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Programvezető: Dr. Merkely Béla, egyetemi tanár

Témavezető: Dr. Tárnoki Dávid László, egyetemi docens

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ASSOCIATION BETWEEN ULTRASOUND MARKERS OF ATHEROSCLEROSIS AND THE GUT MICROBIOME IN TWINS

Ph.D. thesis

Helga Szabó, MD

Doctoral School of Theoretical and Translational Medicine

Semmelweis University

Supervisor: Dávid László Tárnoki, MD, Ph.D.

Official reviewers: Judit Domokos, MD, Ph.D.
Andrea Horváth, MD, Ph.D.

Head of the Complex Examination Committee:
István Karádi, MD, D.Sc., corresponding member of MTA

Members of the Complex Examination Committee:
Henriette Farkas, MD, D.Sc.
Péter Andréka, MD, Ph.D.

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List of Abbreviations

ACVDs: atherosclerotic cardiovascular diseases

AHI: apnoea-hypopnoea index

ApoE−/−: Apolipoprotein E knock-out

BMI: body mass index

CAS: carotid artery atherosclerosis

CCA: common carotid artery

CIH: chronic intermittent hypoxia

CPAP: continuous positive airway pressure

CRP: C-reactive protein

CVDs: cardiovascular diseases

CSF: chronic sleep fragmentation

DBP: diastolic blood pressure

DNA: deoxyribonucleic acid

DZ: dizygotic

ESC: European Society of Cardiology

ESH: European Society of Hypertension

HDL-C: high-density lipoprotein-cholesterol

hs-CRP: high-sensitivity C-reactive protein

IC: intermittent hypercapnia

ICA: internal carotid artery

IH: intermittent hypoxia

IMT: intima-media thickness

LDA: linear discriminant analysis

LDL-C: low-density lipoprotein cholesterol

LEfSe: linear discriminant analysis effect size

MZ: monozygotic

ODI: oxygen desaturation index

OSA: obstructive sleep apnoea

PCAs: principal component analyses

PCoA: principal coordinates analysis

PG: polygraphy

PSG: polysomnography

PWV: pulse wave velocity

RCT: reverse cholesterol transport

rRNA: ribosomal ribonucleic acid

SBP: systolic blood pressure

SCFA: short-chain fatty acid

SD: standard deviation

TIA: transient ischaemic attack

TMAO: trimethylamine N-oxide

TST90%: percentage of total sleep time spent with saturation below 90%

ZO-1: zonula occludens-1

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1. Introduction

Cardiovascular diseases (CVDs) are identified as the prominent cause of mortality worldwide, responsible for roughly 18 million fatalities annually (1). CVDs are a cluster of pathological conditions affecting the heart and related blood vessels, which encompass various disorders such as coronary heart disease, cerebrovascular disease, rheumatic heart disease, and additional maladies. Acute myocardial infarction and strokes collectively contribute to over 80% of all CVD fatalities, and among these fatalities, approximately one-third occur in individuals who are younger than 70 years of age. The presence of carotid artery atherosclerosis (CAS) has been identified as a potential contributor to elevated susceptibility to CVDs (2-4). Moreover, risk factors for the incidence of stroke have been linked to the development and progression of CAS (5). Carotid intima-media thickness (IMT) serves as a significant cardiovascular marker and has been the subject of extensive research. The comparison of genetic versus environmental factors underlying this marker has garnered considerable attention in academic literature. The study conducted by Zhao et al. revealed that there existed a notable influence of genetic factors over the carotid IMT (6). A study was conducted on a sizable Korean twin population to assess the segment-specific genetic impact on carotid IMT, wherein significant heritability values of 0.48, 0.38, and 0.45 were respectively observed for common, carotid bifurcation, and internal carotid artery (ICA) segments. Various established risk factors for cardiovascular disease, such as advanced age, alcohol consumption, diabetes mellitus, hypertension, dyslipidemia, smoking, and elevated levels of high-sensitivity C-reactive protein (hs-CRP) as an inflammatory marker, have been associated with carotid IMT. Several of these factors have demonstrated the ability to affect targeted sections of the carotid artery (7). Whilst genetic variation and environmental risk factors are acknowledged determinants of carotid atherosclerosis, contemporary literature suggests that the structure and heterogeneity of the gastrointestinal microbiome can also significantly impact the emergence of CVDs (8-10).

1.1. Measurement and clinical use of ultrasound markers of atherosclerosis in the carotid artery

At the time of planning the examination, according to the latest guideline of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) published in 2018 (11), the assessment of carotid IMT through carotid ultrasound, in conjunction with the detection of plaques, has been demonstrated to be a valuable tool in the prediction of cardiovascular risk (12, 13). The aforementioned statement holds validity for both the IMT measurements obtained at the carotid bifurcations, which largely signify the presence of atherosclerotic plaque formation, as well as for the IMT measurements collected at the common carotid artery (CCA), which primarily reflect the pathological hypertrophic changes due to hypertension. The existence of a carotid IMT greater than 0.9 mm is deemed anomalous according to literature (14), however, the upper limit of normality is age-dependent. An IMT greater than 1.5 mm or a localized thickening of 0.5 mm or 50% above the adjacent carotid IMT value may strongly suggest the presence of a carotid plaque (15). Irrespective of existing cardiovascular risk factors, the presence of stenotic carotid plaques holds significant predictive utility for both stroke and myocardial infarction (12, 13). Furthermore, it offers improved prognostic accuracy for future myocardial infarction in comparison to IMT (16). The identification of carotid plaques signifies a shift in patient categorization from intermediate to high risk (17, 18). However, routine carotid imaging is not recommended unless deemed clinically necessary (i.e. presence of carotid bruit, past transient ischemic attack (TIA) or cerebrovascular disease, or as part of the examination of individuals with signs of vascular disease).

It is important to note that the new ESC Guidelines on cardiovascular disease prevention in clinical practice were published in 2021, in which carotid artery plaque retained its role in CVD risk reclassification, at the same time, caution must be exercised when using IMT as a tool to enhance risk assessment or prognosticate future CVD occurrences, as there is a dearth of standardized methodologies pertaining to its implementation (19).

1.2. The role of a healthy microbiome and dysbiosis

As Anto et al. (20) reviewed, the gut microbiome comprises a vast array of microbial taxa, including bacteria, viruses, fungi, archaea, and eukaryotes, which co-inhabit the gastrointestinal tract. The gut microbial composition of each person is characterized by distinct and stable features (21). Research suggests that an individual maintains over 60% of the gut microbial phylotypes for a period of two years (20). The predominant bacterial phyla observed within the human gut are *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Collectively, these phyla constitute more than 90% of all bacteria present, with *Bacteroidetes* and *Firmicutes* comprising the majority (23, 24). The *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria* are phyla that are relatively infrequent yet still sufficiently represented. The variability in the observed abundance of bacterial species across different individuals is significant. Nevertheless, the abundance of the major phyla remains relatively constant (25).

The microbiota residing in the gastrointestinal tract plays a vital role in regulating the physiological processes of the host organism. Commensal microorganisms are crucial for the proper anatomical development and physiological processes of human organs, including but not limited to the intestine, brain, and liver. The microbiota located in the gastrointestinal tract confers several advantageous effects on its host, such as the preservation of the cellular layer that covers the intestinal surface, the establishment of both intrinsic and adaptive mechanisms for immune system functionality, the breakdown of otherwise indigestible carbohydrates, the synthesis of secondary bile acid molecules, the management of foreign substances, and the construction of a barrier that hinders the colonization of pathogenic organisms (26). Microbial entities are additionally characterized by their capacity to facilitate metabolism through the acquisition of energy and provision of vital vitamins and short-chain fatty acids (SCFA) to the host organism (27, 28). The elucidation of the gut microbiome's bidirectional relationship to the liver (gut-liver axis) (29) and brain (gut-brain axis) (30) has facilitated a greater understanding of its role in the onset/progression of various ailments.

The current comprehension of the constitution of a healthy gut microbiota originates from investigations conducted on individuals with a healthy condition (31). Overall, a microbiota that is in good health exhibits elevated levels of microbial diversity and is capable of tolerating alterations associated with physiological stressors (32). The definition of a functional group with significant stability, remarkable resistance to stressors, such as immunosuppression, invading infections, and high metabolic pathway redundancy, can be established (33).

The term dysbiosis encompasses a wide range of alterations in the bacterial population composition, deviating from the characteristic pattern observed in individuals exhibiting robust health (34). According to research findings (32), the abundance of beneficial bacteria is anticipated to decrease, and/or the proportion of pathogens in the microbial ecosystem is expected to increase, resulting in reduced microbial diversity. The quantification of the assortment of microbial species within a given community pertains to the diversity of the gut microbiome, whereas the comprehensive count of distinct microbial species present in the gastrointestinal tract is referred to as richness (35). Various measures are used to estimate diversity. Alpha diversity is a term used to describe the "within-sample" diversity, a measure of how diverse a single sample is, usually taking into account the number of different species observed, while beta diversity is a term used to express the differences between samples or environments. One of the alpha diversity metrics is the Shannon index which is an estimator for both species richness and evenness but with weight on the richness (36, 37). Albeit the possibility that gut microbial diversity/richness may not always reflect the activated functional gene pathways (38), it is frequently employed as an indicator of gut health. Atherosclerosis and several risk factors for atherosclerosis, such as diabetes (39, 40), obesity (39, 41), smoking (42), a sedentary lifestyle (43), inflammation (44), and unhealthy dietary habits (45), are all connected with gut dysbiosis. Moreover, the gut epithelium serves a critical function in the host's defense mechanisms by restricting the entry of intestinal contents, microbial components, and whole microorganisms into the bloodstream. The phenomenon of gut dysbiosis has been found to exhibit a positive association with elevated levels of intestinal tight junction permeability, which is colloquially known as "leaky gut" (46-

48). A diminished manifestation of tight junction proteins within the intestinal tract, including zonula occludens-1 (ZO-1), claudin, and occludins, can impede the intestinal barrier via the demise of epithelial cells. Consequently, intestinal permeability is exacerbated (49), thereby promoting gut dysbiosis, which exacerbates atherosclerosis. The correlation between the gastrointestinal microbiome and ailments is an intricate field of study, not characterized by a simple, direct cause-and-effect relationship, but rather a multifaceted association that remains actively investigated.

1.3. The role of individual bacteria in atherosclerosis

As also reviewed by Anto et al. (20), the pathogenesis of atherosclerosis encompasses metabolic and inflammatory components affecting its initiation and progression. The process commences with the occurrence of arterial endothelial denudation, followed by the accumulation of lipids, and ultimately culminates in the mobilization of macrophages and other immune cells to the affected area (50, 51). The identification of deoxyribonucleic acid (DNA) originating from various bacterial species within atherosclerotic plaques provided initial insights into the involvement of microbiota in the pathogenesis of atherosclerosis (52-54). Koren et al. (55) discovered *Chryseomonas* (phylum *Proteobacteria*, later renamed *Pseudomonas luteola*) in all atherosclerotic plaques of 15 individuals studied, as well as *Veillonella* and *Streptococcus* (phylum *Firmicutes*) in the majority of the samples. The identification of various bacterial taxa within plaques has been observed to coincide with their detection within both oral and stool samples of the corresponding individual. This correlation suggests a potential role for these diverse bacterial communities in the development and advancement of atherosclerosis. While there were no discernible distinctions observed in the relative proportions of gut microbial phyla among the groups examined, an association was established between particular bacterial genera and both total serum cholesterol and low-density lipoprotein cholesterol (LDL-C) levels (55). A year later, Karlsson et al. (56) discovered an increased relative abundance of the genus *Collinsella* (Phylum - *Actinobacteria*) in the fecal samples from patients with symptomatic atherosclerosis, while butyrate-producing bacteria *Roseburia* and *Eubacterium* were enriched in controls, implying a

dysbiosis condition in atherosclerotic patients. The authors have detailed the possible significance of functional gene pathways present in the gut microbiome in the development of atherosclerosis. Specifically, the production pathways of peptidoglycan have been discerned to be enriched among individuals affected by atherosclerosis. These results suggest that infectious agents may potentially exert a significant influence on the pathogenesis and progression of atherosclerotic disease in humans. Additionally, consistent with prior research, findings from the investigations conducted by Emoto et al. (57) reveal that the relative prevalence of *Bacteroides*, *Clostridium*, and *Lactobacillales* may serve as indicative factors for the prognosis of coronary artery disease. A subsequent investigation carried out by the aforementioned research group revealed that individuals with coronary artery disease exhibited elevations in both the abundance of *Lactobacillus* and the ratio of *Firmicutes* to *Bacteroidetes* (58). Jie et al. (59) have reported a significant positive correlation between atherosclerosis and an elevated abundance of members belonging to the *Enterobacteriaceae* family while observing a decline in the population of bacteria-producing butyrate. A comprehensive evaluation of 16 prospective observational studies revealed that numerous commensal bacterial populations are correlated with coronary heart disease. Among these, *Bacteroides* and *Prevotella*, present in feces, hold predictive significance for the occurrence of the ailment (60). The utilization of antibiotics and other pharmaceuticals in individuals suffering from CVD or atherosclerosis, whose characteristics do not correspond with those of healthy subjects, constitutes a noteworthy confounding variable in the aforementioned study. Consequently, the assessment of alterations in the microbial populace amongst individuals afflicted with atherosclerosis necessitates judicious attention.

1.4. Atherosclerosis and gut microbiome in patients with obstructive sleep apnoea (OSA)

Obstructive sleep apnea (OSA) is a medical condition characterized by the recurrent collapse of the upper airways during periods of sleep. Studies have found a positive correlation between OSA and a heightened risk of atherosclerosis, as evidenced by prior research (61). The events linked with OSA lead to cerebral arousal, alterations

in intrathoracic pressure, as well as sporadic occurrences of hypoxemia and reoxygenation. The occurrence of these aforementioned events elicits physiological pathways that render patients with OSA susceptible to hypertension and atherosclerosis. Such events include oxidative stress, sympathetic activation, inflammation, hypercoagulability, endothelial dysfunction, and metabolic dysregulation. Simultaneously, the therapeutic procedure of continuous positive airway pressure (CPAP) for OSA has been observed to have a restricted impact on cardiovascular welfare (62). The present gap in therapeutic research involves an exploration of the association between OSA and atherosclerosis, with the objective of identifying modifiable features that could be targeted for treatment.

As we can see above, the incidence of atherosclerosis has been found to have a direct correlation with the composition of the intestinal microbiome and, among other factors, may be influenced by lifestyle determinants. Nevertheless, the consequences of prolonged episodes of intermittent hypoxemia necessitate a thorough assessment while considering the confounding factors of OSA. On the one hand, it has been found that hypoxia exerts an impact on the proliferation of distinct gut bacterial populations (63). On the contrary, the relationship between the aforementioned medical conditions, including hypertension, dyslipidemia, vascular inflammation, and atherosclerosis has been established (61). Consequently, the applicability of research investigating the interplay between atherosclerosis and the gut microbiome cannot be readily extrapolated to those individuals afflicted with OSA.

Yet, so far, just a few findings from investigations on the link between OSA and gut microbiota have been published (64-68), with only two of them concentrating on adults (64, 66). The link between OSA, atherosclerosis, and the gut microbiome has only been studied in a small number of researches, to the best of our knowledge, all in mice animal models (69-72), which showed, among others, that chronic intermittent hypoxia (CIH) and chronic sleep fragmentation (CSF) exert control over the abundance of intestinal microbes, including *Akkermansia mucinphila*, *Clostridium spp.*, *Lactococcus spp.*, and *Bifidobacterium spp.*, as well as functional metabolites, such as tryptophan, free fatty acids, branched amino acids, and bile acids. These factors, in turn, have a significant impact on adipose tissue and hepatic

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lipid metabolism, as well as the level of lipid deposition observed in both tissue and peripheral blood (71).

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2. Objectives

Our main objective was to investigate the association between different ultrasound markers of atherosclerosis and the gut microbiome in the Hungarian twin population.

1) We aimed to compare the clinical parameters of monozygotic (MZ) twins, grouped according to their different carotid IMT values.

2) Our aim was to compare the clinical parameters of OSA patients with verifiable/unverifiable atherosclerosis.

3) We aimed to determine whether there is a difference in the alpha diversity of the gut microbiome of MZ twins with a carotid IMT difference at the phylum level.

4) We set out to determine whether there is a difference in the Firmicutes/Bacteroidetes ratio - one of the indicators of gut dysbiosis - in MZ twins with a carotid IMT difference.

5) We aimed to determine whether there is a difference in the composition of the gut microbiome at the phylum, family, and genus level of MZ twins with a carotid IMT difference.

6) Our goal was to determine the discriminant features that most likely explain the differences between the two groups among OSA patients with verifiable/unverifiable atherosclerosis.

7) We set out to determine whether there is a difference in the alpha diversity of the intestinal microbiome at the phylum and genus level in OSA patients with verifiable/unverifiable atherosclerosis.

8) Our aim was to determine whether there is a difference in the beta diversity of the gut microbiome at the phylum, class, family, and genus level in OSA patients with verifiable/unverifiable atherosclerosis.

9) We set out to determine whether there is a correlation between the maximal carotid IMT and the alpha diversity of the gut microbiome at the phylum and genus level in OSA patients with verifiable/unverifiable atherosclerosis.

10) We aimed to determine whether there is a relationship between the gut bacteria groups at the phylum, class, family, and genus level of OSA patients who have verifiable/unverifiable atherosclerosis.

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11) Our goal was to determine whether there is a difference in the relative abundances of bacteria at the phylum, class, family, and genus level in OSA patients with verifiable/unverifiable atherosclerosis.

12) We set out to determine whether there is a difference between individual gut bacteria among OSA patients with verifiable/unverified atherosclerosis.

3. Methods

The methods detailed below were published in our previous articles (73, 74).

3.1. Subjects

3.1.1. Sample of the carotid IMT discordant twin study

Over a period spanning from October 2018 to April 2020, a cohort of 108 asymptomatic MZ Hungarian twins, comprising 54 pairs, with a mean age of 52.4 ± 14.1 years and 58% of the participants being female, were recruited from the Hungarian Twin Registry (75, 76) to undergo a carotid ultrasound examination. The exclusion criteria utilized for this study included pregnancy, prior carotid surgical intervention, acute infections within the three-week period prior to the study, and pre-existing oncologic illnesses.

The present study involved the measurement of mean and maximal IMT on the left and right CCA. The criterion for inclusion in the investigation was discordance with regards to IMT, which was operationalized as one twin exhibiting a maximal carotid IMT exceeding 0.9 mm and the other twin displaying a maximal IMT below 0.9 mm on either the left or right CCA or on both sides. Out of the 108 twins that were identified, a total of 14 discordant MZ pairs (n = 28, aged 52-73 years with a mean age of 65 ± 6.4 years and a female population of 71%) meeting the specified criteria were examined and subjected to analysis.

Prior to their participation in the research, all participants furnished their informed consent. This study was carried out in strict adherence to the principles and guidelines set forth in the Declaration of Helsinki. Approval for the protocol utilized was granted by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics, under the reference number SE TUKEB 189/2014, with subsequent amendments dated 10th October 2016 and 7th December 2018.

3.1.2. Sample of the OSA and atherosclerosis study

Caucasian subjects participating in the sleep cohort (77) (n = 142, 48 MZ and 23 dizygotic (DZ) twin pairs, mean age 51 ± 15 years) of the voluntary-based Hungarian Twin Registry who was diagnosed with OSA, underwent a carotid ultrasound examination and provided a stool sample for microbiome analysis were included (n = 22, mean age 59 ± 14 years, 55% female). The diagnosis of OSA was done as detailed in chapter 3.2.2.2. The mean and maximal IMT, as well as the existence of plaques, were measured and identified on the left and right CCA and the proximal section of the ICA. Two groups were defined based on the ultrasound examination: OSA patients with and without atherosclerosis. Atherosclerosis was defined as having at least one plaque on either the left, right, or both sides.

Before the commencement of the investigation, the subjects did not receive a diagnosis of OSA nor did they undergo any therapy for OSA management. The exclusion criteria consisted of pregnancy, previous carotid surgery, concurrent acute infection within a period of three weeks preceding the study, underlying presence of an oncologic illness, inflammatory bowel disease, and acute failures of respiratory, cardiac, and renal function.

Among patients with OSA, 16 subjects with atherosclerosis (aged 46–74 years, mean age 63 ± 8.8 years, 56% female) and 6 subjects without atherosclerosis (aged 23–67 years, mean age 47 ± 18.6 years, 50% female) met these criteria and classified into the two groups.

The research investigation was executed in compliance with the Declaration of Helsinki and granted authorization by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (SE TUKEB 30/2014 and SE TUKEB 189/2014, subsequent modifications implemented on October 10th, 2016, and December 7th, 2018).

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3.2. Study designs

Figure 1. shows the summary of the study designs.

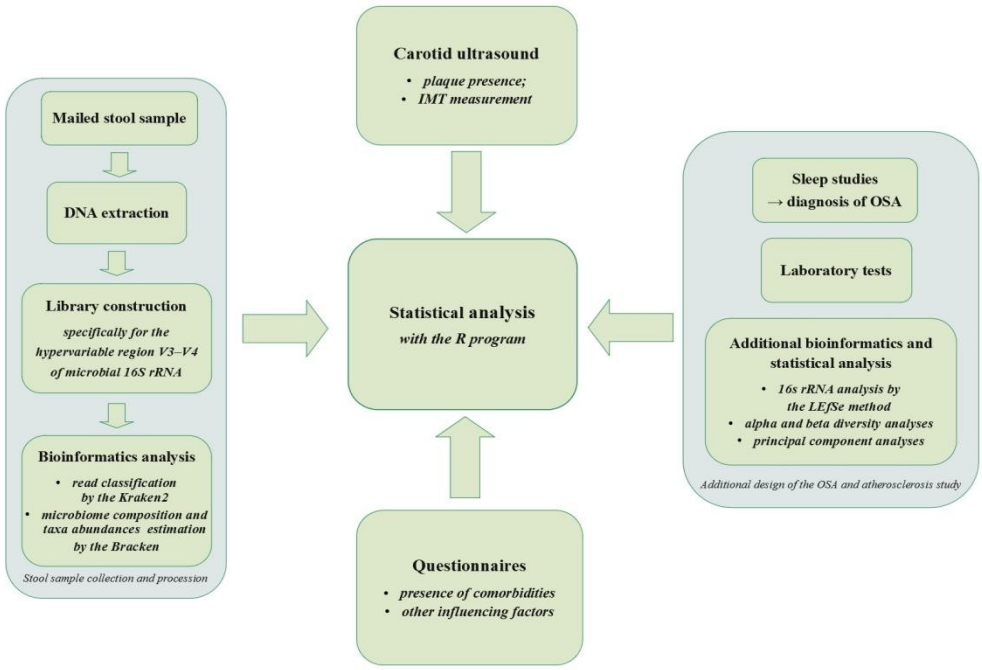

Figure 1. *Summary of the study designs.*

After a carotid ultrasound examination, stool sample collection, and procession, completing questionnaires, and in the case of the OSA study, laboratory tests, and additional bioinformatics analysis, comprehensive statistical analyses were made as detailed in the following chapters.

3.2.1. Common study designs

3.2.1.1. Blood pressure measurements, questionnaires

Blood pressure values were measured in the morning by TensioMed Arteriograph (Medexpert Ltd., Budapest, Hungary). Along with measuring body weight and height, body mass index (BMI) was also computed (OMRON Ltd., Kyoto, Japan). Self-reported questionnaires include the following questions were filled out to assess comorbidities and other important influencing factors:

- Have you been diagnosed with diabetes? If yes, is it treated with medication?
- Have you been diagnosed with hypertension? If yes, is it treated with medication?
- Have you been diagnosed with dyslipidemia? If yes, is it treated with medication?
- Have you been diagnosed with cardiovascular disease? If yes, with what type (ischemic heart disease, angina, myocardial infarction; stroke, TIA; peripheral vascular disease)? Is it treated with medication?
- Do you smoke? Never / previously yes / currently yes. For/from … years; … cigarette/day.
- Do you have coffee? No / Yes: … dose per day.
- Do you do sports? No / If yes, how many times a week: …; how many minutes each time: …

3.2.1.2. Carotid ultrasound

The ultrasound assessment was carried out utilizing the Samsung RS85 instrument (78), in conjunction with a high-resolution linear transducer of LM4-15B (15 MHz). The appearance of plaque was noted in the CCA as well as the proximal portion of the ICA (Figures 2. and 3.). The present study employed a semi-automated method to assess the IMT of the distal wall of the CCA at a precisely defined distance of 0.5-2 cm from the bifurcation. Specifically, the Arterial Analysis software was utilized for the computational analysis, as illustrated in Figure 4. During the process of measurement, the software showcases the particular segment of the vessel wall that has been identified and leverages the capabilities of ultrasonography to display it on the device's screen. This allows the examiner to take charge of the measurement process and make necessary adjustments in order to avoid the possibility of inaccurate data and outcomes that

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may arise from a fully automated measurement approach. In accordance with the 2018 guidelines issued jointly by the European Society of Cardiology and the European Society of Hypertension pertaining to the management of arterial hypertension, an abnormal categorization was assigned to carotid IMT measurements surpassing 0.9 mm (11).

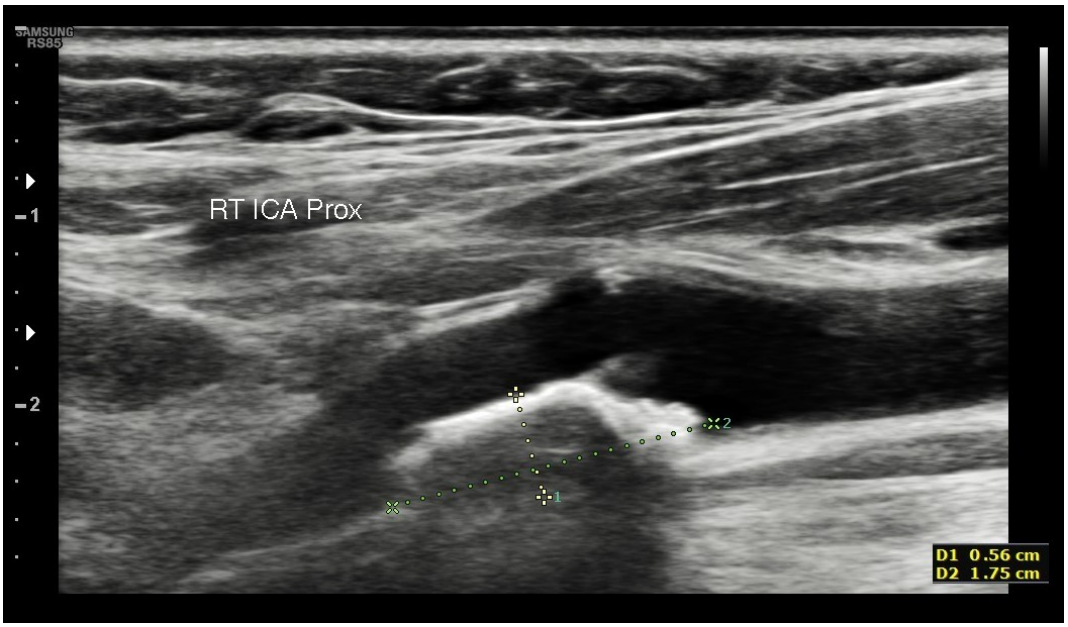

Figure 2. *Longitudinal recording of the proximal part of the right ICA with a plaque detected on carotid ultrasound. ICA: internal carotid artery. Image from the Medical Imaging Centre, Semmelweis University.*

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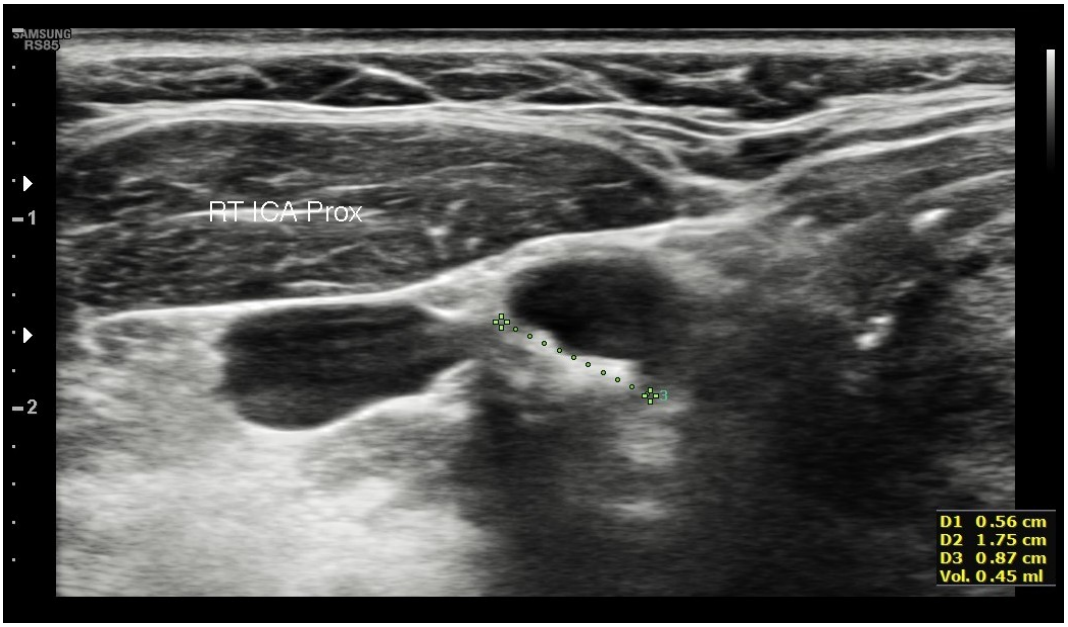

Figure 3. *Axial recording of the proximal part of the right ICA with a plaque detected on carotid ultrasound. ICA: internal carotid artery. Image from the Medical Imaging Centre, Semmelweis University.*

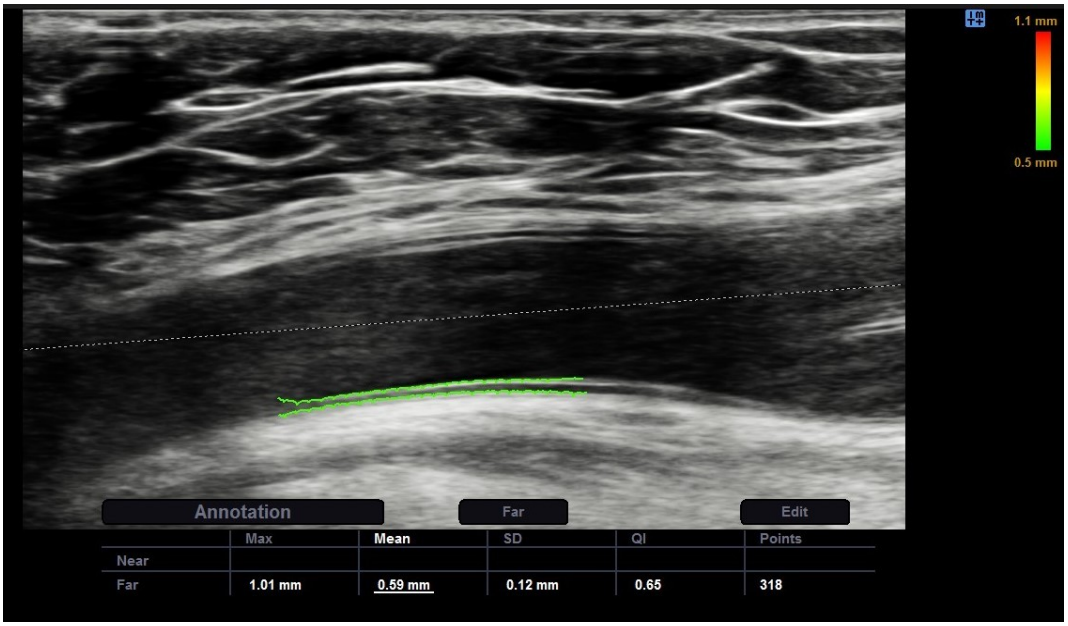

Figure 4. *Automated IMT measurement on the distal wall of the CCA distal segment on carotid ultrasound. CCA: common carotid artery; IMT: intima-media thickness. Image from the Medical Imaging Centre, Semmelweis University.*

3.2.1.3. Stool sample collection and procession

Stool sample collection was made between May 2019 and April 2020. Twin pairs were instructed to comply with the prescribed protocol of delivering their fecal specimens in a container equipped with a preservation solution, ensuring the retention of their original composition. The collected samples were required to be appropriately packaged and dispatched on the same day or, at the latest, within the succeeding day. The study subjects were provided with a comprehensive manual containing detailed guidelines for standardized sampling procedures, along with an illustrative flow chart. Special emphasis was placed on the criticality of minimizing the time lapse between sample collection and restoration, mitigating the possibility of extraneous contamination, and ensuring appropriate quantity, storage, and packaging of samples. In partnership with the Institute of Medical Microbiology at Semmelweis University, all samples were subjected to storage under freezing conditions before being subjected to processing.

After conducting DNA extraction, the library was constructed focusing specifically on the V3–V4 hypervariable region of microbial 16S ribosomal ribonucleic acid (rRNA), adhering to the methodology suggested by Illumina and commonly used in microbiome studies (79). The libraries were assigned with unique index pairs and subjected to quantification and normalization procedures using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Subsequently, the pooled libraries were subjected to sequencing on an Illumina MiSeq platform (Thermo Fischer Scientific, Waltham, MA, USA) via a 600-cycle MiSeq Reagent Kit v3.

3.2.1.4. Bioinformatics and statistical analysis

The bioinformatics analysis of the 16S sequencing data was conducted through methods previously detailed (80). In brief, the FastQC and MultiQC tools were utilized for an evaluation of the quality of the raw reads (81). Subsequently, Trimmomatic was applied for filtering and trimming of low-quality sequences (82), with sequences possessing a minimum length of 50 being exclusively retained. In order to ensure the reliability of the genomic data, low-quality calls

were excluded from the analysis, in addition to the exclusion of the initial 12 base calls that did not meet a minimum Phred score of less than 20 when applying a sliding window size of 5. The classification of reads was carried out using Kraken2 (83, 84), specifically with a k-mer size of 31, in conjunction with the SSU Ref NR 99 database (release 132) sourced from SILVA (85). The Bracken technique was employed to determine the composition of the microbiome and the corresponding taxonomic abundances (86). The Shannon index, a diversity index utilized in statistical analysis to describe the diversity of a population wherein each constituent is a distinct group, was computed in the current study. In this particular case, the groups under investigation represent bacterial taxa.

The present study employed the R program, developed by the R Foundation for Statistical Computing headquartered in Vienna, Austria, to conduct statistical analyses. A p-value equal to or below 0.05 was deemed statistically significant.

3.2.2. Additional design of the OSA and atherosclerosis study

3.2.2.1. Laboratory tests

To quantify triglycerides, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), LDL-C, and C-reactive protein (CRP), venous blood samples were procured.

3.2.2.2. Sleep studies

The sleep study was executed as reported previously (64) using the Somnoscreen Plus Tele PSG and the Somnoscreen RC tools (Somnomedics GmbH, Randersacker, Germany) at the Department of Pulmonology, Semmelweis University from 2014. The manual assessment of sleep stages, movements, and cardiopulmonary events was conducted based on the guidelines provided by the American Academy of Sleep Medicine (87). Apnoea was operationally defined as a reduction in nasal flow exceeding or equal to 90% and lasting for a minimum of 10 seconds. The term hypopnoea is characterized as a reduction of at least 30% in the nasal flow, lasting for a minimum of 10 seconds, that is accompanied by a decrease of at least 3% in oxygen saturation (as observed in both polygraphy (PG) and polysomnography (PSG)) or arousal (as indicated in PSG). Apnoea-

hypopnoea index (AHI), oxygen desaturation index (ODI), and the percentage of total sleep time spent with a saturation level below 90% (TST90%) were calculated. OSA was diagnosed if AHI was ≥5/h.

3.2.2.3. Additional bioinformatics and statistical analysis

The employed approach in the present study for the 16s rRNA analysis consisted of utilizing the linear discriminant analysis effect size (LEfSe) technique (88). This method enables the determination of the features that are most likely to account for dissimilarities between the two groups, and thereby represent potential biomarkers for differentiation. The analysis was performed utilizing the default parameters on the Galaxy server hosted by the Huttenhower lab (http://huttenhower.sph.harvard.edu/galaxy/, accessed on 8 December 2022). The alpha level employed for the factorial Kruskal-Wallis test conducted within groups was set to 0.05, whilst the logarithmic LDA threshold for discriminative features has been established at 2. In order to conduct alpha diversity analysis, the Wilcoxon rank sum test was employed to compare the Shannon indexes of different groups at the phylum and genus levels. The correlation between the Shannon index and maximal CCA IMT was examined at two taxonomic levels. This was achieved through a linear regression model with the inclusion of Shannon indexes, age, and gender as covariates. The regression model was constructed with only age and gender as covariates due to the observation that the majority of the variables did not demonstrate significant differences in the univariate analysis, while also acknowledging the importance of avoiding collinearity. In order to analyze beta diversity, the statistical technique known as principal coordinates analysis (PCoA) was utilized, applying the Bray-Curtis distance measure. This approach converts distances between items into a map-based representation that facilitates visualization of those items. The proximity of objects is positively correlated with their degree of similarity. Specifically, those objects that are positioned in closer proximity to one another exhibit greater levels of resemblance, in contrast to those that are distanced further apart. PCAs were employed to investigate the interrelation among bacterial groupings. PCAs represent a statistical methodology employed to decrease the dimensionality of

significant datasets characterized by a substantial number of dimensions or features per observation, as well as to facilitate their analysis. The PCA was conducted using the Matlab programming language version MATLAB R2020b, utilizing the Fathom toolbox (https://www. usfedu/marine-science/research/matlab-resources/fathom-toolbox-for-matlabaspx, accessed on December 8, 2022). In order to evaluate the proportional distribution of bacteria within both groups, permutation tests were implemented to compare the medians of the respective groups.

4. Results

The results detailed below were published in our previous articles (73, 74).

4.1. Clinical characteristics

4.1.1. Clinical characteristics of the carotid IMT discordant twin study

This study involved the analysis of fourteen pairs of monozygotic twins who were discordant with respect to carotid IMT. The participants included in the analysis had a total number of 28 individuals, with an age range of 52 to 73 years and a mean age of 65 ± 6.4 years Additionally, 71% of the participants were female. Table 1 presents fundamental patient characteristics data, including mean BMI, blood pressure, IMT, and atherosclerosis risk factors, disaggregated by normal/increased IMT grouping.

Table 1. *Basic patient characteristics data in the groups with normal/increased IMT. (73)*

Characteristic	IMT > 0.9 Group (n = 14)	IMT < 0.9 Group (n = 14)
BMI (kg/m^2; mean ± SD)	28.3 ± 3.3	28.3 ± 3.7
Systolic blood pressure (mmHg; mean ± SD)	135.2 ± 16.6	133.2 ± 17.8
Diastolic blood pressure (mmHg; mean ± SD)	79.2 ± 9.9	78.9 ± 10.7
Carotid IMT max (mm; mean ± SD)	0.94 ± 0.16	0.81 ± 0.13
Smoking (n)	1	3
Regular coffee consumption (n)	8	8
Regular sports activities (n)	6	6
Diabetes (n)	3	2
Hypertension (n)	8	4
Dyslipidemia (n)	5	1

BMI: body mass index; IMT: intima-media thickness; SD: standard deviation.

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4.1.2. Clinical characteristics of the OSA and atherosclerosis study

A study conducted among patients diagnosed with OSA consisted of two groups: one with atherosclerosis comprising 16 subjects aged between 46 and 74 years, with a mean age of 63 ± 8.8 years and 56% female participants, and another without atherosclerosis, comprising six subjects aged between 23 and 67 years, with a mean age of 47 ± 18.6 years, and 50% female participants. The statistical analysis of the data collected was performed using t-tests. Table 2 exhibits fundamental patient information organized into distinct clusters comprised of individuals both with and without atherosclerosis. There was no statistically significant difference observed between the two groups.

Table 2. *Descriptive analysis in OSA +/- atherosclerosis groups. (74)*

	OSA + atherosclerosis (n=16)	OSA - atherosclerosis (n=6)	p
Age (years)	63 (9)	47 (19)	0.09
Gender (males%)	44	50	0.81
BMI (kg/m^2)	28.1 (6.2)	26.3 (3.3)	0.39
Smoking (%)	19	33	0.55
Hypertension (%)	63	33	0.27
Cardiovascular disease (%)	13	0	0.16
Diabetes (%)	13	0	0.16
Dyslipidaemia (%)	44	50	0.81
SBP (mmHg)	131 (22)	123 (15)	0.35
DBP (mmHg)	81 (10)	78 (10)	0.48
CRP (mg/L)	3.3 (2.4)	1.5 (1.3)	0.09
Total cholesterol (mmol/L)	5.6 (1.3)	5.7 (1.5)	0.85
LDL-C (mmol/L)	3.1 (1.2)	3.6 (1.4)	0.56
HDL-C (mmol/L)	1.8 (1.6)	1.4 (0.5)	0.44
Triglyceride (mmol/L)	1.5 (0.5)	1.6 (0.8)	0.79
AHI (1/h)	14.1 (10.2)	11.8 (7.6)	0.58
ODI (1/h)	10.9 (7.1)	7.4 (7)	0.33
TST90%	2.9 (4)	2.5 (4.4)	0.87

AHI: apnoea-hypopnoea index; BMI: body mass index; CRP: C-reactive protein; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; ODI: oxygen desaturation index; OSA: obstructive sleep apnoea; SBP: systolic blood pressure; TST90%: percentage of total sleep time spent with saturation below 90%.

4.2. Results of the carotid IMT discordant twin study

4.2.1. Gut microbiome alpha diversity of discordant twin pairs at the phylum level

It was hypothesized that the Shannon index for the phyla would exhibit a greater magnitude in the cohort possessing an IMT below 0.9 mm, relative to the group displaying an IMT above 0.9 In order to evaluate the hypothesis of no significant differences between the two groups, a unidirectional exact permutation test was executed. This test aimed to ascertain the sharp null hypothesis. The results of Table 3 indicate that the null hypothesis, which states that there is no significant association between IMT levels and the Shannon index of the phyla, could not be rejected at a significance level of 0.05 ($p = 0.153$) Consequently, it can be inferred that there may not be a statistically significant relationship between these two variables.

Table 3. *Results of exact permutation tests of Firmicutes/Bacteroidetes ratio and Shannon index of the phyla between the two groups. (73)*

	Mean (IMT>0.9) (n = 14)	**Mean (IMT<0.9) (n = 14)**	**Mean difference**	**P value***
Firmicutes/Bacteroidetes ratio	2.299	1.436	0.863	0.031 (0.018, 0.047)
Shannon index of the phyla	1.35	1.44	-0.09	0.153 (0.124, 0.184)

** p value was estimated using 999 Monte Carlo replications, 99% confidence interval in parenthesis. IMT: intima-media thickness.*

4.2.2. Firmicutes/Bacteroidetes ratio

One hypothesis that was examined was that the Firmicutes/Bacteroidetes ratio would exhibit a higher value among subjects in the high IMT group compared to those in the low IMT group. Based on the findings presented in Table 3, the utilization of an exact permutation test revealed the rejection of the sharp null

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hypothesis that posited the absence of any distinction between the two groups (p = 0.031) The study revealed that an increased Firmicutes/Bacteroidetes ratio was linked to the manifestation of an atherosclerotic phenotype.

4.2.3. Microbial compositions of discordant twin pairs

The microbial composition of each group was analyzed at three distinct taxonomic levels. In both groups, the microbe fractions' median and interquartile range were quantified. The data was then subjected to sorting and subsequent presentation of the top five commonly encountered bacterial compositions for each taxonomy level in Figures 5.-7.

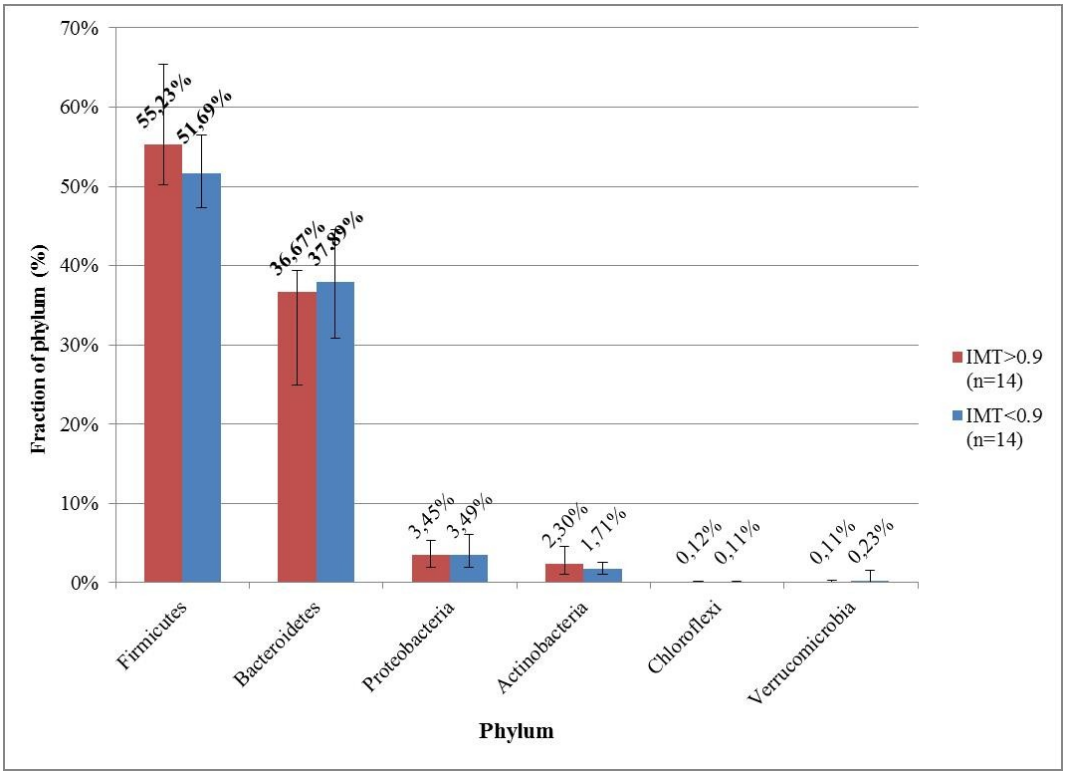

Figure 5. *The 5 most common phyla in the groups with high and normal IMT. The descending order was based on the group with high IMT. The fractions of the most prominent phyla are marked with bold font. IMT: intima-media thickness. (73)*

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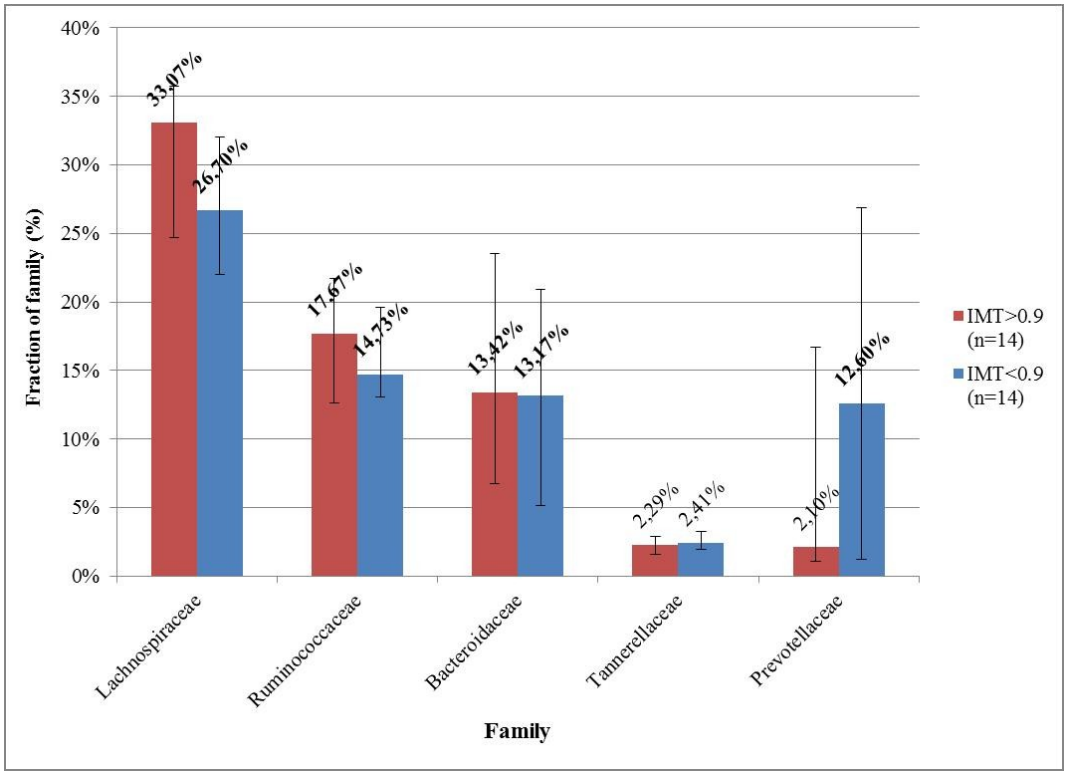

Figure 6. *The 5 most common families in the groups with high and normal IMT. The descending order was based on the group with high IMT. The fractions of the most prominent families are marked with bold font. IMT: intima-media thickness. (73)*

DOI:10.14753/SE.2024.2906

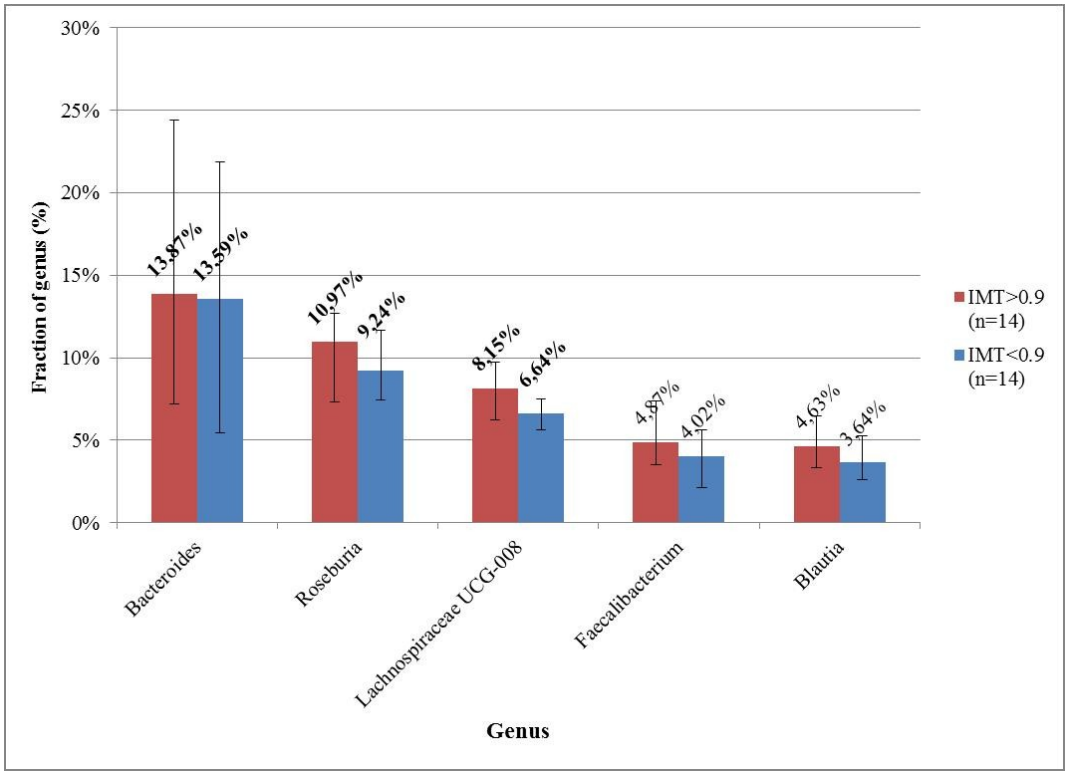

Figure 7. *The 5 most common genera in the groups with high and normal IMT. The descending order was based on the group with high IMT. The fractions of the most prominent genera are marked with bold font. IMT: intima-media thickness. (73)*

Despite similarities in outcomes between the two groups, noteworthy distinctions were also observed.

The phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* exhibited maximum prevalence in both cohorts, as illustrated in Figure 5. *Firmicutes* had a higher percentage of the group with high IMT values, followed by *Bacteroidetes*, and vice versa.

The taxonomic families *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae* were found to be highly prevalent in both groups as evidenced by their ranking as the top three taxonomic families, as depicted in Figure 6. There was a statistically significant increase in the relative abundance of *Prevotellaceae* within the cohort with normal IMT values when compared to the cohort with elevated IMT values.

Bacteroides, *Roseburia*, *Lachnospira*, *Faecalibacterium*, and *Blautia* were the five most important genera in both study groups (Figure 7.).

4.3. Results of the OSA and atherosclerosis study

4.3.1. Result of the LEfSe analysis

Figure 8. shows the result of the LEfSe analysis. The abundance of 25 taxonomic groups was significantly different between the OSA with or without atherosclerosis patients groups.

The following taxa proved to be more abundant in the atherosclerotic group: *Porphyromonas*, *Erysipelotrichaceae*, *Paraprevotella*, *Porphyromonadaceae*, *Erysipelotrichia*, *Epulopiscium*, *Eubacterium*, *Synergistaceae*, *Synergistia*, *Synergistetes*.

The following taxa proved to be more abundant in the non-atherosclerotic group: *Peptostreptococcaceae*, *Romboutsia*, *Lachnospiraceae*, *Coriobacteriales*, *Eubacterium*, *Dysgonomonas*, *Rikenellaceae*, *Tistrella*, *Tistrellaceae*, *Burkholderiaceae*, *Parasutterella*, *Bacteroidaceae*, *Bacteroides*.

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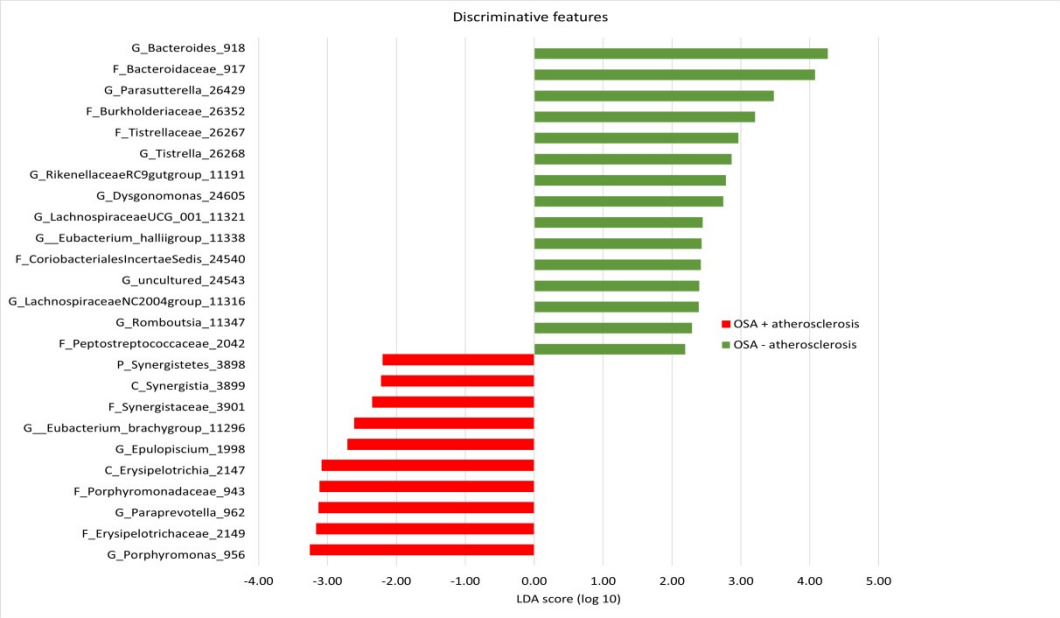

Figure 8. *Discriminant features between the two groups based on the LEfSe method. Red bars represent taxa more abundant in the atherosclerotic group, green bars represent taxa more abundant in the non-atherosclerotic group. LefSe: linear discriminant analysis effect size. (74)*

4.3.2. Results of the alpha diversity analyses

Figure 9. and Figure 10. show the results of alpha diversity analyses at 2 taxonomic levels (phylum and genus). Wilcoxon rank sum tests between the phylum and genus level Shannon indexes of the groups were p = 0.9119 and p = 0.1970, respectively, indicating that there were no notable dissimilarities between the examined groups.

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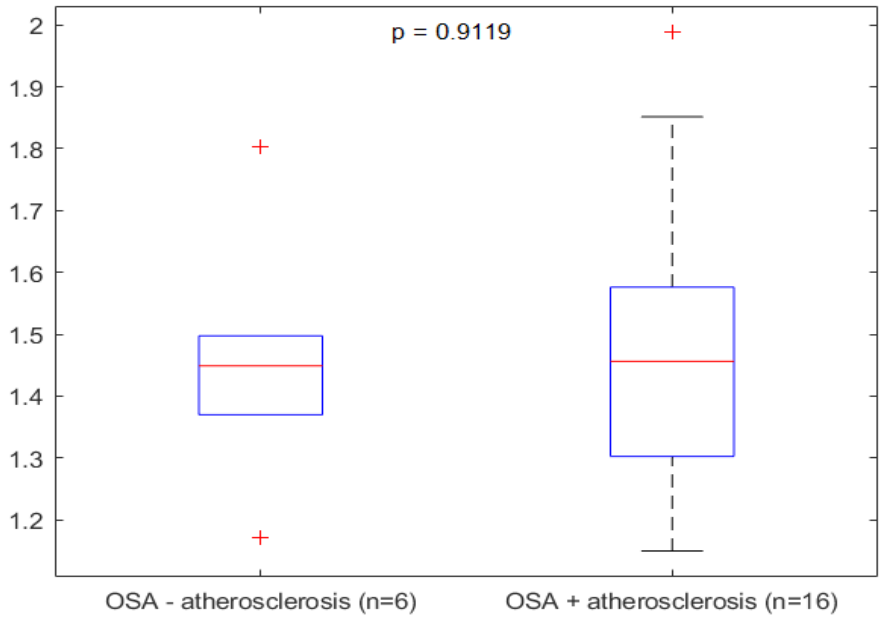

Figure 9. *Shannon alpha diversity box plot analysis at phylum level. (74) Wilcoxon rank sum test indicated no significance between the two study groups.*

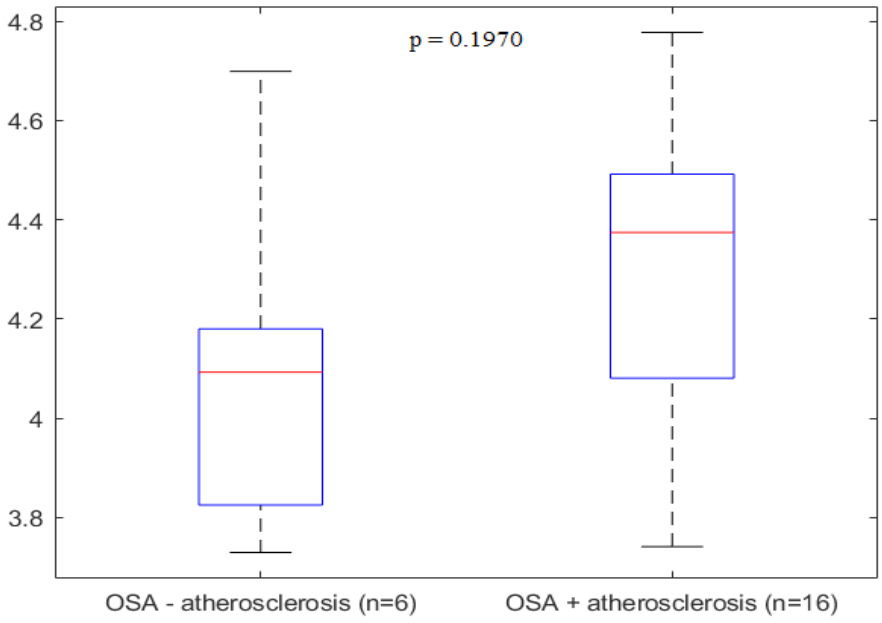

Figure 10. *Shannon alpha diversity box plot analysis at genus level. (74) Wilcoxon rank sum test indicated no significance between the two study groups, however the mean level of Shannon index of the atherosclerosis + group was higher.*

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4.3.3. Results of the beta diversity analyses

Figures 11.-14. show the results of beta diversity analyses at 4 taxonomic levels (phylum, class, family, and genus). In the PCoA space, no discernible clusters were observed. The group exhibiting atherosclerosis displayed a greater level of variation. It is noteworthy that this group's sample size was considerably larger than the other group, consisting of 16 versus 6 individuals.

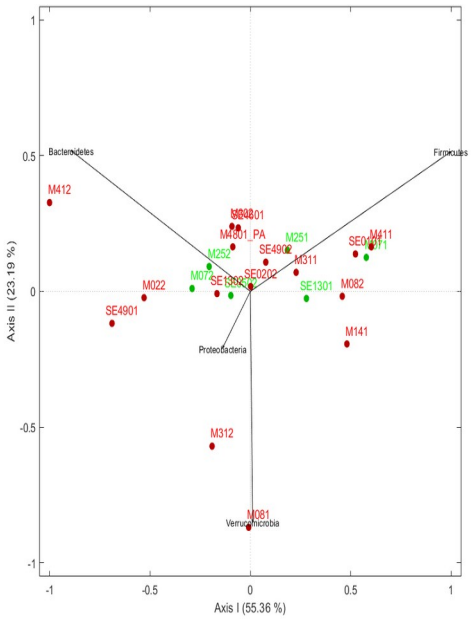

Figure 11. *Beta diversity analyses at the phylum level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCoA: principal coordinates analysis. (74) No obvious clustering showed in the PCoA space, however, the group with atherosclerosis appeared to be more varied.*

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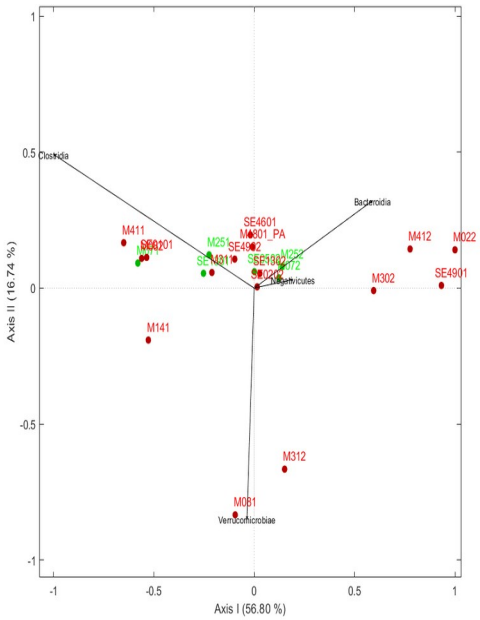

Figure 12. *Beta diversity analyses at the class level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCoA: principal coordinates analysis. (74) No obvious clustering showed in the PCoA space, however, the group with atherosclerosis appeared to be more varied.*

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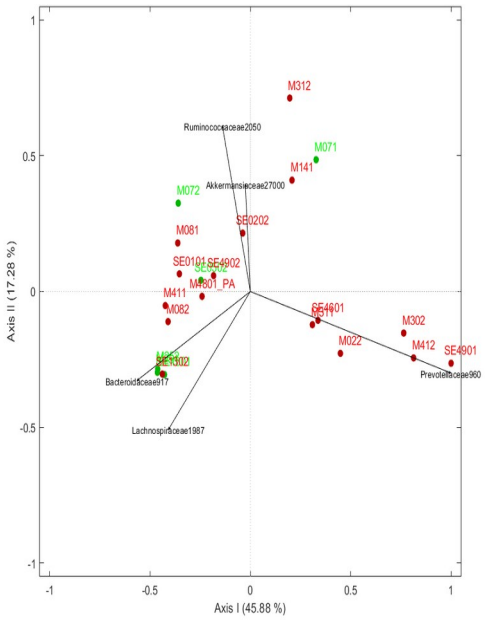

Figure 13. *Beta diversity analyses at the family level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCoA: principal coordinates analysis. (74) No obvious clustering showed in the PCoA space, however, the group with atherosclerosis appeared to be more varied.*

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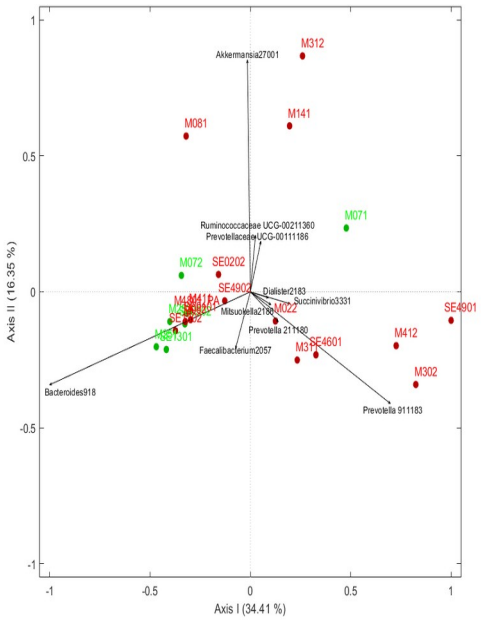

Figure 14. *Beta diversity analyses at the genus level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCoA: principal coordinates analysis. (74) No obvious clustering showed in the PCoA space, however, the group with atherosclerosis appeared to be more varied.*

4.3.4. Result of the correlation between maximal CCA IMT and microbiome alpha diversity

We conducted two linear regression analyses with the bilateral mean of maximal CCA IMT at two taxonomic levels, while considering gender, age, and the Shannon index at the phylum and genus levels as independent predictor variables. The findings are presented in Table 4. The maximal CCA IMT exhibited a strong association with the diversity at the phylum level: a decline in diversity was found to be directly proportional to an elevation in IMT. The association was considered adjusted because age and gender were included in the regression. Age affected regression as well.

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Table 4. *Correlation of maximal CCA IMT and microbiome alpha diversity at 2 taxonomic levels. (74)*

	Dependent variable:	
	Maximal CCA IMT	
	Phylum level	**Genus level**
Male gender	−0.046 (0.053) p = 0.401	0.006 (0.062) p = 0.929
Age	0.009 *** (0.002) p = 0.0001	0.008 *** (0.002) p = 0.002
Shannon index	−0.275 ** (0.119) p = 0.033	0.011 (0.100) p = 0.916
Note:	** p < 0.05; *** p < 0.01	

CCA IMT: common carotid artery intima-media thickness.

The correlation between the Shannon index at the phylum taxonomic level and maximal CCA IMT is shown in Figure 15. as an effect plot based on regression.

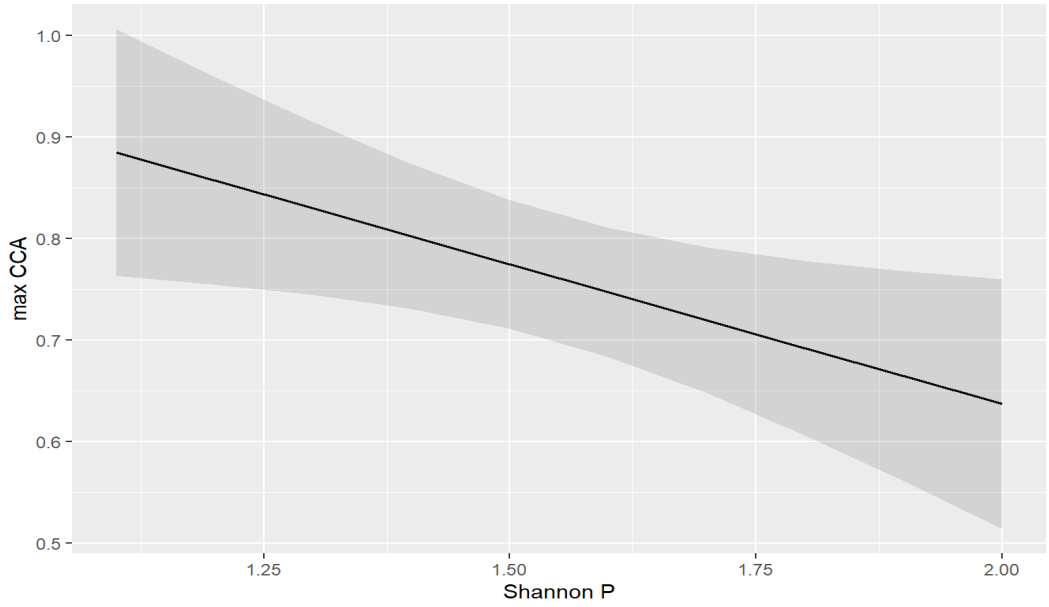

Figure 15. *The correlation between the Shannon index at the phylum taxonomic level and maximal CCA IMT. CCA IMT: common carotid artery intima-media thickness. (74) As maximum CCA IMT increases, phylum-level diversity decreases.*

4.3.5. Results of the principal component analyses

The results of PCAs are shown in Figure 16.-19. The PCA space did not exhibit apparent clustering. No noteworthy descriptor variables (bacterial groups) were apparent.

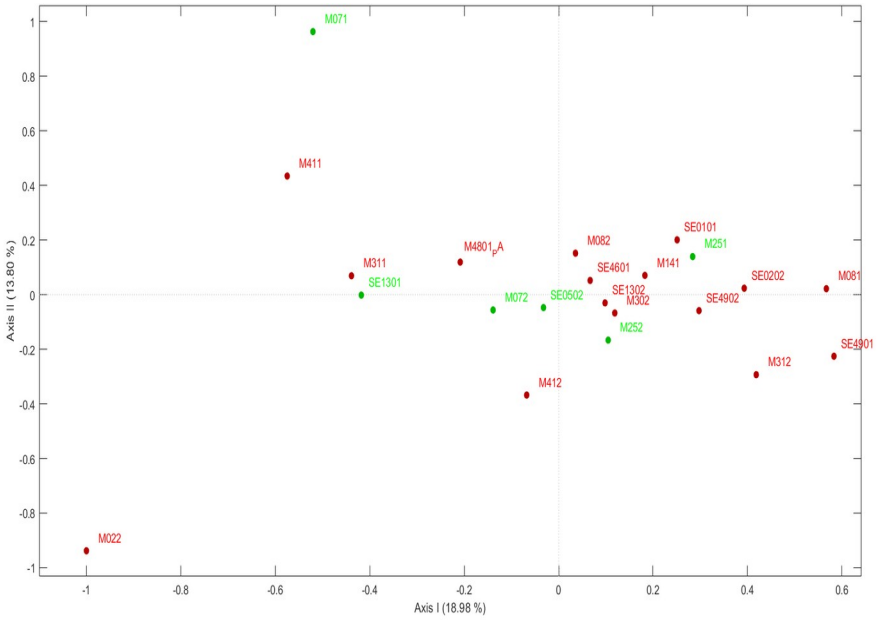

Figure 16. *The results of PCA at the phylum level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCA: principal component analysis. (74) No obvious clustering appeared in the PCA space.*

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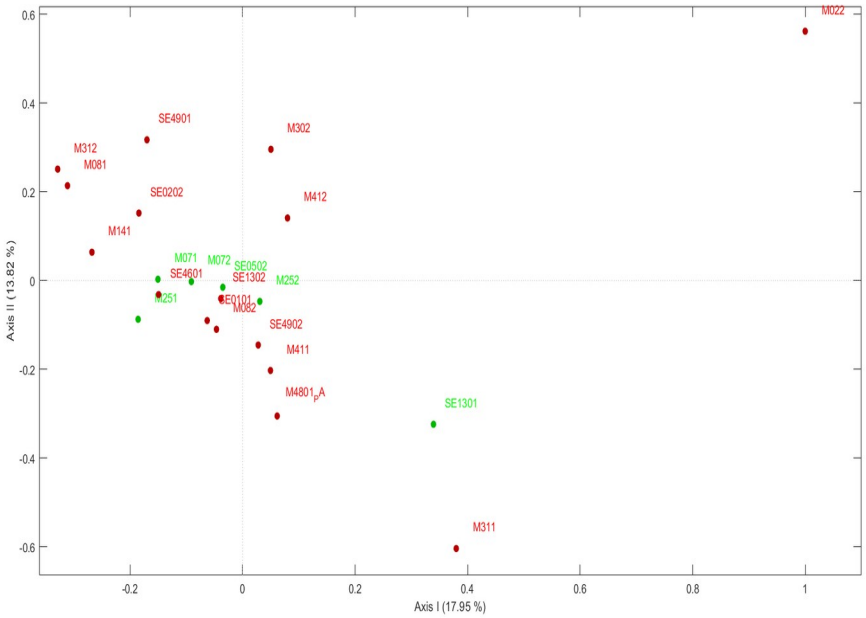

Figure 17. *The results of PCA at the class level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCA: principal component analysis. (74) No obvious clustering appeared in the PCA space.*

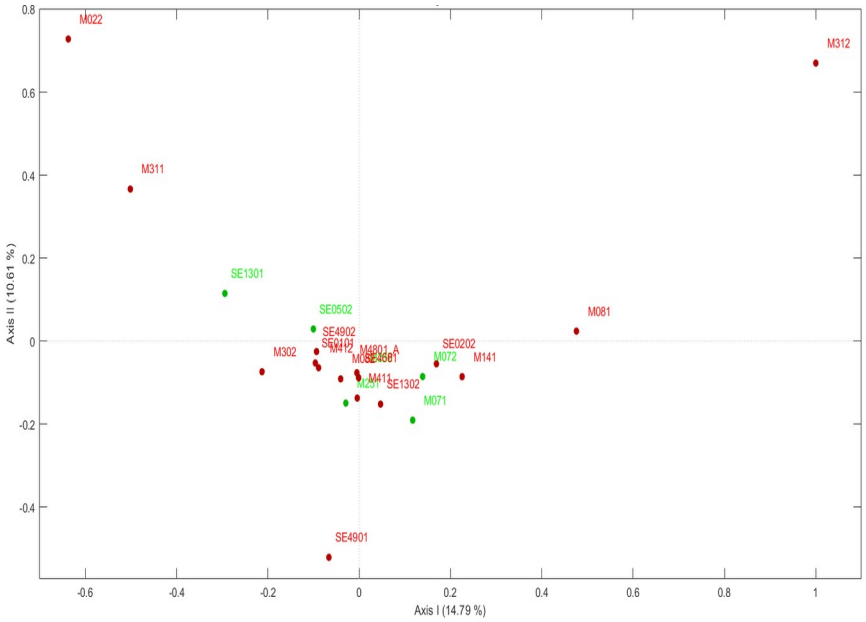

Figure 18. *The results of PCA at the family level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCA: principal component analysis. (74) No obvious clustering appeared in the PCA space.*

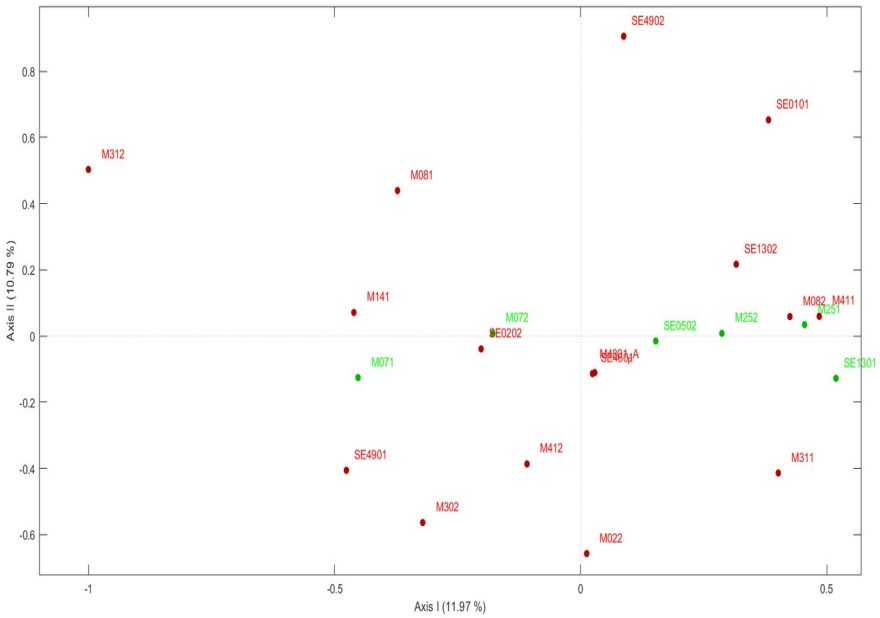

Figure 19. *The results of PCA at the genus level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis. SE_, M_: names of patient samples. PCA: principal component analysis. (74) No obvious clustering appeared in the PCA space.*

4.3.6. Results of the relative abundances of bacteria

Figures 20.-23. show taxonomic categories with more than 1% relative abundance on average through the samples.

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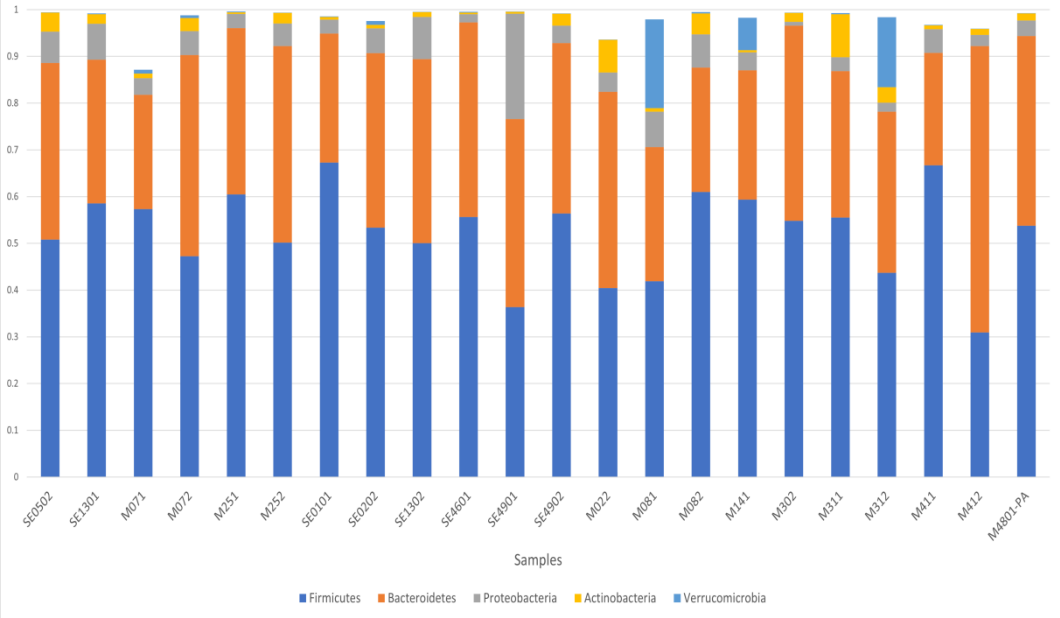

Figure 20. *The relative abundances of bacteria at the phylum level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group. SE_, M_: names of patient samples. (74)*

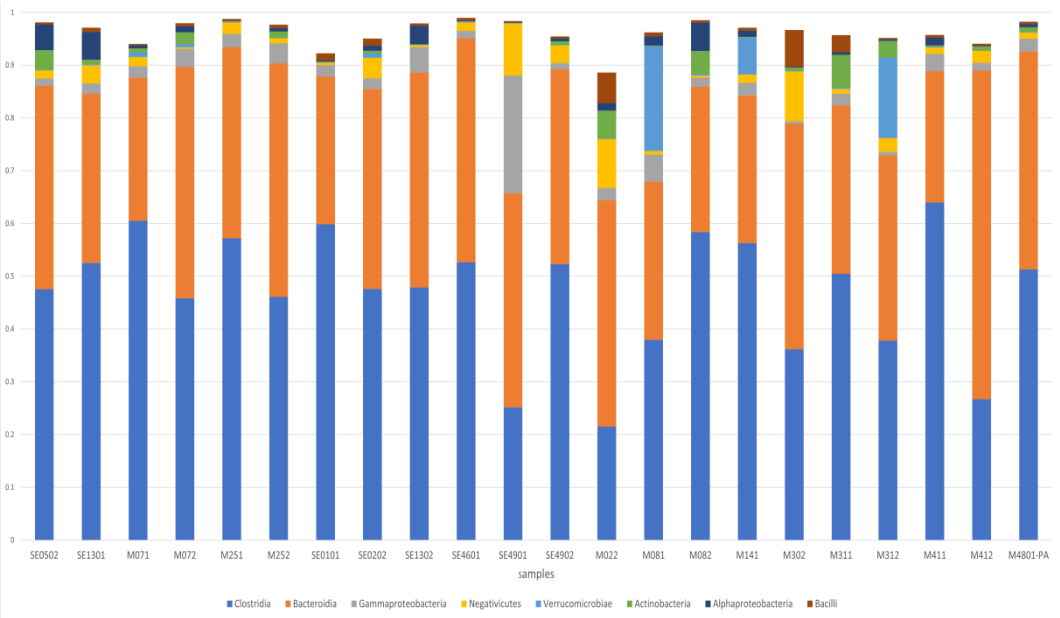

Figure 21. *The relative abundances of bacteria at the class level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group. SE_, M_: names of patient samples. (74)*

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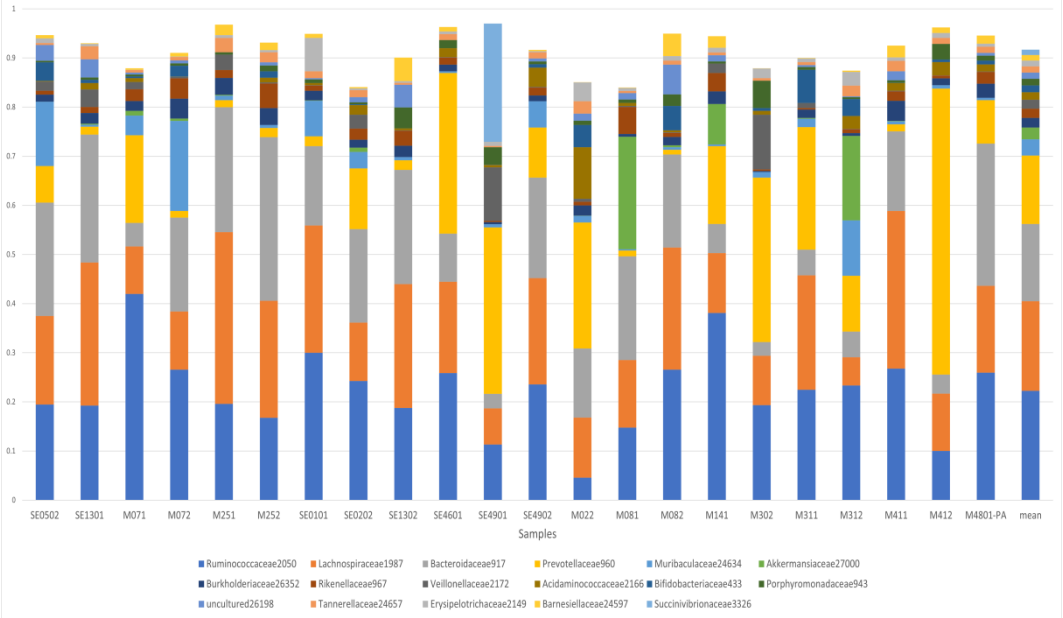

Figure 22. *The relative abundances of bacteria at the family level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group. SE_, M_: names of patient samples. (74)*

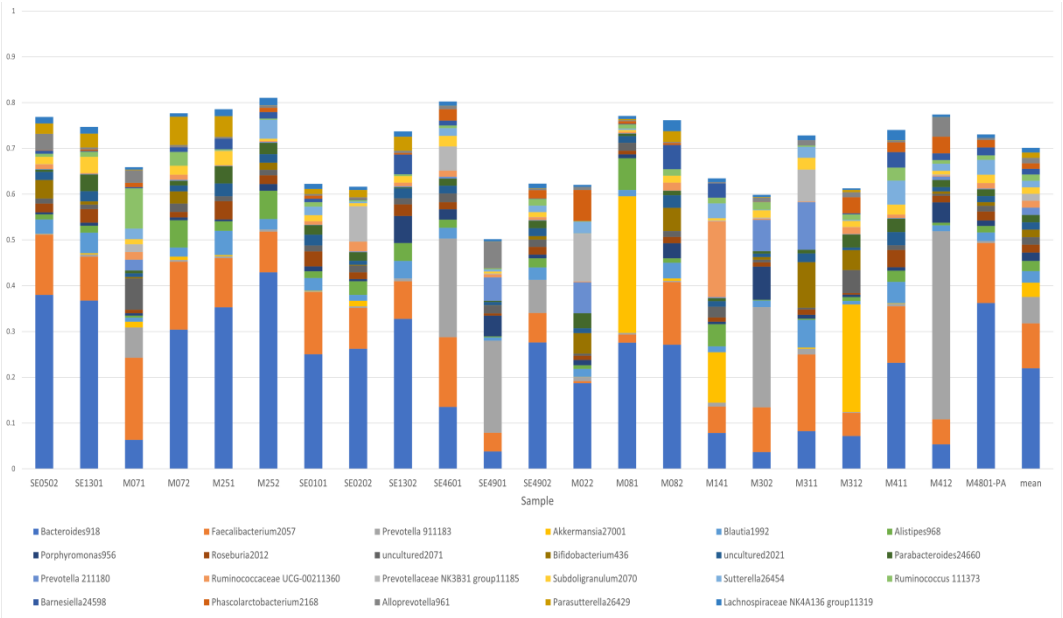

Figure 23. *The relative abundances of bacteria at the genus level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group. SE_, M_: names of patient samples. (74)*

4.3.7. Results of the analysis of the difference between individual bacteria

Using the difference between the median fractional reads of the bacteria in the atherosclerotic and non-atherosclerotic samples, we conducted permutation testing to see if there was a difference between the two samples for each microbe. Since many tests were performed, we used the Benjamini-Hochberg approach to correct the probability of error from the multiple hypothesis testing, using $p < 0.1$ more acceptable p-values. We discovered significantly lower *Peptostreptococcaceae* levels in atherosclerotic individuals (Table 5. and Figure 24.).

Table 5. *The result of the permutation test for the Peptostreptococcaceae family. (74)*

	Median abundance (Non-Atherosclerotic)	Median abundance (Atherosclerotic)	ΔMedian	p-value
Peptostreptococcaceae	0.002360	0.000950	0.00141	<0.00001

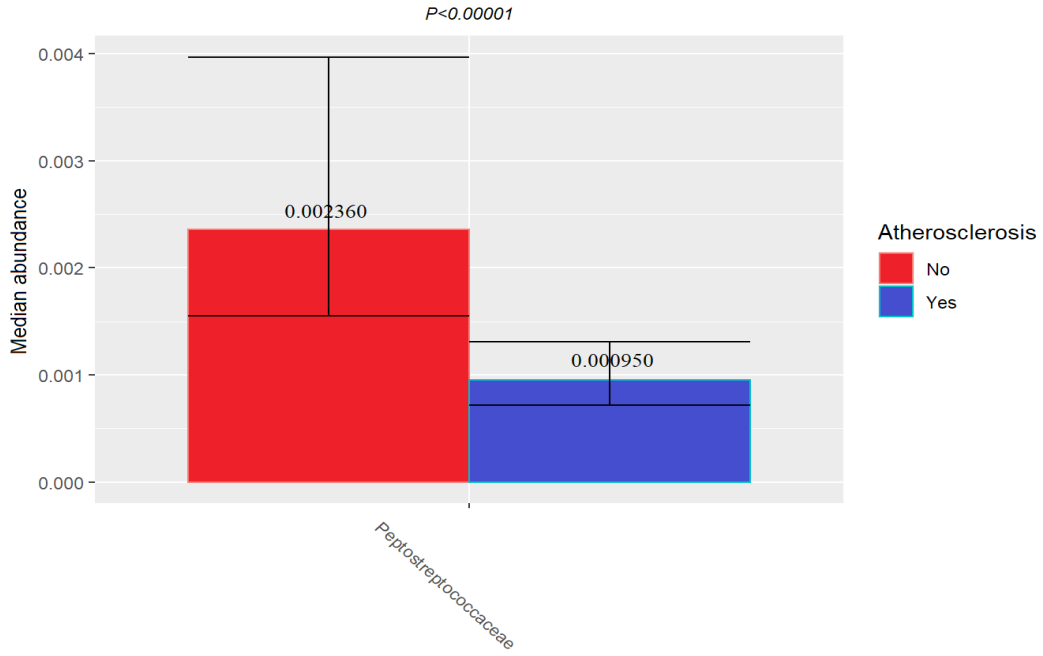

Figure 24. *The median abundance and the interquartile range of the fractions of Peptostreptococcaceae. (74)*

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4.3.8. Results of the candidate microbes without correction

In the absence of statistical correction for multiple comparisons and using a significance threshold of $p < 0.05$ for the permutation test, the present study identified several candidate microbes at the genus level in two distinct groups. More specifically, in the non-atherosclerotic group, the identified genera were *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella*, while in the atherosclerotic group, the corresponding genera were *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae* (Figure 25.).

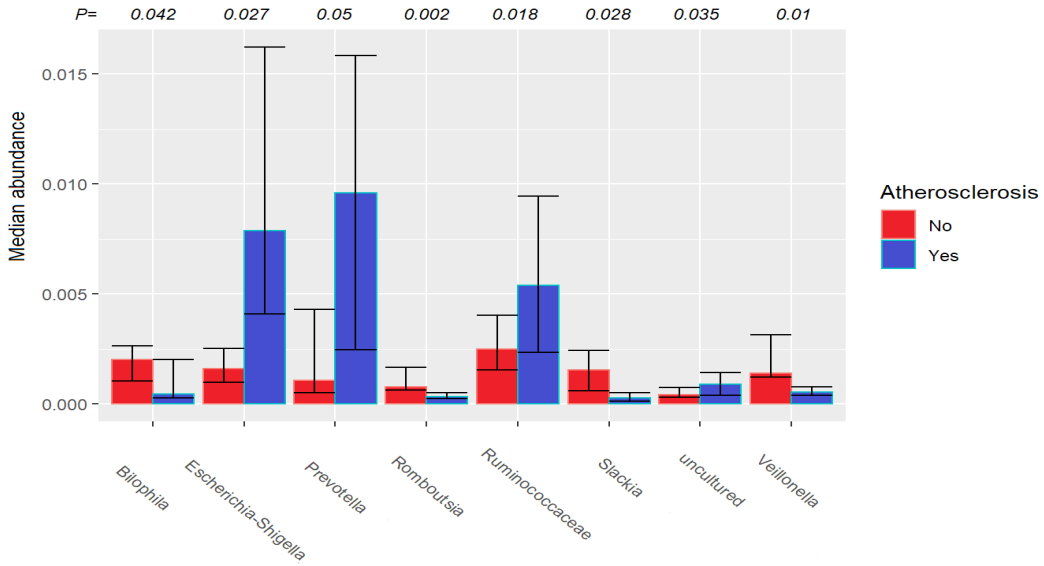

Figure 25. *The median abundance of candidate microbes at the genus taxonomic level without correction in the two groups. (74)*

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5. Discussion

5.1. Carotid IMT and gut microbiome diversity

The gut microbiome has been shown to affect hypertension and atherosclerosis through a multitude of pathways (89). The carotid-femoral pulse wave velocity (PWV), a measure of arterial stiffness, was examined in 617 middle-aged women from the TwinsUK cohort (90), and its connection with the gut microbiome composition and contemporaneous serum metabolomics data was assessed. After adjusting for covariates, PWV was found to be adversely correlated with gut microbiome alpha diversity, implying that gut microbiome diversity is inversely related to arterial stiffness in women. The preceding link is further supported by the Moscow Study, in which enterotyping revealed two clusters distinguished by alpha diversity, with IMT being higher in the cluster with lesser diversity (adj. $p < 0.001$) (91). While we did not find an association of lower alpha diversity with higher IMT in our carotid IMT discordant twin study, this can be argued by 1) the relatively low number of participants compared to other studies, and 2) the different study population: although the gender ratio was nearly the same, the geographical location (Hungary vs. Moscow and Moscow Region) and the age of the participants (mean age 65 ± 6.4 vs. 52 ± 13 years, and aged 52–73 vs. 25–76 years) differed significantly (9); in our OSA and atherosclerosis study, we identified a link between a greater maximal CCA IMT and reduced phylum-level diversity, which is consistent with earlier research.

5.2. Carotid IMT and Firmicutes/Bacteroidetes ratio

The human gut microbiota is dominated by two bacterial phyla, *Firmicutes* and *Bacteroidetes*, which account for more than 90% of the entire community, as well as additional subdominant phyla such as *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* (28, 92). This composition is relatively unaffected by acute perturbations since its plasticity allows it to recover to its initial composition quickly (93). We were able to support this conclusion because the most prevalent phyla were the same in each of our studies: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*,

Actinobacteria, and *Verrucomicrobia* vs. *Chloroflexi* - the latter in the group with IMT > 0.9. However, there may be variances in their proportions.

There are conflicting results in the literature about the Firmicutes/Bacteroidetes ratio as a possible hallmark for obesity (94-103), on which we could not take a position, since there was no significant difference in BMI values between the two groups in our carotid IMT discordant twin study, but an increased Firmicutes/Bacteroidetes ratio was discovered in conjunction with higher IMT, independent of BMI, reinforcing its importance as a marker of atherosclerotic phenotype. Furthermore, we must be cautious because some lifestyle-related factors known to alter microbiota composition (e.g., dietary preferences (104)) were not investigated in the current study; thus, the role of these factors must be considered when evaluating the results.

5.3. Atherosclerosis and *Prevotellaceae*

There are conflicting findings in the scientific literature regarding the relationship between *Prevotella* and cardiometabolic and cardiovascular disorders. *Prevotella* abundance was substantially associated with obesity in the Moscow Study (91), and *Prevotella* was enriched or showed a rising trend in middle-aged, eastern Polish males with abnormal levels of total and LDL-C values (105). At the same time, as a result of atherosclerosis, patients with stroke and TIA had fewer commensal or beneficial genera, such as *Bacteroides*, *Prevotella*, and *Faecalibacterium*, and more opportunistic pathogens, such as *Enterobacter*, *Megasphaera*, *Oscillibacter*, and *Desulfovibrio* (106). On the one hand, in parallel with this latter, in our carotid IMT discordant twin study, a significantly higher proportion of the *Prevotellaceae* family in the group with normal IMT values compared to the group with high IMT values was found. On the other hand, parallel to the former, in our OSA and atherosclerosis study, we found the *Prevotella* genus as one of the candidate microbes in the OSA+atherosclerotic group. In an OSA study conducted by Ko et al., 113 patients were diagnosed with PSG and tested by 16S rRNA pyrosequencing (65). As a result, three enterotypes, *Bacteroides*, *Ruminococcus*, and *Prevotella*, were identified in OSA patients, and a link between the *Prevotella* enterotype and substantially altered arousal-related metrics or sleep stages was discovered. Furthermore, in order to comprehend the pathogenic involvement of certain bacteria in the development of

atherosclerosis, we must investigate the processes that they produce. Trimethylamine can be produced in the gut by high animal protein/L-carnitine diets, which can then be converted to trimethylamine N-oxide (TMAO) in the liver by the activity of Flavin monooxygenase 3. TMAO may contribute to atherosclerosis by enhancing foam cell production, reducing reverse cholesterol transport (RCT), and exerting pro-thrombotic effects (20). While enriched *Prevotella* proportions were associated with higher plasma TMAO levels (39), another study discovered that TMAO relative abundance was positively correlated with *Prevotella copri* (107). Further metagenomic analyses with a large number of samples may help in the future to clarify the above contradictory results.

5.4. Atherosclerosis and *Peptostreptococcaceae* in subjects with OSA

In OSA+atherosclerotic patients, we found a considerably reduced level of *Peptostreptococcaceae*, which contradicts earlier findings. Xue et al. investigated whether intermittent hypoxia (IH) or intermittent hypercapnia (IC) changes in the metabolome and microbiota generate a pro-atherosclerotic condition following 4-week IH or IC conditions using computer-assisted image processing to calculate the amount of atherosclerosis and 16S rRNA sequencing for microbiome profiling. They discovered that *Peptostreptococcaceae* increased in relative abundance two weeks before the appearance of atherosclerosis, suggesting that *Peptostreptococcaceae* contributes to the development of IH- and IC-induced atherosclerosis in Apolipoprotein E knock-out (ApoE−/−) mice (72). In another ApoE−/− mouse study that looked at the development of atherosclerosis and atherosclerotic plaques using different diets, *Peptostreptococcaceae* levels, as well as *Ruminococcaceae*, *Clostridiaceae*, *Enterobacteriaceae*, and *Streptococcaceae* levels, were found to be significantly higher in mice with atherosclerosis than in control mice (108). In humans (30 omnivores and 23 vegetarian or vegan healthy adults), Koeth et al. discovered that an omnivorous diet and TMAO production, which is important in the progression of atherosclerosis, are positively correlated with the abundance of the *Peptostreptococcaceae* family (39, 109, 110). However, the first two studies focused on specific groups of mice, whereas the third report focused on diet and microbiome

metabolites. Furthermore, in the first case, mice were solely exposed to IH or IC. Therefore, more research is needed to determine the disparities in the results.

5.5. Atherosclerosis and further candidate microbes in subjects with OSA

We reported the candidate microbes at the genus level in the non-atherosclerotic OSA group: *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella*; and in the atherosclerotic OSA group: *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae*. *Prevotella* was already discussed in detail in Chapter 5.3. Another bacteria to highlight is *Ruminococcaceae* since it has also been implicated in other studies on a similar topic. As mentioned in Chapter 5.3., in the study of Ko et al., *Ruminococcus* was among the three enterotypes in OSA patients (65). In addition, another report from this study described the *Ruminococcus* enterotype to be the most dangerous to OSA patients by examining stool samples from 93 participants (66). Furthermore, in IH-conditioned ApoE-/- mice, Xue et al. found higher relative abundances of pro-atherosclerotic *Ruminococcaceae*, *Coriobacteriaceae*, and *Lachnospiraceae* and decreased relative abundances of anti-atherosclerotic *Erysipelotrichaceae* (72). *Escherichia-Shigella*, the third prominent microbe in the atherosclerotic OSA group, is a member of the *Enterobacteriaceae* family. Jie et al. used shotgun sequencing to investigate the composition of the intestinal microbiome in relation to CVDs with a total of 405 participants (218 atherosclerotic cardiovascular diseases (ACVDs) patients and 187 healthy controls, not checking for OSA status) and discovered a higher proportion of *Enterobacteriaceae* and *Streptococcus spp.* in ACVDs patients compared to the control group (59). It should be noted that this latter study is the only one of those mentioned that performed shotgun sequencing, in contrast to the others, which used 16S rRNA sequencing.

Veillonella, one of our non-atherosclerotic OSA candidate microbes, is a propionate-producing bacteria (111). SCFAs are the primary end products of microbial enzymatic conversion of dietary complex carbohydrates to monosaccharides in the gut. The primary SCFAs produced during this process are acetate, propionate, and butyrate, which account for 90% of the total SCFA produced by gut bacteria (112). They may help prevent atherosclerosis by maintaining the intestinal barrier and lowering overall systemic inflammation (20). Furthermore, propionate may reduce

lipogenesis, serum cholesterol levels, and carcinogenesis in different tissues (1113). With further research, the aforementioned prominent species may become unique targets.

5.6. Limitations

5.6.1. Common limitations

The first and foremost of our limitations is the rather small sample size of the study (in both cases); type II error may have rendered certain differences insignificant. Second, the quality of the incoming sample due to postal delivery and possibly partially different individual sampling practices, despite that, participants got detailed sampling instructions. All samples obtained that were of poor quality were repeated individually. Third, similar to other research of this type and topic, we used 16S rRNA sequencing instead of the much more expensive whole-genome shotgun sequencing, which would have allowed for improved detection of bacterial species, higher detection of diversity, and improved gene prediction (1114). Fourth, we lack diet data, which could have an impact on our findings. Fifth, because we used self-reported questionnaires, we were not aware of any false or suppressed data or conditions. At the same time, the questionnaires did not include overly intimate or intrusive questions in accordance with the general values; additionally, for each question, we gave the respondent the option to refuse the given answer, thereby avoiding distortions caused by false data to the greatest extent possible.

5.6.2. Additional limitations of the carotid IMT discordant twin study

Another limitation of this study was the lack of blood samples, which prevented researchers from investigating the effect of classical atherosclerotic risk factors (e.g., cholesterol and glucose levels) on the examined associations.

5.6.3. Additional limitations of the OSA and atherosclerosis study

An additional limitation of this study was that discordant twin analysis for atherosclerosis was not possible as there were only 1 discordant MZ pair in addition to 6 concordant twin pairs (4 pairs with carotid atherosclerosis) and 8 patients as one member of a twin pair (7 with carotid atherosclerosis). Thus, we

did not examine twinning, we compared the atherosclerotic and non-atherosclerotic samples as if they were independent subjects; nonetheless, this may have influenced our results due to the effect of genetics. Furthermore, due to a lack of individuals with moderate to severe OSA in the sample, analysis by OSA severity was not possible. Moreover, because none of the patients used CPAP, it is impossible to say how the therapy altered the gut bacteria or whether it influenced atherosclerosis.

6. Conclusions

As far as we are aware, this is the first twin study to investigate the impact of the gut microbiome on carotid IMT in discordant twins; as well as the first pilot study to analyse the association between the intestinal microbiome and atherosclerosis in adult patients with OSA, with and without atherosclerosis.

We reported an increased maximal CCA IMT in association with decreased phylum-level diversity, parallel to previous studies, confirming the link between atherosclerosis and dysbiosis.

We also confirmed findings of other studies in which increased Firmicutes/Bacteroidetes ratio was reported in subjects with subclinical atherosclerosis represented by increased carotid IMT, further strengthening its role as a possible marker of atherosclerosis phenotype.

While we found a substantially greater fraction of the *Prevotellaceae* family in the group with normal IMT values compared to the group with high IMT values, on the other hand in our OSA and atherosclerosis study, we found *Prevotella* genus as one of the candidate microbes in the OSA+atherosclerotic group. Since the current literature also disputes the effects of *Prevotella*, to clarify, further metagenomic analyses with a large number of samples may help in the future.

Peptostreptococcaceae levels were found to be significantly lower in atherosclerotic OSA subjects, which contradicts previous research, therefore, this result must be treated with reservations, and further, larger studies must be performed in order to clarify what exactly the role of this bacteria is.

Other candidate microbes that appeared at the genus level in the non-atherosclerotic OSA group: *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella*; and in the atherosclerotic OSA group: *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae*, all of which may play important roles in the treatment of atherosclerosis, the repair of dysbiosis, and the maintenance of beneficial intestinal flora.

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7. Summary

Carotid artery atherosclerosis is known to be associated with an increased risk for CVDs, which are the leading cause of death globally. Factors determining its development include genetic variation and environmental risk factors, as well as the composition and diversity of the gut microbiome. Although these associations are already known, many questions remain unanswered to fully understand how the microbiome contributes to atherosclerosis and CVD. By studying MZ twins - since their genome is nearly the same - the genetic factors can be mostly ruled out; therefore, the role of common and unique environmental factors can be explored. Therefore, we aimed to investigate the correlations between different ultrasound markers of atherosclerosis, such as increased carotid IMT and plaque presence, and the gut microbiome in the Hungarian twin population. In our first study, 14 MZ twin pairs discordant for carotid IMT participated and underwent a comprehensive carotid ultrasound examination and a stool sample collection. Increased Firmicutes/Bacteroidetes ratio was associated with increased IMT, further strengthening its role as a possible marker of atherosclerosis phenotype. In the group with normal IMT values, a substantially higher fraction of *Prevotellaceae* was observed in contrast with subjects having subclinical atherosclerosis, suggesting its protective role against atherosclerosis, which could not be confirmed in our OSA study. In our second study, 22 patients with OSA, 16 with and 6 without carotid atherosclerosis were involved, who besides a comprehensive carotid ultrasound examination and a stool sample collection, underwent a diagnostic sleep examination and a blood test. An increased maximal CCA IMT was significantly associated with decreased phylum-level diversity, confirming the link between atherosclerosis and dysbiosis. The level of *Peptostreptococcaceae* was significantly lower in atherosclerotic subjects, which contradicts previous research. We also reported some other candidate microbes in the atherosclerotic and non-atherosclerotic OSA groups, which may have a role in the restoration of dysbiosis or the treatment of atherosclerosis. Although there are many limiting factors in our studies, we trust that our results will provide the basis for further large-sample research to understand the role of the gut microbiome in atherosclerosis, even in subgroups, such as patients with OSA.

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9. Bibliography of the candidate's publications

9.1. Publications related to the current Ph.D. thesis

- **Szabo H**, Hernyes A, Piroska M, Ligeti B, Fussy P, Zoldi L, Galyasz S, Makra N, Szabo D, Tarnoki AD, Tarnoki DL. Association between Gut Microbial Diversity and Carotid Intima-Media Thickness. Medicina (Kaunas). 2021;57(3). doi: 10.3390/medicina57030195. **(IF: 2.948)**

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9.2. Publications not related to the current Ph.D. thesis

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