may provide opportunities for future treatment. Different treatment strategies based on modulating gut microbiota composition have been investigated in other diseases such as inflammatory bowel disease and diabetes.[53–55] Fecal microbiota transplantation (FMT) aims to restore gut microbiota composition by administering microbiota from healthy donors to diseased subjects through a nasoduodenal tube.[55] In obese subjects, FMT has been shown to alter brain dopamine

transporter binding, thus pointing towards a gut-brain connection.[56] Nonetheless, FMT is logistically challenging and the effects of transplantation fade over time.[57,58] Another strategy includes the use of prebiotics (often fiber supplements) aimed to promote the growth of certain microbes, or probiotics, supplements of beneficial strains.[59] A meta-analysis showed positive effects on cognition of *Bifidobacterium* and *Lactobacillus* probiotics in patients with MCI.[60] However, beneficial butyrate-producing species are often strictly anaerobic or oxygen sensitive, complicating culturing and probiotic production.[61] A third strategy is to directly target microbial pathways such as SCFA production, by interventions with high fiber intake or by administering SCFAs including acetate or sodium butyrate.[62,63]

Concluding, we found associations between gut microbiota composition and AD pathology in our memory clinic cohort. Lower abundance of SCFA-producing microbes was associated with higher odds of AD pathology. SCFAs are known to have peripheral immunomodulatory potential, providing a putative target for treatment.

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Conflicts of interest

Charlotte E. Teunissen received grants from the European Commission, the Dutch Research Council (ZonMW), Association of Frontotemporal Dementia/Alzheimer's Drug Discovery Foundation, The Weston Brain Institute, Alzheimer Netherlands. Charlotte E. Teunissen has a collaboration contract with ADx Neurosciences, performed contract research or received grants from Probiodrug, Biogen, Esai, Toyama, Janssen prevention center, Boehringer, AxonNeurosciences, Fujirebio, EIP farma, PeopleBio, and Roche. Frederik Barkhof is a consultant for Biogen-Idec, Bayer-Schering, Merck-Serono, Roche, Combinostics and IXICO; has received sponsorship from European Commission–Horizon 2020, National Institute for Health Research–University College London Hospitals Biomedical Research Centre, Novartis, and Merck; and serves on the editorial boards of *Radiology, Neuroradiology, Multiple Sclerosis Journal*, and *Neurology*. Philip Scheltens has

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Author contributions

WF, CT, FB, PS and CD contributed to conception and design of the study. HH, FL, AD, ML and BV collected the data. RK was responsible for the sequencing of the samples. BV performed the statistical analyses. WF, MN and MM contributed to the interpretation of the results. BV wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

References

1. Heneka, M. T. *et al.* Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405 (2015).
2. Levy, M., Kolodziejczyk, A. A., Thaiss, C. A. & Elinav, E. Dysbiosis and the immune system. *Nat. Rev. Immunol.* 17, 219–232 (2017).
3. van Olst, L. *et al.* Contribution of Gut Microbiota to Immunological Changes in Alzheimer's Disease. *Front. Immunol.* 12, 683068 (2021).
4. Saresella, M. *et al.* Alterations in Circulating Fatty Acid Are Associated With Gut Microbiota Dysbiosis and Inflammation in Multiple Sclerosis. *Front Immunol* 11, 1390 (2020).
5. Moccia, M. *et al.* Single-Arm, Non-randomized, Time Series, Single-Subject Study of Fecal Microbiota Transplantation in Multiple Sclerosis. *Front. Neurol.* 11, 978 (2020).
6. Ley, R. E., Peterson, D. A. & Gordon, J. I. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124, 837–848 (2006).
7. Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J. & Duncan, S. H. The influence of diet on the gut microbiota. *Pharmacol. Res.* 69, 52–60 (2013).

8. Gentile, C. L. & Weir, T. L. The gut microbiota at the intersection of diet and human health. *Science* (80) 362, 776–780 (2018).
9. Cerf-Bensussan, N. & Gaboriau-Routhiau, V. The immune system and the gut microbiota: Friends or foes? *Nat. Rev. Immunol.* 10, 735–744 (2010).
10. Deleu, S., Machiels, K., Raes, J., Verbeke, K. & Vermeire, S. Short chain fatty acids and its producing organisms: An overlooked therapy for IBD? *EBioMedicine* 66, 103293 (2021).
11. Zhang, L. *et al.* Altered Gut Microbiota in a Mouse Model of Alzheimer's Disease. *J. Alzheimer's Dis.* 60, 1241–1257 (2017).
12. Sun, J. *et al.* Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl. Psychiatry* 9, 1–13 (2019).
13. Kim, M. S. *et al.* Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model. *Gut* 69, 283–294 (2020).
14. Cox, L. M. *et al.* Calorie restriction slows age-related microbiota changes in an Alzheimer's disease model in female mice. *Sci. Rep.* 9, 1–14 (2019).
15. Fernando, W. M. A. D. B. *et al.* Sodium Butyrate Reduces Brain Amyloid-β Levels and Improves Cognitive Memory Performance in an Alzheimer's Disease Transgenic Mouse Model at an Early Disease Stage. *J. Alzheimer's Dis.* 74, 91–99 (2020).
16. Vogt, N. M. *et al.* Gut microbiome alterations in Alzheimer's disease. *Sci. Rep.* **7**, 1–11 (2017).
17. Zhuang, Z. Q. *et al.* Gut Microbiota is Altered in Patients with Alzheimer's Disease. *J. Alzheimer's Dis.* 63, 1337–1346 (2018).
18. Haran, J. P. *et al.* Alzheimer's disease microbiome is associated with dysregulation of the anti-inflammatory P-glycoprotein pathway. *MBio* 10, e00632-19 (2019).
19. Liu, P. *et al.* Altered microbiomes distinguish Alzheimer's disease from amnestic mild cognitive impairment and health in a Chinese cohort. *Brain. Behav. Immun.* 80, 633–643 (2019).
20. Ueda, A. *et al.* Identification of Faecalibacterium prausnitzii strains for gut microbiome-based intervention in Alzheimer's-type dementia. *Cell Reports Med.* 2, 100398 (2021).
21. Cattaneo, A. *et al.* Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiol. Aging* 49, 60–68 (2017).
22. van der Flier, W. M. *et al.* Optimizing Patient Care and Research: The Amsterdam Dementia Cohort. *J. Alzheimer's Dis.* 41, 313–327 (2014).
23. van der Flier, W. M. & Scheltens, P. Amsterdam Dementia Cohort: Performing Research to Optimize Care. *J. Alzheimer's Dis.* 62, 1091–1111 (2018).
24. Slot, R. E. R. *et al.* Subjective Cognitive Impairment Cohort (SCIENCe): study design and first results. *Alzheimer's Res. Ther.* 10, 1–13 (2018).
25. Albert, M. S. *et al.* The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's Dement.* **7**, 270–279 (2011).
26. McKhann, G. *et al.* Clinical diagnosis of Alzheimer's disease. *Neurology* 34, 939–939 (1984).
27. Tombaugh, T. N. & McIntyre, N. J. The Mini-Mental State Examination: A

Comprehensive Review. *J. Am. Geriatr. Soc.* 40, 922–935 (1992).
28. Fadrosh, D. W. *et al.* An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2, 1–7 (2014).
29. Mulder, C. *et al.* Amyloid-β(1–42), Total Tau, and Phosphorylated Tau as Cerebrospinal Fluid Biomarkers for the Diagnosis of Alzheimer Disease. *Clin. Chem.* 56, 248–253 (2010).
30. Tijms, B. M. *et al.* Unbiased Approach to Counteract Upward Drift in Cerebrospinal Fluid Amyloid-β 1–42 Analysis Results. *Clin. Chem.* 64, 576–585 (2018).
31. Willemse, E. A. J. *et al.* Diagnostic performance of Elecsys immunoassays for cerebrospinal fluid Alzheimer's disease biomarkers in a nonacademic, multicenter memory clinic cohort: The ABIDE project. *Alzheimer's Dement. Diagnosis, Assess. Dis. Monit.* 10, 563 (2018).
32. Scheltens, P. *et al.* Atrophy of medial temporal lobes on MRI in 'probable' Alzheimer's disease and normal ageing: diagnostic value and neuropsychological correlates. *J. Neurol. Neurosurg. Psychiatry* 55, 967–972 (1992).
33. Rhodius-Meester, H. F. M. *et al.* MRI visual ratings of brain atrophy and white matter hyperintensities across the spectrum of cognitive decline are differently affected by age and diagnosis. *Front. Aging Neurosci.* 9, 117 (2017).
34. Pasquier, F. *et al.* Inter-and intraobserver reproducibility of cerebral atrophy assessment on mri scans with hemispheric infarcts. *Eur. Neurol.* 36, 268–272 (1996).
35. Fazekas, F., Chawluk, J. B., Alavi, A., Hurtig, H. I. & Zimmerman, R. A. MR signal abnormalities at 1.5 T in Alzheimer's dementia and normal aging. *Am. J. Roentgenol.* 8, 421–426 (1987).
36. Wardlaw, J. M. *et al.* Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. *Lancet Neurol.* 12, 822–838 (2013).
37. Faith, D. P. Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10 (1992).
38. Hill, M. O. Diversity and Evenness: A Unifying Notation and Its Consequences. *Ecology* 54, 427–432 (1973).
39. Chen, T. & Guestrin, C. XGBoost: A scalable tree boosting system. in *Proceedings of the ACM SIGKDD International Conference on Knowledge Discovery and Data Mining* 785–794 (2016). doi:10.1145/2939672.2939785.
40. Wang, X.-W. & Liu, Y.-Y. Comparative study of classifiers for human microbiome data. *Med. Microecol.* 4, 100013 (2020).
41. Mirzayi, C. *et al.* Reporting guidelines for human microbiome research: the STORMS checklist. *Nat. Med.* 27, 1885–1892 (2021).
42. Braniste, V. *et al.* The gut microbiota influences blood-brain barrier permeability in mice. *Sci. Transl. Med.* (2014) doi:10.1126/scitranslmed.3009759.
43. Xaio, H., Banks, W. A., Niehoff, M. L. & Morley, J. E. Effect of LPS on the permeability of the blood–brain barrier to insulin. *Brain Res.* 896, 36–42 (2001).
44. Moissl-Eichinger, C., Willing, B. P., Burke, C. M. & Lukiw, W. J. Bacteroides fragilis Lipopolysaccharide and Inflammatory Signaling in Alzheimer's Disease. *Front. Microbiol.* 7, 1544 (2016).
45. Shen, Y. *et al.* Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. *Cell Host Microbe* 12, 509–520 (2012).
46. Wang, Y. *et al.* A commensal bacterial product elicits and modulates

migratory capacity of CD39+ CD4 T regulatory subsets in the suppression of neuroinflammation. *Gut Microbes* 5, 552–561 (2014).
47. Venegas, D. P. *et al.* Short chain fatty acids (SCFAs)mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10, 277 (2019).
48. Wenzel, T. J., Gates, E. J., Ranger, A. L. & Klegeris, A. Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. *Mol. Cell. Neurosci.* 105, 103493 (2020).
49. Verberk, I. M. W. *et al.* Serum markers glial fibrillary acidic protein and neurofilament light for prognosis and monitoring in cognitively normal older people: a prospective memory clinic-based cohort study. *Lancet Heal. Longev.* 2, e87–e95 (2021).
50. Claesson, M. J. *et al.* Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488, 178–184 (2012).
51. Poehlman, E. T. & Dvorak, R. V. Energy expenditure, energy intake, and weight loss in Alzheimer disease. *Am. J. Clin. Nutr.* 71, 650S-655S (2000).
52. Doorduijn, A. S. *et al.* Energy intake and expenditure in patients with Alzheimer's disease and mild cognitive impairment: the NUDAD project. *Alzheimers. Res. Ther.* 12, 1–8 (2020).
53. Vieira, A. T., Fukumori, C. & Ferreira, C. M. New insights into therapeutic strategies for gut microbiota modulation in inflammatory diseases. *Clin. Transl. Immunol.* 5, e87 (2016).
54. Meijnikman, A. S., Gerdes, V. E., Nieuwdorp, M. & Herrema, H. Evaluating Causality of Gut Microbiota in Obesity and Diabetes in Humans. *Endocr. Rev.* 39, 133–153 (2018).
55. Groot, P. de *et al.* Faecal microbiota transplantation halts progression of human new-onset type 1 diabetes in a randomised controlled trial. *Gut* 70, 92–105 (2021).
56. Hartstra, A. V. *et al.* Infusion of donor feces affects the gut–brain axis in humans with metabolic syndrome. *Mol. Metab.* 42, 101076 (2020).
57. Kootte, R. S. *et al.* Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. *Cell Metab.* 26, 611-619.e6 (2017).
58. Chen, J., Zaman, A., Ramakrishna, B. & Olesen, S. W. Stool Banking for Fecal Microbiota Transplantation: Methods and Operations at a Large Stool Bank. *Front. Cell. Infect. Microbiol.* 11, 281 (2021).
59. Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R. & Rastall, R. A. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616 (2019).
60. Zhu, G., Zhao, J., Zhang, H., Chen, W. & Wang, G. Probiotics for Mild Cognitive Impairment and Alzheimer's Disease: A Systematic Review and Meta-Analysis. *Foods* 10, 1672 (2021).
61. Andrade, J. C. *et al.* Commensal Obligate Anaerobic Bacteria and Health: Production, Storage, and Delivery Strategies. *Front. Bioeng. Biotechnol.* 8, 550 (2020).
62. Wijdeveld, M., Nieuwdorp, M. & IJzerman, R. The interaction between microbiome and host central nervous system: the gut-brain axis as a potential new therapeutic target in the treatment of obesity and cardiometabolic disease. *Expert Opin. Ther.*

Targets 24, 639–653 (2020).
63. Bouter, K. E. C. *et al.* Differential metabolic effects of oral butyrate treatment in lean versus metabolic syndrome subjects article. *Clin. Transl. Gastroenterol.* 9, (2018).

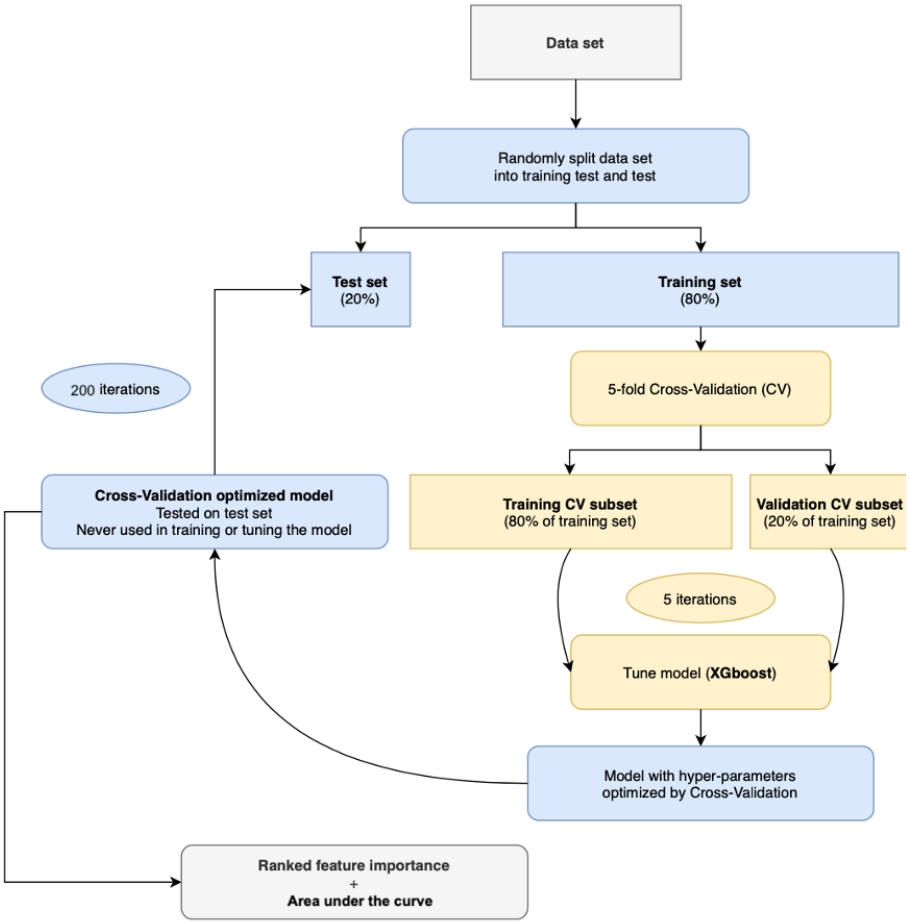
Supplements

Supplement 1: Bioinformatic pipeline

16S rRNA primers were removed from the sequencing reads using seqtk (v.1.3). The reads were subsequently processed using dada2 (v.1.18) as follows. After examining read quality profiles, 50 bases were trimmed from the 5' end of the forward reads, and 60 bases from the 5' of the reverse reads, respectively. The reads were truncated at the first base with a Q score lower than 4, then quality filtered using 2 maximum expected error for the forward reads and 4 maximum expected errors for the reverse reads, allowing for no ambiguous bases. The filtered reads were used to learn the error rates and to infer Amplicon Sequence Variants (ASVs) separately for the forward and the reverse reads. Forward and reverse ASVs were merged allowing no mismatching bases and requiring a minimum overlap of 20 bases. ASVs shorter than 350 bp, longer than 500 bp, and chimeric ASVs were removed. An ASV table was constructed for the remaining ASVs. ASV taxonomy was then assigned using the dada2 *assignTaxonomy* function and the SILVA database (v.138) allowing up to 3 multiple species-level assignments.[1,2] The ASV table and taxonomy were integrated using the phyloseq R package (v.1.34.0). The ASV table was rarefied to 20,000 counts per sample.[3] Of 175 sequenced samples, 5 had insufficient counts (<20,000) and were excluded at the rarefaction stage.

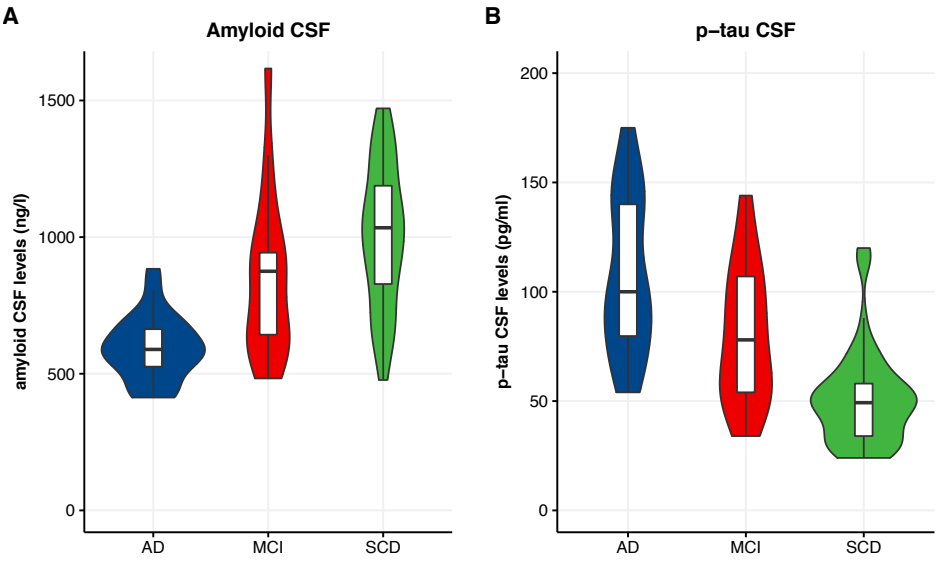
1. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al.* (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* doi:10.1093/nar/gks1219.
2. Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods.* doi:10.1038/nmeth.3869.
3. McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE.* doi:10.1371/journal.pone.0061217.

Supplement 2: Machine learning design



Schematic overview of the machine learning design.

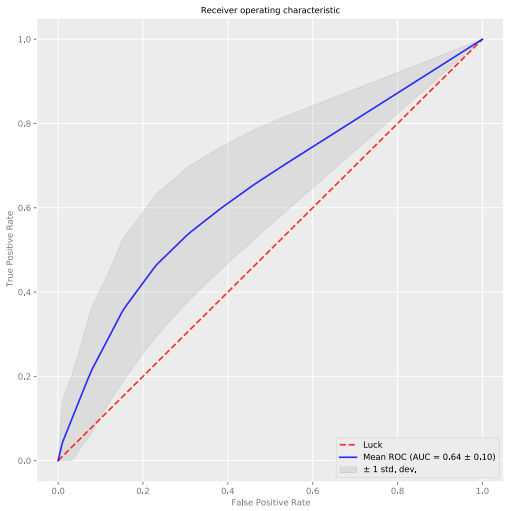
Supplement 3: Distribution of CSF biomarkers



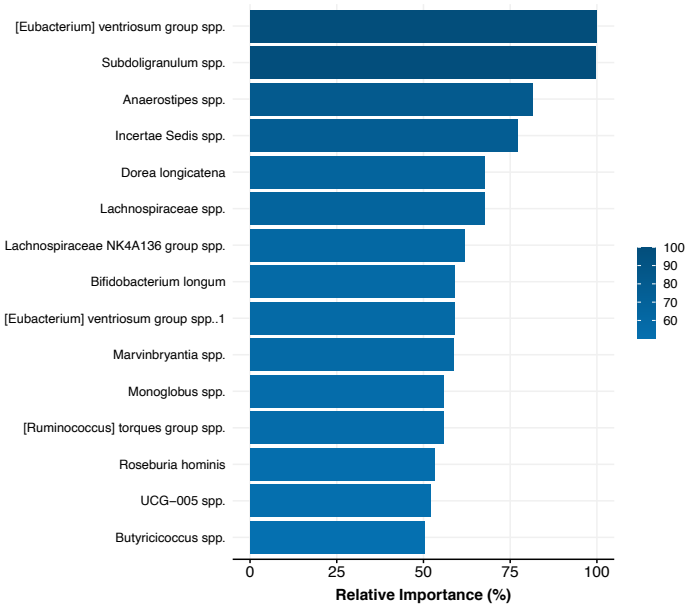
Distribution of (A) amyloid and (B) p-tau CSF levels per diagnosis group.

Supplement 4: Machine learning results amyloid and p-tau CSF model

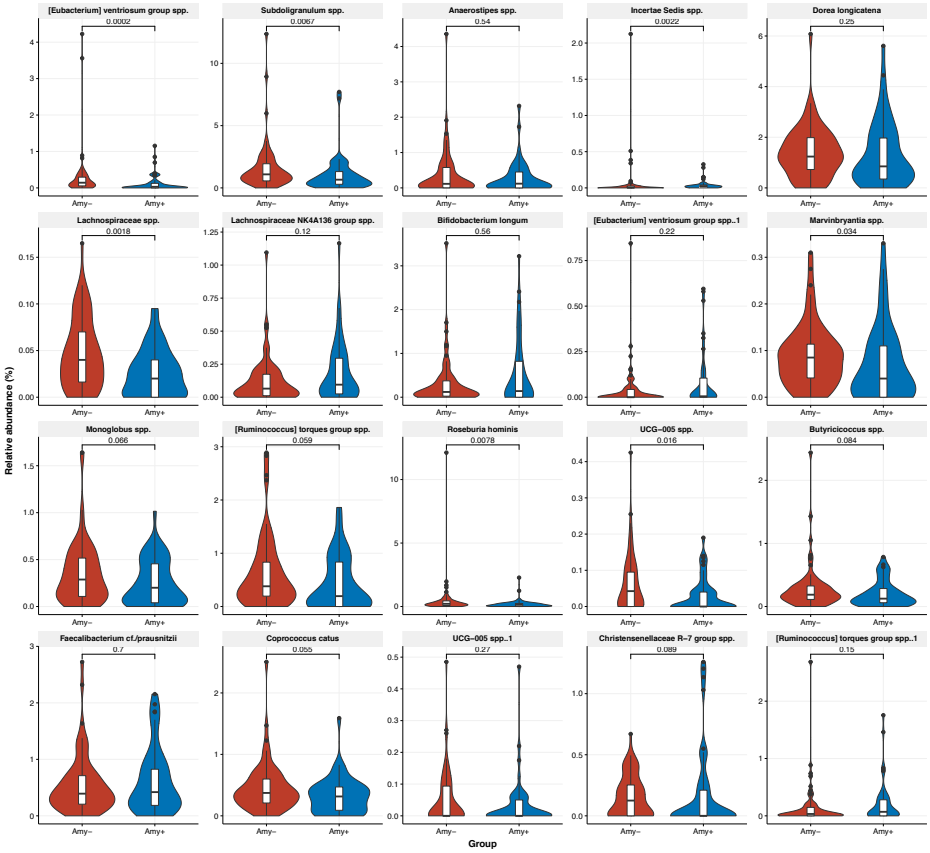
Amyloid CSF



AUC for machine learning model predicting amyloid CSF status from microbiota composition.

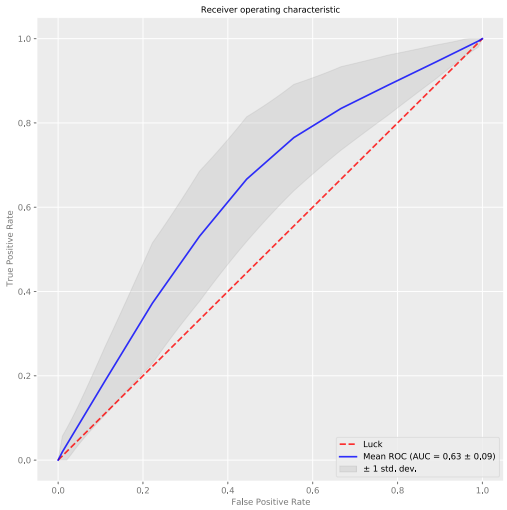


Feature importance of highest ranked predictors of the machine learning model for amyloid CSF status

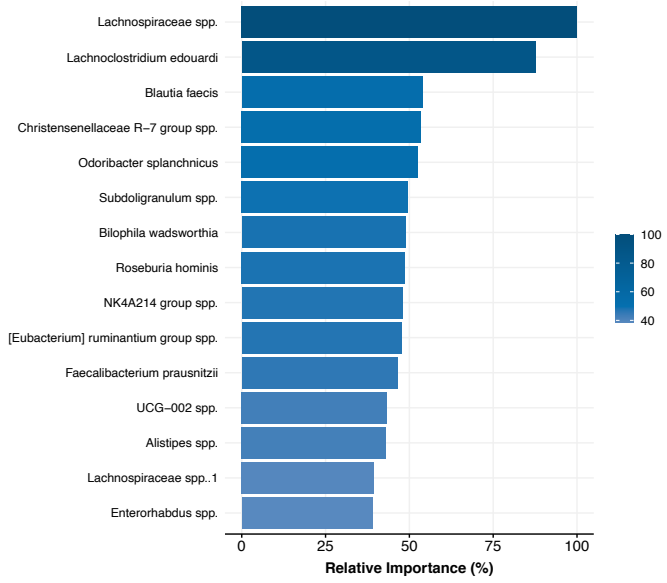


Violin plots with differences in 20 highest ranked predictors between amyloid positive (Amy+) and amyloid negative (Amy-) subjects. Differences between groups were tested with Mann-Whitney U tests.

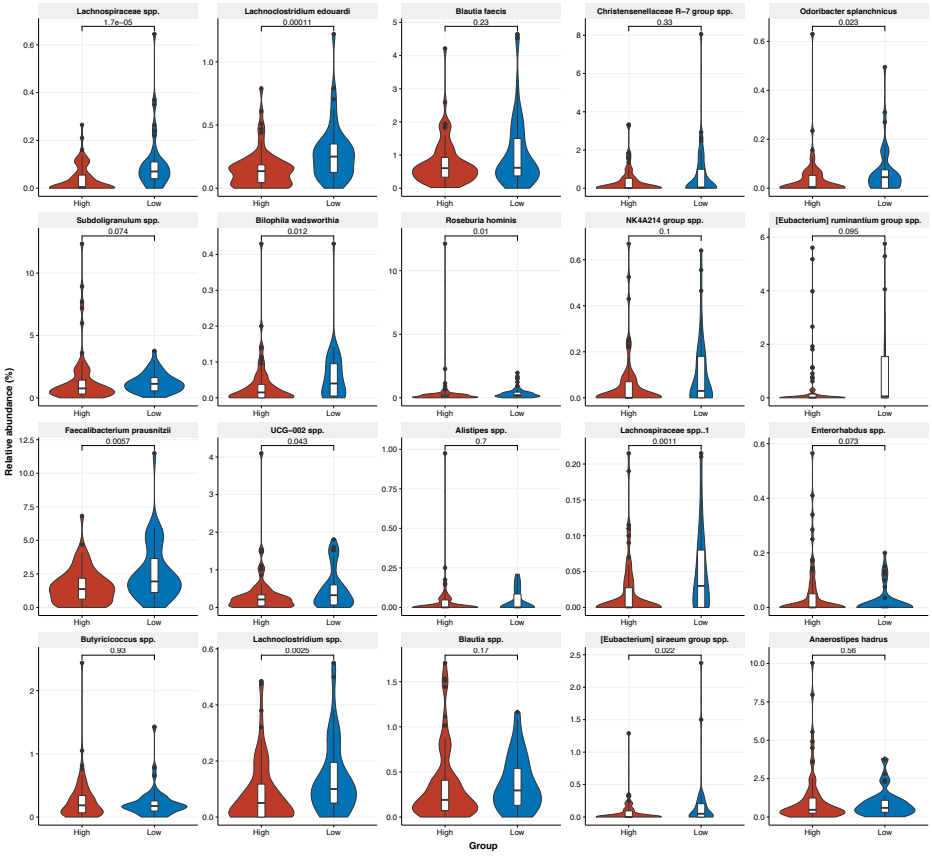
p-tau CSF



AUC for machine learning model predicting p-tau CSF status from microbiota composition.



Feature importance of highest ranked predictors of the machine learning model for p-tau CSF status.



Violin plots with differences in 20 highest ranked predictors between high and low p-tau subjects. Differences between groups were tested with Mann-Whitney U tests.

9

Summary and discussion

Summary

In this thesis, we explored the role of gut microbiota and its metabolites in two public health challenges: hypertension (**Part I**) and Alzheimer's disease (**Part II**).

Part I: Hypertension

In **Chapter 2**, we examined the relationship between gut microbiota and blood pressure across different ethnic groups in the HELIUS cohort. We found that bacteria that are known for short chain fatty acid (SCFA) production were associated with lower blood pressure. However, when we assessed fecal SCFA levels, we noticed elevated concentrations in individuals with high blood pressure.The findings suggest that gut microbiota and SCFAs may play a role in the development of hypertension, either as a causal factor or as a consequence of the condition.

Chapter 3 provides a comprehensive review of the current literature on the role of gut microbiota in hypertension and atherosclerosis. It highlights the complex interactions between gut microbiota and various physiological systems involved in blood pressure regulation and atherosclerosis, including the immune system, the renin-angiotensin-aldosterone system, and the autonomous nervous system. Mechanisms by which gut microbiota might specifically affect blood pressure are SCFA, lipopolysaccharides and the gut-brain axis. Overall, the existing literature suggests that gut microbiota may be a promising target for the prevention and treatment of hypertension and atherosclerosis, while challenges ahead include the translation of the findings to humans.

Chapter 4 investigates the effects of oral butyrate, a SCFA, on blood pressure in patients with hypertension in a randomized placebo-controlled trial. We found that treatment with butyrate for four weeks increased daytime systolic blood pressure compared to placebo. These are likely to be direct effects of butyrate, since oral butyrate only increased plasma butyrate yet no other SCFA levels. Although existing intervention studies with multiple SCFA showed their blood pressure lowering potential, this study showed that not all SCFA have beneficial effects on human hypertension. More mechanistic studies are needed to explain the differential effects of SCFA in humans.

Chapter 5 explored the associations of plasma metabolites with blood pressure. Our machine learning analyses showed that formylmethionine was a top predict-

ing metabolite for systolic and diastolic blood pressure, and a very consistent predictor across age, sex and ethnicity. Next, we investigated the in vitro effects of this metabolite on endothelial cells. We found that formylmethionine induced endothelial dysfunction, and specifically, that it suppressed eNOS, disrupted the endothelial barrier and increased oxidative stress. We hypothesize that formylmethionine is a mitochondrial damage-associated molecular pattern (DAMP) that has a role in blood pressure regulation.

In **Chapter 6**, we revisit the metabolomics analyses from chapter 6 through the lens of sex differences. We found that plasma metabolite profiles are associated with blood pressure and autonomic cardiovascular control in a sex-specific manner. Sphingomyelins and conjugated bile acids were more predictive of BP in men, while metabolites from acylcarnitine and catecholamine pathways were better predictors in women. In addition, we could predict heart rate variability from metabolite profiles in men, but not in women, further underscoring potential sex differences in blood pressure physiology. Several of the best predicting metabolites were associated with gut microbiota composition. This could indicate that inventions targeted at the gut microbiota might have sex-specific effects, since plasma metabolites derived from the microbiota are differently associated with blood pressure for men and women.

In summary, we investigated the role of gut microbiota and plasma metabolites in hypertension. First, we found cross-sectional associations of SCFA-producing microbes, faecal SCFA levels and blood pressure. Next, we confirmed that butyrate, a SCFA, modulated blood pressure with a clinical trial in hypertensive patients. However, in contrast to existing human intervention studies on SCFA, butyrate had blood pressure increasing effects. In our plasma metabolomics analyses, we identified a novel metabolite that was associated with higher blood pressure: formylmethionine. This metabolite could be a bacterial product, but is more likely a mitochondrial metabolite that may have a role in blood pressure regulation. In the sex-stratified analyses of the metabolomics data, we showed that plasma metabolites are associated with blood pressure in a sex-specific manner. These analyses resulted in several leads for further mechanistic studies on sex differences in hypertension.

Part II: Alzheimer's disease

Chapter 7 examined the association between nutritional status and structural brain changes in patients with subjective cognitive decline (SCD), mild cognitive impairment (MCI) and Alzheimer's disease (AD) dementia in the NUDAD cohort. In this cross-sectional analysis, we found that lower indicators of nutritional status were associated with more atrophy on MRI, specifically global cortical atrophy and medial temporal atrophy. The study suggests that poor nutrition may contribute to the development and progression of Alzheimer's disease through its effects on brain structure, although this needs confirmation of interventional longitudinal studies. The findings highlight the importance of maintaining good nutritional status in patients with Alzheimer's disease.

In **Chapter 8**, we investigated the relationship between gut microbiota composition and biomarkers of AD pathology in the NUDAD cohort. We found modest associations between gut microbiota composition and cerebrospinal fluid AD biomarkers such as amyloid-beta and tau proteins. Specifically, higher abundance of several SCFA-producing microbes was associated with lower odds of abnormal CSF amyloid and/or p-tau levels. This association could be explained by the immunomodulatory potential of SCFA as shown by previous in vitro and in vivo studies. However, this was a cross-sectional analysis and therefore no conclusions on causality should be drawn. Future studies could focus on the relation between plasma and faecal SCFA and inflammatory markers such as glial fibrillary acidic protein (GFAP) in AD.

In conclusion, the studies of this second part of this thesis shed light on the potential impact of nutritional status and gut microbiota composition on Alzheimer's disease pathology. Since these associations were subtle, it seems more likely the gut microbiome has a disease-modulating rather than a causal role.

Discussion

This thesis explored the potential contribution of the gut microbiota and plasma metabolome in the context of two major public health concerns: hypertension and Alzheimer's disease. Through a range of approaches, including population-based cohorts, observational studies, clinical trials, and in vitro work, my research uncovered new perspectives that can guide future investigations in these areas. In this discussion, I would like to reflect on the topics touched upon in this thesis and put the findings in a broader context.

Reflections on gut microbiota research

The field of gut microbiome research is rapidly expanding, with numerous studies demonstrating changes in the gut microbiota composition in a variety of diseases, including inflammatory bowel disease, diabetes, depression, and Parkinson's disease.[1–4] However, this field is also facing challenges such as publication bias, which skews literature towards positive findings, and a general overabundance of publications, which causes single publications to be lost among the multitudes.[5] As the field advances, it seems increasingly unlikely that the gut microbiome is causally related to all of these diseases. Thus, rather than merely demonstrating an association between the microbiome and a disease (as we also did in Chapter 2 and 8), there is a growing emphasis on proving causality, identifying underlying mechanisms and capturing (some of) the complexity of the microbiome.

Interventions on gut microbiota composition are crucial for establishing causality. Fecal microbiota transplantation (FMT) has shown promise in the context of several diseases, especially *C. difficile* infections.[6] However, FMT is neither a long-term nor a scalable solution for large patient populations, since the process of FMT is logistically complex and effects might only be effective on the short term (weeks to months).[7] Supplementing specific combinations of gut microbes through probiotics may be a solution, but it requires determining which microbiota are beneficial. This is a challenge because engraftment of supplemented bacteria is not often measured or reported, and when analyzed, has substantial variation between subjects and studies.[8] At the same time, our understanding of the metabolic interactions between bacteria is lagging behind, while a better insight into these interactions could greatly facilitate targeting interventions. If the effects of gut microbiota are mediated by certain metabolites, supplementing these metabolites (postbiotics) might be sufficient to modify disease outcomes. Alternatively, dietary modifications to stimulate or lower production of specific

metabolites could be a strategy to target the metabolic effects of the microbiome.

Intuitively, the gut microbiome has a larger effect on organs that are in close proximity. Hence, inflammatory bowel syndrome is heavily impacted by the gut microbiome, while this is much less the case in neurodegenerative diseases like Alzheimer's disease (AD). This observation was also supported by the findings of this thesis, which found only a modest association between gut microbiota composition and AD biomarkers. However, even a small impact can be significant for conditions such as blood pressure and AD. The 4.4% of systolic blood pressure variance explained by gut microbiota composition, for example, could translate to a significant impact on cardiovascular mortality on a population-level in the longer term. Similarly, even a small impact on AD is relevant, as there is currently no treatment available to slow down the neurodegenerative process.

Beyond the focus of this thesis, though also for the diseases studied here, we should acknowledge the complexity of the gut microbiome. The impact of viruses and fungi cannot be ignored in this context. A recent publication with data obtained from the HELIUS study showed that subjects with metabolic syndrome have a distinct gut virome composition.[9] Moreover, the gut microbiota has a spatial dimension that is not accurately reflected in fecal samples, as microbial taxa distributions vary along the gastrointestinal tract. Bacterial diversity is, for instance, lowest in the small intestine and increases towards the colon. This spatial gradient could bias microbiota analyses, since the material in fecal samples might better reflect the gut microbiota composition in the distal colon than in the small intestine.[10] An ingestible collection device designed to collect samples along the gastrointestinal tract could provide more insight into the distribution of microbes and metabolites.[11] Additionally, diurnal fluctuations of the gut microbiome – to some extent caused by the diurnal rhythmicity of food intake - might result in slight differences in composition and function when sampling at different times of the day.[12] Thus, the field of microbiome research still had great methodological strides to make in order to deepen our understanding.

One prominent recent methodological advance is in sequencing approaches. In this thesis, we used 16S ribosomal RNA (rRNA) sequencing of fecal samples to determine gut microbiota composition as opposed to shotgun sequencing. 16S sequencing targets a specific region of the 16S rRNA gene present in bacteria and archaea. Many copies of the 16S gene target region are generated through PCR amplification and sequenced to identify the bacterial and archaeal taxa present in the original sample. However, due to variations in the conserved region of the 16S gene, primer binding affinity might differ for different microbial taxa. Certain taxa show mismatches in these conserved regions relative to the 16S primer

sequences, thus resulting in their underrepresentation in the sequence data. This can lead to biased estimates of microbial diversity and abundance in the original sample.[13] Shotgun metagenomics avoids this bias, while also enabling analysis of the functional capacities of the microbiome however at significant higher costs. Because of the use of 16S rRNA sequencing in this thesis, we cannot be sure that the short chain fatty acid (SCFA)-producing microbes discussed here are actually capable of SCFA production, because we lacked other genetic information on these microbes than the 16S gene. In summary, shotgun sequencing, repeated sampling and novel sampling methods using ingestible devices could aid in unraveling the ecological complexity of the microbiome in health and disease.

Gut microbiota, short chain fatty acids and blood pressure

Hypertension is the most important modifiable risk factor for cardiovascular morbidity and mortality, yet blood pressure control has not significantly improved over the last decade on a global scale. Despite several effective antihypertensive drugs being available, only a minority of hypertensive patients have sufficiently controlled blood pressure. To expedite progress in this field and improve blood pressure control, we need to improve preventive strategies and accessibility of interventions. Targeting the gut microbiota presents an attractive approach that can be achieved through dietary modifications, prebiotics, or probiotics with limited side effects. Therefore, we set out to study the effects of the microbiota-derived butyrate with a randomized placebo-controlled clinical trial, in which oral butyrate supplementation, surprisingly, increased blood pressure. This finding may seem contradictory to previous cross-sectional studies, including the HELIUS study discussed in this thesis, which reported higher abundance of butyrate-producing microbes to be associated with lower blood pressure. However, cross-sectional studies cannot establish the direction of the association. The slightly higher fecal butyrate concentrations in subjects with high blood pressure in the cross-sectional study could be a result of relatively lower absorption rate of butyrate, which might be a protective mechanism in this context.

Our current understanding of the pharmacokinetics of butyrate is limited, particularly regarding its absorption and elimination upon ingestion. While colonic absorption predominantly takes place through passive mechanisms, active co-transport with hydrogen or sodium ions becomes increasingly important along the gastrointestinal tract.[14] Once absorbed, butyrate enters the portal and then systemic circulation and is likely eliminated through renal excretion.[15] However, the expected increase in plasma levels following oral administration and the

half-life of butyrate remain unclear. To elucidate the mechanisms of the systemic effects of butyrate, it is essential to conduct pharmacokinetic studies. These studies should assess the flux of butyrate between compartments to take into account the endogenous production of butyrate. Unfortunately, securing funding for such studies in the medical field poses significant challenges, despite their pivotal role in improving the success rates of later phase clinical trials. Addressing this funding gap and recognizing the importance of pharmacokinetic and dose optimization research are crucial steps toward enhancing the effectiveness and safety of medical interventions in later clinical trial phases.

There are several other directions that we can explore to investigate the effects of butyrate in hypertension. Given that other clinical trials have shown that a combination of SCFAs can lower blood pressure, we should consider the proportions of different SCFAs in our research. The next step to uncover the interaction of SCFA could be to compare the effect of different combinations of SCFAs with *in vitro* experiments. Additionally, the efficacy of interventions with SCFA-enriched prebiotics as reported in previous clinical trials could be improved by omitting butyrate from these supplements. Lastly, the ongoing analysis of peripheral blood mononuclear cells (PBMC) from patients in the BEAM trial could provide more insights on the effects of butyrate on the inflammatory phenotype.

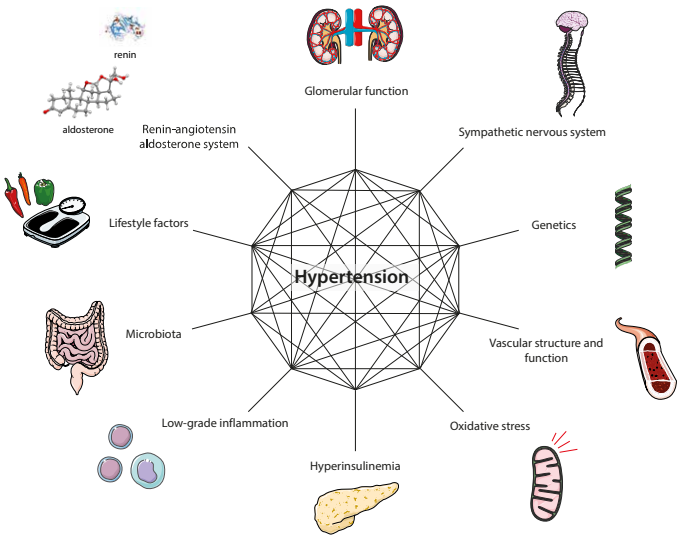
Overall, the studies on gut microbiota, short chain fatty acids and blood pressure in this thesis show that gut microbiota can modulate blood pressure. Therefore, one can argue that we should include this as one of the factors in the pathophysiology of hypertension, as described by the mosaic theory (**Figure 1**).

Novel metabolites in hypertension

We have identified a novel metabolite associated with blood pressure in our metabolomics analyses: formylmethionine (fMet). Our *in vitro* study of the effects of fMet on endothelial cells showed that fMet has pro-inflammatory effects and might affect mechanisms controlling peripheral vascular resistance, such as the production of nitric oxide. Follow-up experiments with vascular smooth muscle cells are currently underway to investigate how fMet influences the contractile pathways of these cells. However, blood pressure regulation is more complex and involves different neurohumoral systems. *In vivo* studies are therefore needed to uncover the acute and chronic effects of fMet on this interplay, for example in spontaneous hypertensive rats.

In a broader perspective, our findings on fMet illustrate that the mitochondria of endothelial cells could contribute to hypertension, since fMet

Figure 1: Mosaic theory of hypertension



Schematic overview of the contributing factors in the pathophysiology of hypertension, including the gut microbiota.

is more likely a mitochondrial damage-associated molecular pattern (DAMP) rather than a microbial or dietary product. The role of mitochondrial dysfunction in hypertension is however still relatively unexplored.[16] Human mitochondria likely originated from endocytosis of a proteobacterium by another prokaryotic cell in the evolutionary process. As a result, there are many similarities between mitochondrial and bacterial protein synthesis.[17] Mitochondrial products released upon tissue injury are very similar to microbial pathogen-associated molecular patterns (PAMPs) resulting from infection. The release of mitochondrial DAMPs by human cells elicits a neutrophil-mediated inflammatory response through formylreceptor-1 and Toll-like receptor 9 – the same receptors that PAMPs use.[18] This explains why traumatic injury can result in systemic inflammatory response syndrome (SIRS) resembling sepsis. Our metabolomics analyses in the HELIUS cohort underscore that these mitochondrial products also contribute to low-grade systemic inflammation implicated in hypertension and atherosclerosis pathophysiology.[19]

Our metabolomics analyses yielded many other blood pressure-predicting metabolites, of which I would like to highlight a few. Nitric oxide pathway metabolites such as citrulline and arginine were high-ranked predictors of blood pressure in younger subjects, while lipid metabolites (conjugated bile acids,

acylcarnitines and long chain fatty acids) and carbohydrates (glucose, arabinose) were better predictors of blood pressure in older subjects. This could reflect the differences in pathophysiology of hypertension between these groups, as it seems that vascular tissue regulation of peripheral resistance is more important in young subjects. In contrast, the best predicting metabolites in older HELIUS subjects hint to underlying processes of atherosclerosis and (pre-)diabetic comorbidity, emphasizing the importance of early prevention strategies.

The sex-stratified analyses showed that catecholamines are better predictors of blood pressure in women while sphingomyelins and secondary bile acids were higher ranked predictors in men. The evidence of sex differences in autonomic nervous system function is limited and needs more scrutiny in order to explain why catecholamine products are better blood pressure predictors in women. In addition, further research is needed to investigate the sex-specific effects of bile acids and sphingomyelins. Levels of these metabolites are known to be different between men and women – in men they are higher for bile acids and lower for sphingomyelins– yet it is unclear how these would affect blood pressure differently. It would therefore be interesting to investigate the sex-specific effects of these metabolites on vascular function using *in vivo* and *in vitro* models. It is crucial to also be aware of sex differences in these models: not only regarding the sex of animals for *in vivo* studies, but also *in vitro* regarding the sex of cell lines and the use of estrogenic components in cell culture media.

We also found that phenylalanine was associated with lower heart rate variability, but only in men. Other studies found that phenylalanine induces cardiac senescence in mice, yet we were the first to show a relation of this metabolite with autonomous nervous system function in humans. In line with our finding, the phenylalanine-derived phenylacetylglutamine (PAG) has been shown to interact with adrenergic receptors.[20] Phenylacetylglutamine can be synthesized from dietary phenylalanine by gut microbiota, and circulating phenylalanine is metabolized by the hepatic enzyme phenylalanine hydroxylase.[21] How the relation between dietary phenylalanine, the gut microbiota and the liver is different between men and women remains to be uncovered.

Alzheimer's disease, nutrition and gut microbiota

Alzheimer's disease is a complex disorder with a high degree of heritability, estimated to be between 60% and 80%.[22] The *APOE* ε4 risk allele is known to play an important role in the development of the disease, but recent genome-wide association studies have identified numerous other risk genes, including *CD33*,

IL34, and *TREM2*, which are involved in neuroinflammation and microglial function.[23] These findings highlight the central role of neuroinflammation in Alzheimer's pathogenesis.

The gut microbiome may also play a role in disease progression through its immunomodulatory potential.[24] Our research has revealed a modest association between the gut microbiota and AD pathology biomarkers, suggesting a possible contribution to the inflammatory profile that exacerbates the disease. In 2019, a study investigating the effects of sodium oligomannate (GV-971) on cognitive function showed promising results, with the microbiome potentially playing a role in mediating these effects.[25] However, the validity of the methods and findings has been questioned, highlighting the need for further investigation to uncover the underlying mechanisms.[26] Unfortunately, a global multicenter phase 3 clinical trial exploring the effects of oligomannate was discontinued due to the SARS-CoV-2 pandemic, delaying the validation of these findings.[27]

Lifestyle factors, including diet, have been estimated to contribute to around 40% of dementia risk.[22] In investigating the impact of nutrition and gut microbiota on AD using cross-sectional data, it is important to establish the direction of the associations that we find between these and AD pathology. By examining changes in nutritional status and gut microbiota composition over time in patients at different stages of the disease, the NUDAD study provides valuable insights. However, intervention studies are needed to establish causality and determine the effectiveness of lifestyle modifications.

Although evidence for the effect of lifestyle interventions on AD pathology is limited, there is growing interest in multidomain interventions that combine healthy diet, exercise, cognitive training, and social activities. The FINGER study, a large-scale randomized controlled trial in Finland, reported that such an intervention reduced the risk of cognitive impairment through such an intervention. This approach is being investigated in 25 countries as part of the World-Wide FINGER network, and personalized prevention strategies are also being explored in projects such as the Dutch ABOARD project.

In summary, while the gut microbiome may not have a major role in the treatment of AD, it is important to investigate its potential as a contributing factor to the disease and as a target for prevention strategies. The combination of lifestyle interventions and pharmacological strategies may hold promise in preventing or slowing the progression of AD.

Reflections on diversity in this thesis

Despite growing recognition of the importance of inclusion and diversity in the scientific community, many studies are performed in predominantly white, male populations, thereby limiting the external validity of novel mechanisms and therapies. This hinders the progress of our understanding of diseases and perpetuates health disparities. In my thesis, I highlighted the opportunities of increasing diversity in research, yet I also have to acknowledge the limitations of my own projects in this regard.

The BEAM study is one of the many clinical trials that excluded premenopausal women due to their menstrual cycles. The rationale for this decision was based on limited evidence showing that sex hormone cycles would impact the microbiome and blood pressure.[28,29] Although sex is used as a covariate in many microbiome papers, evidence on sex differences in microbiome composition itself after adjusting for confounders is very limited.[30–32] In hindsight, the disadvantage of the lack of representation might not outweigh the advantages of excluding this group. Going forward, it is necessary that we ensure adequate inclusion of women and other underrepresented groups in clinical research to ensure that our findings are applicable to all individuals.

In the projects using data from the HELIUS cohort in Amsterdam, we used ethnicity categories to investigate the associations between microbiota and blood pressure across different ethnic groups. However, we must be cautious about interpreting differences between ethnic groups as solely being due to biological factors, as ethnicity is a social construct that is shaped by complex social and cultural factors. When stratifying or adjusting for ethnicity, it becomes challenging to disentangle the specific factors at play. In the context of the microbiome, differences in diet are likely to be of significant importance. Additionally, disparities in socioeconomic status may contribute to ethnic variations. To enhance mechanistic understanding and mitigate potential racial bias, it is advisable to consider variables such as diet or socioeconomic status rather than relying solely on ethnicity.

Oversimplification of ethnicity into a few categories might in some cases even contribute to health disparities. An example is the correction for race in the calculation of estimated glomerular filtration rate (eGFR), which was proposed by an American study that observed higher creatinine levels at a similar renal filtration function in African-Americans.[33] Besides the fact that race is a social construct, this association was attributed to a higher muscle mass, although the literature is inconclusive about ethnic differences in body composition.[34] The eGFR

formula with a positive adjusting factor for black patients has likely overestimated renal function in many cases, leading to a lower referral rate for specialist care or transplanations.[35] Yet the formula was included in many guidelines whereafter it was applied to a much broader range of ethnicities (i.e. all black and multiracial patients).[35–37] This is example underscores why we should explain the procedures and justifications for both including ethnicity in our analyses and assuming that genetics is the most probable cause of the group differences we find.

As scientists, we have a responsibility to promote diversity, equity, and inclusion in all aspects of our research. Major publishers such as Nature now recognize the importance of transparency and rigor when reporting on ethnicity and sex in medical research, and we must follow their lead in ensuring that our research reflects the diversity of our populations.[38,39]

Concluding remarks

In conclusion, this thesis has provided valuable insights into the potential role of gut microbiota and plasma metabolites in hypertension and AD. Through our work, we have demonstrated the influence of gut microbiota, specifically through SCFA, in modulating blood pressure in hypertension, and have identified novel plasma metabolites like fMet that shed new light on the involvement of mitochondria in this condition. Additionally, the (modest) associations between nutritional status, gut microbiota, and AD pathology biomarkers could reflect a potential contribution to neurodegeneration and neuroinflammation in AD. We eagerly await ongoing intervention studies for further evidence on the direction of these associations. Ultimately, this thesis underscores the potential of the gut microbiota and plasma metabolome as innovative approaches to address these two significant public health concerns with complex pathophysiology. It is my hope that these findings serve as a foundation for future research projects aimed at understanding and leveraging the interplay between gut microbiota, plasma metabolites, and the pathogenesis of hypertension and AD.

References

1. Halfvarson, J. *et al.* Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol* (2017) doi:10.1038/nmicrobiol.2017.4.
2. Valles-Colomer, M. *et al.* The neuroactive potential of the human gut microbiota in quality of life and depression. *Nat Microbiol* (2019) doi:10.1038/s41564-018-0337-x.
3. Sampson, T. R. *et al.* Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell* 167, 1469-1480. e12 (2016).
4. Forslund, K. *et al.* Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* (2015) doi:10.1038/nature15766.
5. Walter, J., Armet, A. M., Finlay, B. B. & Shanahan, F. Establishing or Exaggerating Causality for the Gut Microbiome: Lessons from Human Microbiota-Associated Rodents. *Cell* 180, 221–232 (2020).
6. Mullish, B. H. *et al.* The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium difficile infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* 67, 1920–1941 (2018).
7. Kootte, R. S. *et al.* Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. *Cell Metab* 26, 611-619.e6 (2017).
8. Ianiro, G. *et al.* Variability of strain engraftment and predictability of microbiome composition after fecal microbiota transplantation across different diseases. *Nat Med* 28, 1913–1923 (2022).
9. de Jonge, P. A. *et al.* Gut virome profiling identifies a widespread bacteriophage family associated with metabolic syndrome. *Nat Commun* 13, 3594 (2022).
10. Tropini, C., Earle, K. A., Huang, K. C. & Sonnenburg, J. L. The gut microbiome: Connecting spatial organization to function. *Cell Host Microbe* 21, 433–442 (2017).
11. Shalon, D. *et al.* Profiling the human intestinal environment under physiological conditions. *Nature* 1–11 (2023) doi:10.1038/s41586-023-05989-7.
12. Thaiss, C. A. *et al.* Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* 159, 514–529 (2014).
13. McLaren, M. R., Willis, A. D. & Callahan, B. J. Consistent and correctable bias in metagenomic sequencing experiments. *eLife* 8, e46923 (2019).
14. Parada Venegas, D. *et al.* Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol* 10, 277 (2019).
15. Newmark, H. L., Lupton, J. R. & Young, C. W. Butyrate as a differentiating agent: pharmacokinetics, analogues and current status. *Cancer Letters* 78, 1–5 (1994).
16. Puddu, P., Puddu, G. M., Cravero, E., De Pascalis, S. & Muscari, A. The Putative Role of Mitochondrial Dysfunction in Hypertension. *Clin Exp Hypertens* 29, 427–434 (2007).
17. Timmis, J. N., Ayliffe, M. A., Huang, C. Y. & Martin, W. Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nat Rev Genet* 5, 123–135 (2004).
18. Zhang, Q. *et al.* Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* (2010) doi:10.1038/nature08780.
19. Wenceslau, C. F. *et al.* Mitochondrial damage-associated molecular patterns and vascular function. *Eur Heart J* 35, 1172–1177 (2014).

20. Nemet, I. *et al.* A Cardiovascular Disease-Linked Gut Microbial Metabolite Acts via Adrenergic Receptors. *Cell* 180, 862-877.e22 (2020).
21. Romano, K. A. *et al.* Gut Microbiota-Generated Phenylacetylglutamine and Heart Failure. *Circ Heart Failure* 16, e009972 (2023).
22. Livingston, G. *et al.* Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. *Lancet* 396, 413–446 (2020).
23. Scheltens, P. *et al.* Alzheimer's disease. *Lancet* 397, 1577–1590 (2021).
24. van Olst, L. *et al.* Contribution of Gut Microbiota to Immunological Changes in Alzheimer's Disease. *Front Immunol* 12, 683068 (2021).
25. Wang, X. *et al.* Sodium oligomannate therapeutically remodels gut microbiota and suppresses gut bacterial amino acids-shaped neuroinflammation to inhibit Alzheimer's disease progression. *Cell Res* 29, 787–803 (2019).
26. More, Unfortunately, on the Chinese Alzheimer's Drug Approval. https://www.science.org/content/blog-post/more-unfortunately-chinese-alzheimer-s-drug-approval.
27. Green Valley (Shanghai) Pharmaceuticals Co., Ltd. *A Phase 3, Multi-center, Randomized, Double-blind, Parallel-group, Placebo-controlled Clinical Trial to Evaluate the Efficacy and Safety of Sodium Oligomannate (GV-971) in Treatment of Mild to Moderate Alzheimer's Disease (GREEN MEMORY: GREen Valley 971 EvaluatioN Memory)*. https://clinicaltrials.gov/ct2/show/NCT04520412 (2022).
28. Markle, J. G. M. *et al.* Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. *Science* 339, 1084–1088 (2013).
29. Baker, S. E., Limberg, J. K., Ranadive, S. M. & Joyner, M. J. Neurovascular control of blood pressure is influenced by aging, sex, and sex hormones. *Am J Physiol Regul Integr Comp Physiol* 311, R1271–R1275 (2016).
30. Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–215 (2018).
31. Kurilshikov, A. *et al.* Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat Genet* 53, 156–165 (2021).
32. Lopera-Maya, E. A. *et al.* Effect of host genetics on the gut microbiome in 7,738 participants of the Dutch Microbiome Project. *Nat Genet* 54, 143–151 (2022).
33. Levey, A. S. *et al.* A New Equation to Estimate Glomerular Filtration Rate. *Ann Int Med* 150, 604–612 (2009).
34. Hsu, J., Johansen, K. L., Hsu, C., Kaysen, G. A. & Chertow, G. M. Higher Serum Creatinine Concentrations in Black Patients with Chronic Kidney Disease: Beyond Nutritional Status and Body Composition. *Clin J Am Soc Nephrology* 3, 992 (2008).
35. Vyas, D. A., Eisenstein, L. G. & Jones, D. S. Hidden in Plain Sight — Reconsidering the Use of Race Correction in Clinical Algorithms. *N Engl J Med* 383, 874–882 (2020).
36. Cerdeña, J. P., Plaisime, M. V. & Tsai, J. From race-based to race-conscious medicine: how anti-racist uprisings call us to act. *Lancet* 396, 1125–1128 (2020).
37. Eneanya, N. D., Yang, W. & Reese, P. P. Reconsidering the Consequences of Using Race to Estimate Kidney Function. *JAMA* 322, 113–114 (2019).
38. Why Nature is updating its advice to authors on reporting race or ethnicity. *Nature* 616, 219–219 (2023).
39. Accounting for sex and gender makes for better science. *Nature* 588, 196–196 (2020).

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Appendices

Nederlandse samenvatting

In dit proefschrift heb ik de rol van de darmmicrobiota en de bijbehorende metabolieten onderzocht in twee belangrijke gezondheidsproblemen: hypertensie (**Deel I**) en de ziekte van Alzheimer (**Deel II**).

Deel I: Hypertensie

In **Hoofdstuk 2** hebben we de relatie tussen darmmicrobiota en bloeddruk onderzocht in verschillende etnische groepen van de HELIUS-studie. We vonden een verband tussen de samenstelling van het darmmicrobioom en bloeddruk, maar dit verband was niet in alle groepen even sterk. We vonden daarnaast dat bacteriën die bekend staan om de productie van korteketenvetzuren (short chain fatty acids; SCFA) geassocieerd waren met een lagere bloeddruk. Fecale SCFA concentraties waren echter hoger bij studiedeelnemers met een hogere bloeddruk. Een verklaring voor deze ogenschijnlijk tegenstrijdige bevindingen zou kunnen zijn dat de opname van SCFA relatief efficiënter verloopt bij hogere SCFA-productie. De bevindingen suggereren dat de darmmicrobiota en SCFA een rol kunnen spelen bij de ontwikkeling van hypertensie, als oorzakelijke factor of als gevolg van de aandoening.

In **Hoofdstuk 3** hebben we een uitgebreid overzicht gegeven van de huidige literatuur over de rol van darmmicrobiota bij hypertensie en atherosclerose. Hierin hebben we de complexe interacties benadrukt tussen de darmmicrobiota en verschillende fysiologische systemen die betrokken zijn bij de regulatie van de bloeddruk en atherosclerose, waaronder het immuunsysteem, het renine-angiotensine-aldosteronsysteem en het autonome zenuwstelsel. Mechanismen waarmee de darmmicrobiota specifiek de bloeddruk zou kunnen beïnvloeden zijn SCFA, lipopolysacchariden, veranderingen in darmpermeabiliteit en de darm-hersenas. Over het algemeen laat de bestaande literatuur zien dat de darmmicrobiota veelbelovend kunnen zijn voor de preventie en behandeling van hypertensie en atherosclerose, maar ook dat de vertaling van de bevindingen van diermodellen naar mensen uitdagend blijft.

In **Hoofdstuk 4** hebben we de effecten van capsules met butyraat, een SCFA, op bloeddruk onderzocht in een gerandomiseerd, placebogecontroleerd onderzoek bij patiënten met hypertensie. Behandeling met butyraat gedurende vier weken verhoogde de systolische bloeddruk overdag in vergelijking met placebo. Dit zijn

waarschijnlijk directe effecten van butyraat, aangezien de butyraatbehandeling alleen de plasmaconcentraties van butyraat verhoogde en niet de concentraties van andere SCFA. Hoewel bestaande interventiestudies met voedingsvezels en meerdere SCFA juist bloeddrukverlagende effecten lieten zien, toonde dit onderzoek aan dat niet alle SCFA gunstige effecten hebben op hypertensie bij de mens. Verdere mechanistische studies zijn nodig om de tegengestelde effecten van SCFA bij mensen te verklaren.

In **Hoofdstuk 5** onderzochten we de associaties van plasmametabolieten met bloeddruk. Onze machine learning analyses toonden aan dat formylmethionine de beste voorspellende metaboliet was voor de systolische bloeddruk. Het was ook een zeer consistente voorspeller voor bloeddruk in verschillende groepen van leeftijd, geslacht en etniciteit. Vervolgens onderzochten we de *in vitro* effecten van deze metaboliet op endotheelcellen. We ontdekten dat formylmethionine endotheliale disfunctie verzoorzaakt, waaronder een vermindering van eNOS, verstoring van de endotheelbarrière en toename van oxidatieve stress. Onze hypothese is dat formylmethionine een mitochondriële damage-associated molecular pattern (DAMP) is die een rol speelt bij de regulering van de bloeddruk.

In **Hoofdstuk 6** bekeken we de analyses uit hoofdstuk 5 door de lens van sekseverschillen. We ontdekten dat er sekseverschillen zijn in de verbanden tussen plasmametabolietenprofielen en zowel bloeddruk als hartritmevariabiliteit. Sphingomyelinen en geconjugeerde galzuren waren meer voorspellend voor bloeddruk bij mannen, terwijl metabolieten uit acylcarnitine- en catecholaminepathways betere voorspellers waren bij vrouwen. Bovendien konden we hartslagvariabiliteit voorspellen uit metabolietprofielen bij mannen, maar niet bij vrouwen, wat verder benadrukt dat er potentieel sekseverschillen zijn in de fysiologie van bloeddrukregulatie. Een aantal metabolieten waren geassocieerd met de samenstelling van de darmmicrobiota. Dit laat zien dat interventies gericht op de darmmicrobiota seksespecifieke effecten kunnen hebben, aangezien plasmametabolieten afkomstig van de microbiota anders geassocieerd zijn met bloeddruk bij mannen en vrouwen.

Samenvattend hebben we in dit deel van mijn proefschrift de rol onderzocht van darmmicrobiota en plasmametabolieten bij hypertensie. Allereerst vonden we cross-sectionele verbanden tussen SCFA-producerende bacteriën, fecale SCFA concentraties en bloeddruk. Vervolgens bevestigden we dat butyraat, een SCFA, effect had op de bloeddruk met een klinische interventiestudie bij hypertensieve

patiënten. Butyraat had echter bloeddrukverhogende effecten, in tegenstelling tot eerdere studies met voedingsvezels en SCFA, die bloedddrukverlagende effecten hebben laten zien. In onze metabolietenanalyses hebben we een nieuwe metaboliet gevonden die geassocieerd was met hogere bloeddruk: formylmethionine. Dit zou een bacterieel product kunnen zijn, maar is meer waarschijnlijk een mitochondriaal product dat een rol zou kunnen spelen bij de regulering van de bloeddruk. Daarnaast hebben we laten zien dat het verband tussen plasma metabolieten en bloeddruk seksespecifiek is. Deze analyses resulteerden in verschillende aanknopingspunten voor verder mechanistisch onderzoek naar sekseverschillen in hypertensie.

Deel II: Zieke van Alzheimer

Hoofdstuk 7 analyseerden we de associatie tussen voedingsstatus en structurele veranderingen in de hersenen bij patiënten met subjectieve cognitieve achteruitgang (SCD), milde cognitieve stoornissen (MCI) en de ziekte van Alzheimer (AD) dementie in de NUDAD-cohort. In deze cross-sectionele analyse vonden we dat lagere indicatoren voor voedingsstatus geassocieerd waren met meer atrofie op MRI, in het bijzonder globale corticale atrofie en mediale temporale atrofie. Dit onderzoek laat zien dat slechte voeding zou kunnen bijdragen aan de ontwikkeling en progressie van de ziekte van Alzheimer middels effecten op de hersenstructuur, hoewel dit verder bevestigd moet worden door interventiestudies. De bevindingen benadrukken opnieuw het belang van het behouden van een goede voedingsstatus bij patiënten met de ziekte van Alzheimer.

In **Hoofdstuk 8** onderzochten we de relatie tussen de samenstelling van de darmmicrobiota en biomarkers van AD-pathologie in de NUDAD-cohort. We vonden bescheiden associaties tussen de samenstelling van de darmmicrobiota en AD-biomarkers in het hersenvocht, zoals amyloïd-bèta en tau-eiwitten. Specifiek hadden deelnemers met een groter aantal SCFA-producerende bacteriën minder vaak abnormale AD-biomarkers in het hersenvocht. Dit verband kan worden verklaard door de immunomodulerende eigenschappen van SCFA zoals eerdere *in vitro* en *in vivo* studies hebben aangetoond. Dit was echter een cross-sectionele analyse en daarom kunnen we geen oorzakelijke conclusies trekken. Voor toekomstig onderzoek zou het interessant zijn om te kijken naar de relatie tussen plasma- en fecale SCFA en ontstekingsmarkers zoals *glial fibrillary acid protein* (GFAP) bij patiënten met AD.

Samenvattend hebben we in de hoofdstukken in het tweede deel van dit proefschrift gekeken naar de mogelijke impact van voedingsstatus en de samenstelling van de darmmicrobiota op de pathologie van de ziekte van Alzheimer. De verbanden hiertussen bleken subtiel te zijn, en daarom lijkt het waarschijnlijker dat het darmmicrobioom geen oorzakelijk, maar eerder een ziektemodulerend effect heeft.

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List of publications

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Verhaar, B.J.H., Hendriksen, H.M.A., de Leeuw, F. A., Doorduijn, A.S., van Leeuwenstijn, M., Teunissen, C.E., Barkhof, F., Scheltens, P., Kraaij, R., van Duijn, C.M., Nieuwdorp, M., Muller, M., van der Flier, W.M. (2021). *Gut Microbiota Composition Is Related to AD Pathology.* Frontiers in Immunology, 12, 794519.

Verhaar, B.J.H., Prodan, A., Nieuwdorp, M., Muller, M (2020). *Gut Microbiota in Hypertension and Atherosclerosis: A Review.* Nutrients, 12, 2982.

Verhaar, B.J.H., Collard, D., Prodan, A., Levels, J.H.M., Zwinderman, A.H., Bäckhed, F., Vogt, L., Peters, M.J.L., Muller, M., Nieuwdorp, M., van den Born, B.J.H. (2020). *Associations between gut microbiota, faecal short-chain fatty acids, and blood pressure across ethnic groups: the HELIUS study.* European Heart Journal, 41(44), 4259–4267.

Verhaar, B.J.H., de Leeuw, F.A., Doorduijn, A.S., Fieldhouse, J.L.P., van de Rest, O., Teunissen, C.E., van Berckel, B.N.M., Barkhof, F., Visser, M., de van der Schueren, M.A.E., Scheltens, P., Kester, M.I., Muller, M., van der Flier, W.M. (2020). *Nutritional status and structural brain changes in Alzheimer's disease: The NUDAD project.* Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring, 12(1), e12063.

Other publications

van Olst, L., Roks, S.J., Kamermans, A., **Verhaar, B.J.H.**, van der Geest, A.M., Muller, M., van der Flier, W.M., de Vries, H.E. (2021). *Contribution of gut microbiota to immunological changes in Alzheimer's disease.* Frontiers in Immunology, 12, 683068.

Fieldhouse, J.L.P., Doorduijn, A.S., de Leeuw, F.A., **Verhaar, B.J.H.**, Koene, T., Wesselman, L.M., de van der Schueren, M.A.E., Visser, M., van de Rest, O., Scheltens, P., Kester, M.I., van der Flier, W.M. (2020). *A suboptimal diet is associated with poorer cognition: The NUDAD project.* Nutrients, 12(3), 703.

Verhaar, B.J.H., Vernooij, M.W., Biessels, G.J., Muller, M. (2018). *Risico's van vitamine K-antagonisten bij cerebrale microbloedingen.* Nederlands Tijdschrift voor Geneeskunde, 162, D1790.

Overwater, I.E., **Verhaar, B.J.H.**, Lingsma, H.F., Bindels-de Heus, G.C.B., van den Ouweland, A.M., Nellist, M., ten Hoopen, L.W., Elgersma, Y., Moll, H.A., de Wit, M.C.Y. (2017). *Interdependence of clinical factors predicting cognition in children with tuberous sclerosis complex.* Journal of Neurology, 264, 161-167.

Portfolio

PhD candidate:	Barbara J.H. Verhaar
PhD period:	April 2019 - January 2023
PhD supervisors:	Prof.dr. M. Muller, Prof.dr. M. Nieuwdorp Prof.dr. W.M. van der Flier, Dr. J.H.M. Levels

PhD training

Course	Year	ECTS
ACS PhD afternoons	2019-2022	1.0
ACS Education Committee	2019-2022	1.0
Vascular Medicine Journal Clubs	2020-2022	2.0
Diabetes Meeting	2019-2022	2.0
Lab Meetings (Experimental Vascular Medicine)	2019-2022	1.0
Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK)	2019	1.5
Nederlandse Hartstichting Papendal course Vascular Biology & Pathology	2019	2.0
Data Management Plan training	2020	1.0
Scientific Integrity	2020	2.0
Winterschool - Data analyses in R	2020	3.0
John Hopkins Data Science Specialisation - Online Courses (Coursera)	2020-2021	5.0

Conferences and meetings

Conference	Year	ECTS
ACS PhD retreat, oral presentation	2019	1.0
ACS Symposium	2019	-
NUDAD consortium meeting: presentation	2020	1.0
Dementie Update	2020	-
Alzheimer Association International Conference (AAIC) online, poster presentation	2020	1.0
European Society of Cardiology (ESC) congress (online), poster presentation	2020	1.0
Heart-Brain connection (HBCx) consortium meeting: presentation	2021	1.0
Alzheimer Association International Conference (AAIC) online, poster presentation and oral presentation	2021	1.0

Conference	Year	ECTS
European Society of Cardiology (ESC) congress (online), poster presentation	2021	1.0
Nederlands Hypertensie Congres, oral presentation	2022	1.0
Cell Symposium "Metabolites in Signaling and Disease", poster presentation	2022	1.0
Leducq consortium meeting; presentation	2022	1.0
European Society of Hypertension (ESH) conference, oral presentation	2022	1.0
Leducq consortium meeting; presentation	2022	1.0
European Society of Hypertension (ESH) conference, oral presentation	2023	1.0
Leducq consortium meeting: presentation	2023	1.0

Teaching and supervision

Item	Year	ECTS
Lecturing minor Cardiovascular Aging	2018	1.0
Organizing minor Cardiovascular Aging	2019	2.0
Lecturing minor Cardiovascular Aging	2019	1.0
Organizing minor Cardiovacular Aging	2020	2.0
Lecturing minor Cardiovascular Aging	2020	1.0
Supervising bachelor students in writing bachelor theses	2019-2020	2.0
Supervising master students in writing master theses	2019-2020	2.0

Grants and awards

Item	Year
ACS Out of the Box grant	2021
Wetenschapsprijs Nederlandse Hypertensie Vereniging	2022
Accomodation grant ESH	2022
Tatal Zein award (Best Oral Presentation) ESH	2022
Accomodation grant ESH	2023

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Barbara
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About the author

Barbara Johanna Helena Verhaar was born on January 18th 1993 in Amsterdam, the Netherlands. In 2010, she graduated from the Minkema College in Woerden and started medical school at the University of Amsterdam. After her bachelor's degree, she continued her academic journey with the master program Vitality and Ageing at Leyden Academy on Vitality and Ageing in Leiden. Thereafter, she continued medical school in Amsterdam and graduated in 2019 with an honours program certificate. During her research internship, she worked on a project on nutritional status and MRI characteristics of Alzheimer patients in the Alzheimer center of the Amsterdam UMC - location VUmc.

She started her PhD program under the supervision of professors Majon Muller, Max Nieuwdorp, and Wiesje van der Flier, and dr. Han Levels. Her research covered diverse projects related to the gut microbiome, plasma metabolites, hypertension, and Alzheimer's disease, culminating in this thesis. She also broadened her expertise with a research visit to the laboratory of Arash Haghikia in Charité Berlin in the summer/autumn of 2022.

Currently, she works as a postdoctoral researcher within the Public and Occupational Health and Vascular Medicine departments of the Amsterdam UMC, and aspires to become a clinical microbiologist.

She lives in Woerden together with Andrei, and lots of plants.