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Article

The Relationship between Atherosclerosis and Gut Microbiome in Patients with Obstructive Sleep Apnoea

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Abstract: Background: Obstructive sleep apnoea (OSA) and gut dysbiosis are known risk factors for atherosclerosis. However, only very few studies have been focused on the relationship between OSA, atherosclerosis, and the intestinal microbiome, all in animal models. Methods: Twenty-two patients with OSA, 16 with and 6 without carotid atherosclerosis were involved in the study. After a diagnostic sleep examination, the intima media thickness (IMT) was measured and plaques were found using carotid ultrasound. Blood was also drawn for metabolic profile, and a stool sample was provided for 16S ribosomal RNA microbiome investigation. Results: An increased maximal common carotid artery (CCA) IMT was significantly associated with decreased phylum-level diversity. The level of *Peptostreptococcaceae* was significantly lower in atherosclerotic subjects. Some other candidate microbes appeared in the two groups at the genus level as well: *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella* in the non-atherosclerotic group; and *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae* in the atherosclerotic group. Conclusions: This is the first pilot research to analyze the association between the gut microbiome and atherosclerosis in adult patients with OSA with and without carotid atherosclerosis. Dysbiosis and individual bacteria may contribute to the development of carotid atherosclerosis in patients with OSA. Further investigations are necessary to reveal a more precise background in a larger sample.

Keywords: gut microbiome; alpha diversity; intima-media thickness; carotid atherosclerosis; ultrasound; sleep apnoea; sleep disorder

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1. Introduction

Obstructive sleep apnoea (OSA), a disorder marked by recurrent collapse of the upper airways during sleep, is a known risk factor for atherosclerosis [1]. Yet, continuous positive airway pressure (CPAP) therapy for OSA has a limited impact on cardiovascular outcomes [2]. Exploring the relationship between OSA and atherosclerosis in order to identify potentially treatable characteristics is thus an unmet clinical need.

Atherosclerosis development has been directly correlated with the intestinal microbiome's composition [3]. We found a link between an increased Firmicutes/Bacteroidetes ratio and increased intima-media thickness (IMT), a marker of subclinical atherosclerosis [4]. Furthermore, when participants with subclinical atherosclerosis were compared

to those with normal IMT levels, a significantly higher proportion of *Prevotellaceae* was discovered. Despite the fact that the Moscow study claimed that a higher IMT was discovered in the cluster with less diversity, we did not find a statistically significant difference in alpha diversity between the two groups [5]. Individual species have been studied to see if *Akkermansia muciniphila* has anti-atherogenic properties [6]. Furthermore, the genus *Collinsella* was concentrated in individuals with symptomatic atherosclerosis, whereas *Roseburia* and *Eubacterium* were enriched in healthy participants [7]. Atherosclerosis and the gut microbiome have a link that is thought to be influenced by lifestyle factors, most particularly the diet [3]. However, in the presence of confounding OSA, the effect of chronic intermittent hypoxaemia must be considered. On one hand, hypoxaemia affects the growth of certain gut bacteria [8]. On the other hand, it directly correlates with atherosclerosis via hypertension, dyslipidaemia, and vascular inflammation [1]. Therefore, studies on atherosclerosis and gut microbiome cannot directly be translated to patients with OSA.

So far, only a few research teams have published their findings from studies on the relationship between OSA and the intestinal microbiota [9–13], with only two of them focusing on adults [9,10]. 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 [14–17].

This study's aim was to explore the relationship between adult OSA patients' gut microbiome and atherosclerosis which is essential to find gut dysbiosis-targeted treatments that may be used as an adjunct to traditional OSA treatment and may result in advantages for OSA morbidities.

2. Materials and Methods

Study Participants

Caucasian subjects participating in the sleep cohort [18] (n = 142, 48 monozygotic, MZ and 23 dizygotic, DZ twin pairs, mean age 51 ± 15 years) of the voluntary-based Hungarian Twin Registry [19,20] who was diagnosed with OSA, underwent a carotid ultrasound examination and provided a stool sample for microbiome analysis were included. On the left and right common carotid artery (CCA) and the proximal segment of the internal carotid artery (ICA), the mean and maximal IMT, in addition to the presence of the plaques, were measured and detected. Based on the ultrasound examination, two groups were formed: OSA patients with and without atherosclerosis. Atherosclerosis was defined as if one person had at least one plaque either on the left, right, or both sides.

Prior to the study, none of the subjects had been diagnosed with OSA or received any sort of OSA treatment. Pregnancy, prior carotid surgery, acute infection within three weeks of the study, underlying oncologic disease, inflammatory bowel disease, and acute respiratory, cardiac, and renal failure were among the exclusion criteria.

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.

Laboratory tests, blood pressure measurements, questionnaires

Venous blood was collected by a professional nurse to measure triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and C-reactive protein (CRP). Blood pressure was also measured, in the morning. Self-report questionnaires were used to assess the presence of comorbidities such as hypertension, cardiovascular disease, diabetes, dyslipidemia, and other important influencing factors like smoking. Along with measuring body weight and height, BMI was also computed.

Sleep Studies

The sleep study was executed as published previously [9] using the Somnoscreen Plus Tele PSG and the Somnoscreen RC devices (Somnomedics GmbH, Randersacker, Germany). A 90% reduction in nasal airflow that lasts at least 10 s is referred to as apnoea. A nasal airflow reduction of 30% or more that lasts for at least 10 s accompanied by either arousal (on PSG) or a decrease in oxygen saturation of at least 3% (on both the PG and PSG) was

referred to as hypopnoea. The proportion of the total sleep time that was below 90% of saturation (TST90%), the oxygen desaturation index (ODI), and the apnea-hypopnea index (AHI) were all calculated. If the AHI was 5/h or more, OSA was identified.

Carotid Ultrasound

The ultrasound scan used a high-resolution linear LM4-15B (15 MHz) transducer and a Samsung RS85 device in accordance with the previously described technique [4]. Plaque presence was detected along the CCA and the proximal segment of ICA, and semi-automated IMT measurements on the distal wall of the CCA distal segment were made at a distance of 0.5–2 cm from the bifurcation using the Arterial Analysis software. During the measurement, the software displays the specific vessel wall segment it detects and uses for the analysis on the screen of the ultrasound machine, which the examiner has the opportunity to override and modify, avoiding possible erroneous data and results from the fully automatic measurement.

Stool Sample Collection and Procession, Bioinformatics and Statistical Analysis

The procedure described in earlier publications [4,21] was followed for the collection and processing of the stool samples and for the bioinformatics and statistical analysis. The participants received a detailed instruction manual, with a flow chart, for the purpose of standardized sampling, in which the importance of the short time between sampling and return, the prevention of external contamination, the quantity, proper storage, and packaging of the sample were highlighted. All samples received were stored and frozen prior to processing. For the hypervariable region, V3–V4 of microbial 16S ribosomal RNA a library extraction was conducted after DNA extraction. Prior to sequencing on an Illumina MiSeq platform (Thermo Fischer Scientific, Waltham, MA, USA), the libraries were tagged with individual index pairs, verified using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and pooled. The raw reads' quality was evaluated using FastQC and MultiQC [22]. Only sequences with a minimum length of 50 were kept after the low-quality sequences were filtered and trimmed with Trimmomatic [23]. Low-quality calls and the initial 12 base calls were eliminated (Phred score < 20, sliding window size 5). A Kraken2 was applied to carry out the read categorization [24,25], with k-mer size 31 against the SSU Ref NR 99 database (release 132) of SILVA [26]. Bracken [27] was used to evaluate the taxa abundances and microbiome composition. The Shannon index was calculated, which is a diversity index used by statistics to summarize the diversity of a population in which each member belongs to a unique group; in our case, the groups are bacteria.

Version 4.05 of the R program (R Foundation for Statistical Computing, Vienna, Austria) was applied for statistical analysis. A p-value of 0.05 or less was considered significant. For 16s rRNA analysis, the linear discriminant analysis (LDA) effect size (LEfSe) method [28] was used, which determines the features most likely to explain differences between the two groups (potential biomarkers for differentiating them). It was performed with default parameters on the Galaxy server of the Huttenhower lab (http://huttenhower.sph.harvard.edu/galaxy/, accessed on 3 November 2022). The alpha level for the factorial Kruskal–Wallis test among the groups was 0.05 and the threshold for the logarithmic LDA score for discriminative features was 2. For alpha diversity analysis, the Wilcoxon rank sum test was used between the phylum and the genus level Shannon indexes of the groups. For beta diversity analysis, the principal coordinates analysis (PCoA) based on the Bray–Curtis distance measure was applied, which is a statistical method that converts data on distances between items into map-based visualization of those items. The association between the Shannon index and maximal CCA IMT at two taxonomic levels was analyzed using linear regression including Shannon indexes, age, and gender as covariates. Since most covariates did not show a significant difference in the univariate analysis and also to prevent collinearity, we did not include all covariates in the regression, only age and gender. To find the relationship between the bacterial groups, principal component analyses (PCAs) were performed, which is a statistical technique for reducing the dimensionality of a large dataset containing a high number of dimensions/features per observation and analyze it. The PCA was performed in Matlab programming language (MATLAB R2020b) with

the use of the Fathom toolbox (https://www.usf.edu/marine-science/research/matlab-resources/fathom-toolbox-for-matlab.aspx, accessed on 3 November 2022). The relative abundances of bacteria between the two groups were compared using permutation tests to compare the medians of the two groups.

3. Results

Table 1 displays basic patient data in groups with and without atherosclerosis. No significant difference was found between the two groups.

Table 1. Descriptive analysis in the two groups.

	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
Dyslipidemia (%)	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%.

Figure 1 shows the result of the LEfSe analysis. 25 taxonomic groups were significant.

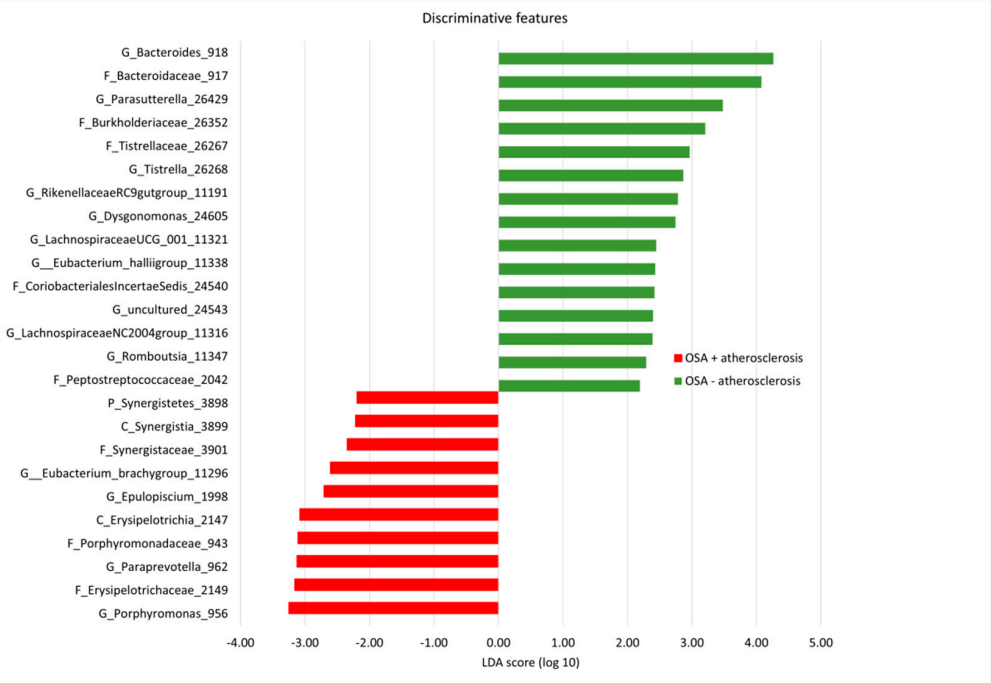

Figure 1. Discriminant features between the two groups based on the LEfSe method. Red bars represent taxa more abundant in the atherosclerotic group, green bars represent taxa more abundant in the non-atherosclerotic group.

Figures 2 and 3 show the results of alpha diversity analyses at 2 taxonomic levels (phylum and genus); and Figures 4–7 show the results of beta diversity analyses at 4 taxonomic levels (phylum, class, family, and genus).

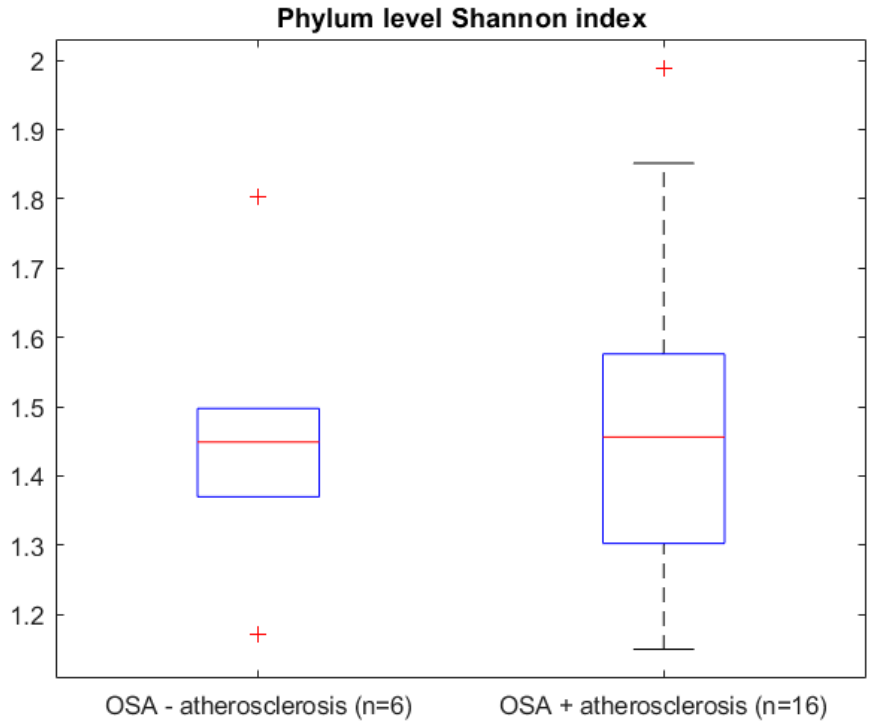

Figure 2. Wilcoxon rank sum test between the phylum level Shannon indexes of the groups.

Wilcoxon rank sum test between the phylum level Shannon indexes of the groups: $p = 0.9119$, no significant difference between the groups (with alpha = 0.05).

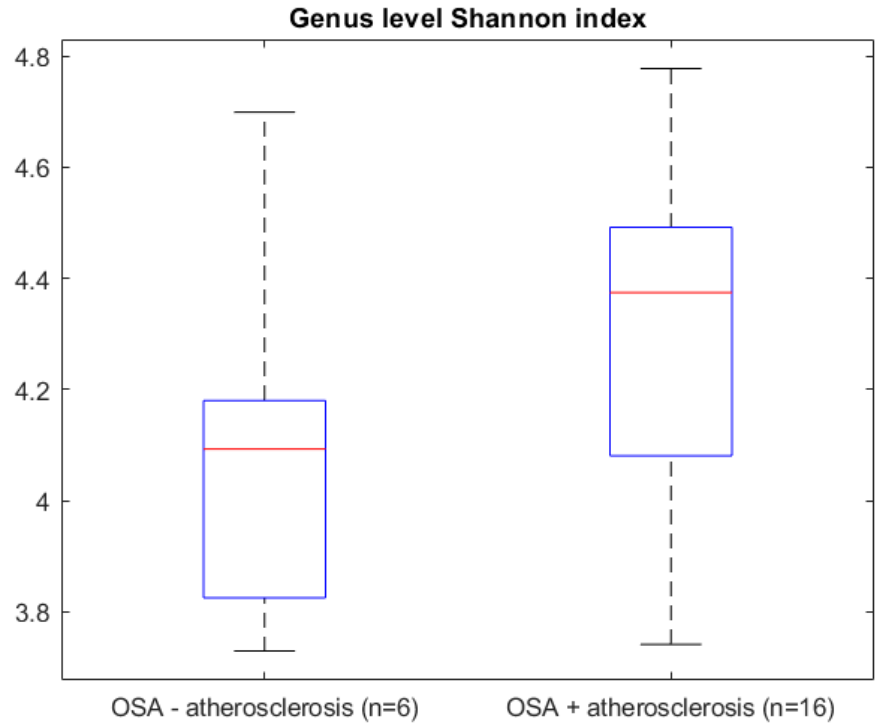

Figure 3. Wilcoxon rank sum test between the genus level Shannon indexes of the groups.

Wilcoxon rank sum test between the genus level Shannon indexes of the groups: $p = 0.1970$, no significant difference between the groups (with alpha = 0.05).

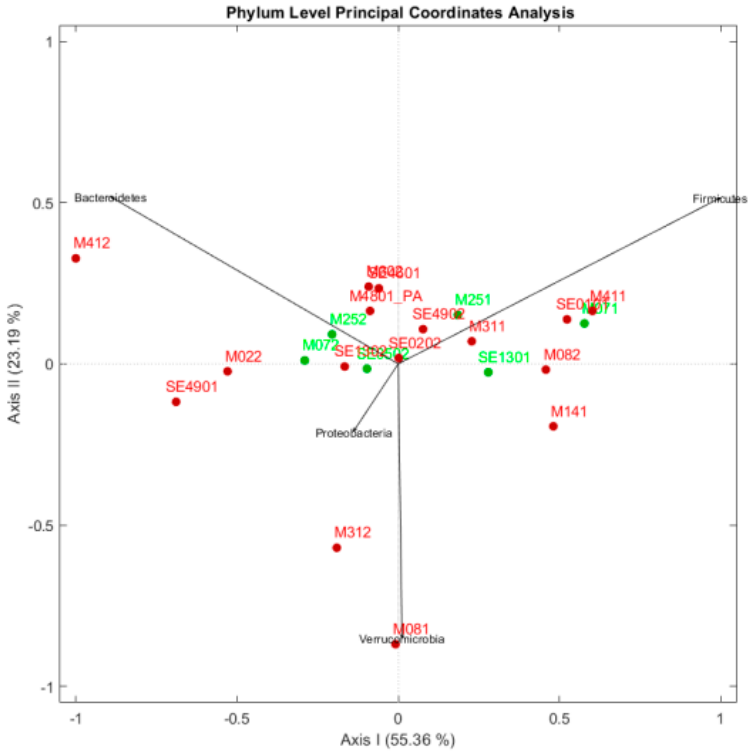

Figure 4. Beta diversity analyses at the phylum level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

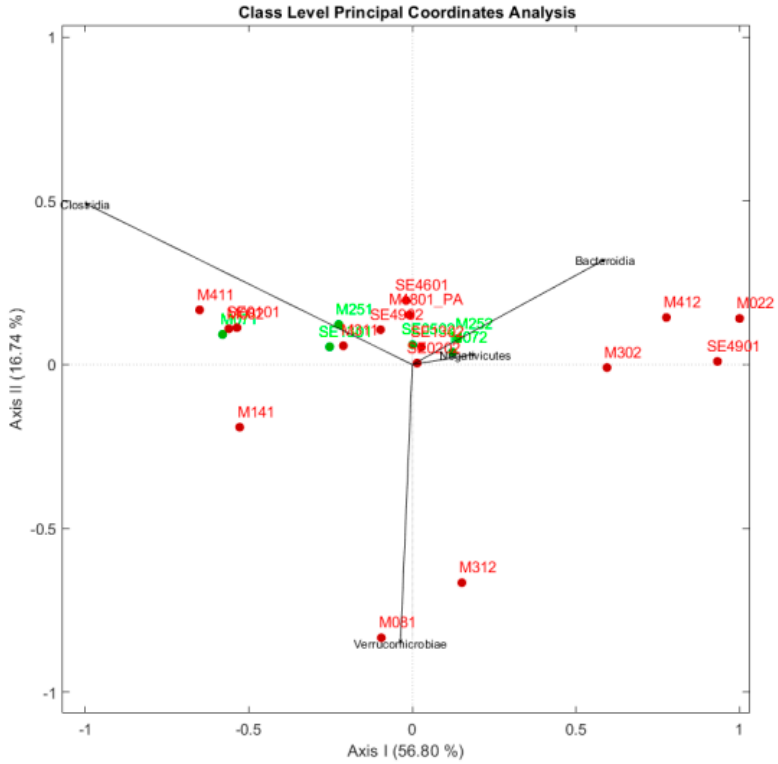

Figure 5. Beta diversity analyses at the class level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

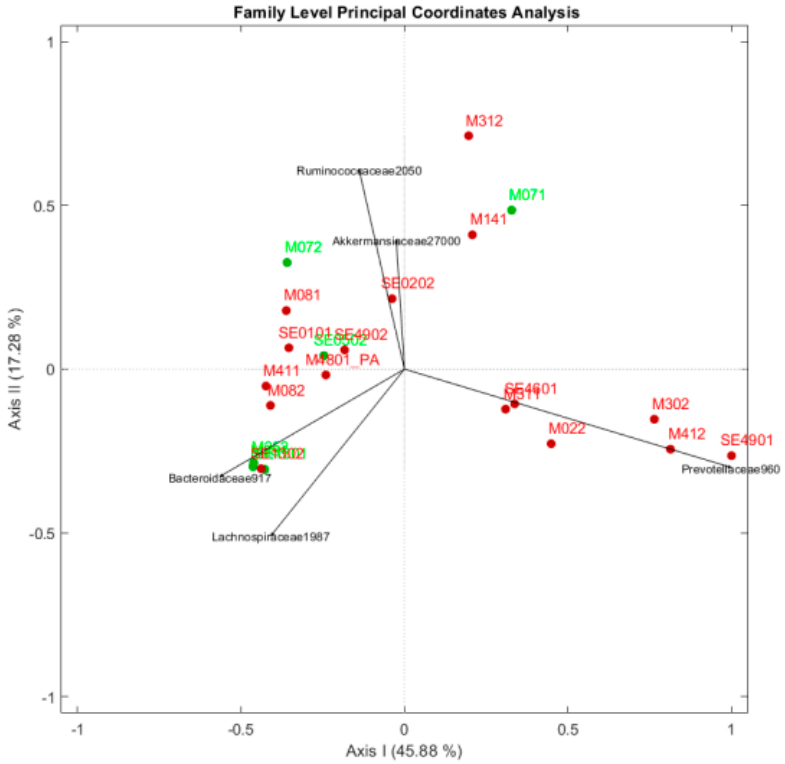

Figure 6. Beta diversity analyses at the family level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

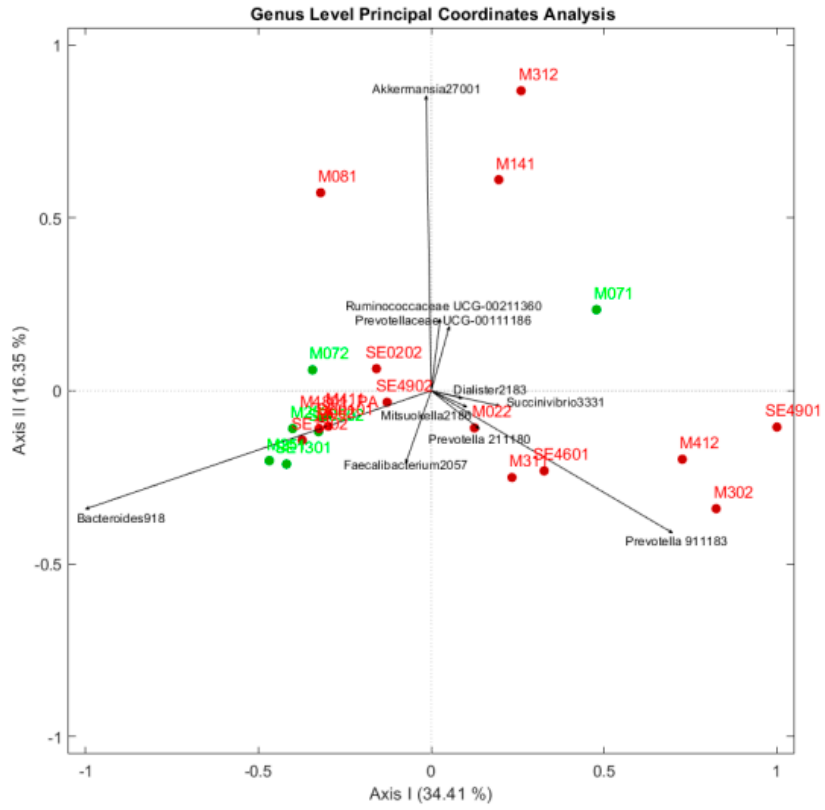

Figure 7. Beta diversity analyses at the genus level: PCoA based on Bray–Curtis distance measure. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

There was no visible clustering in the PCoA space. Atherosclerosis positive group seemed to be more variable (of note, its sample size is much bigger than the other group, 16 vs. 6 samples).

Next, we performed 2 linear regressions on the bilateral mean of maximal CCA IMT at 2 taxonomic levels, where the predictor variables were gender, age, and the Shannon index at the taxonomic levels of phylum and genus, respectively (Table 2.). Maximal CCA IMT is significantly associated with phylum-level diversity: a decrease in diversity correlates with an increase in IMT. As age and gender are included in the regression, the correlation is considered to be corrected. The effect of age also appears in regression.

Table 2. Correlation of maximal CCA IMT and microbiome alpha diversity at 2 taxonomic levels.

	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_P	−0.275 ** (0.119) $p = 0.033$	
Shannon_G		0.011 (0.100) $p = 0.916$
Constant	0.636 *** (0.196) $p = 0.005$	0.230 (0.427) $p = 0.597$
Observations	22	22
R^2	0.620	0.508
Adjusted R^2	0.557	0.426
Residual Std. Error (df = 18)	0.107	0.122
F Statistic (df = 3; 18)	9.809 *** $p = 0.0005$	6.203 *** $p = 0.005$
Note:	** $p < 0.05$; *** $p < 0.01$	

CCA IMT: common carotid artery intima-media thickness; Shannon_G: Shannon index at the genus level; Shannon_P: Shannon index at the phylum level.

The correlation between Shannon index at the phylum taxonomic level and maximal CCA IMT is shown in Figure 8 as an effect plot based on regression.

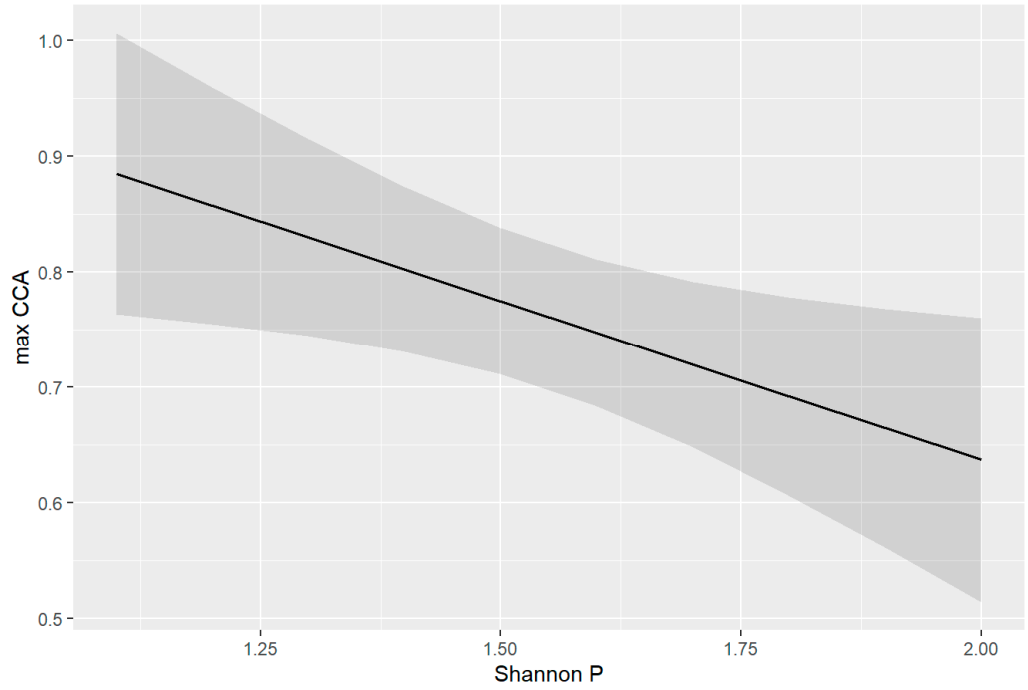

Figure 8. The correlation between Shannon index at the phylum taxonomic level and maximal CCA IMT.

The results of PCAs are shown in Figures 9–12.

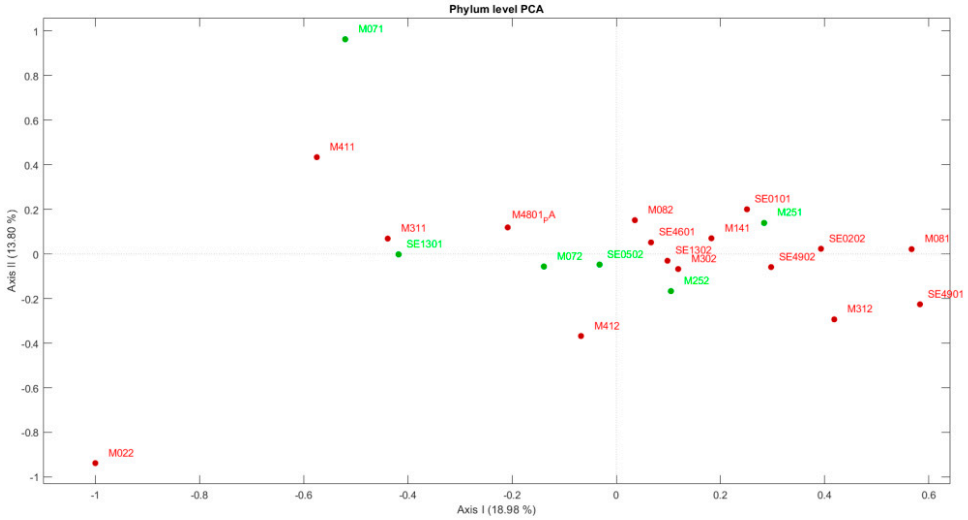

Figure 9. The results of PCA at the phylum level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

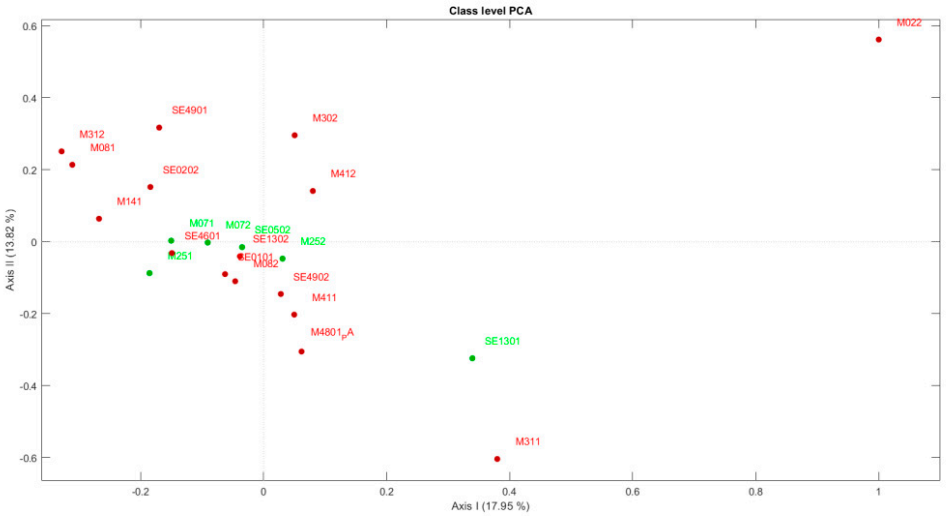

Figure 10. The results of PCA at the class level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

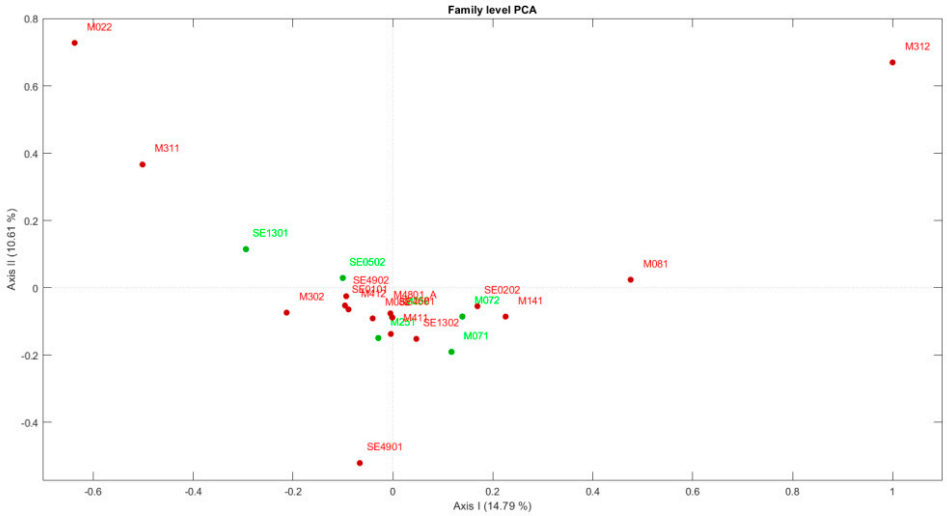

Figure 11. The results of PCA at the family level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

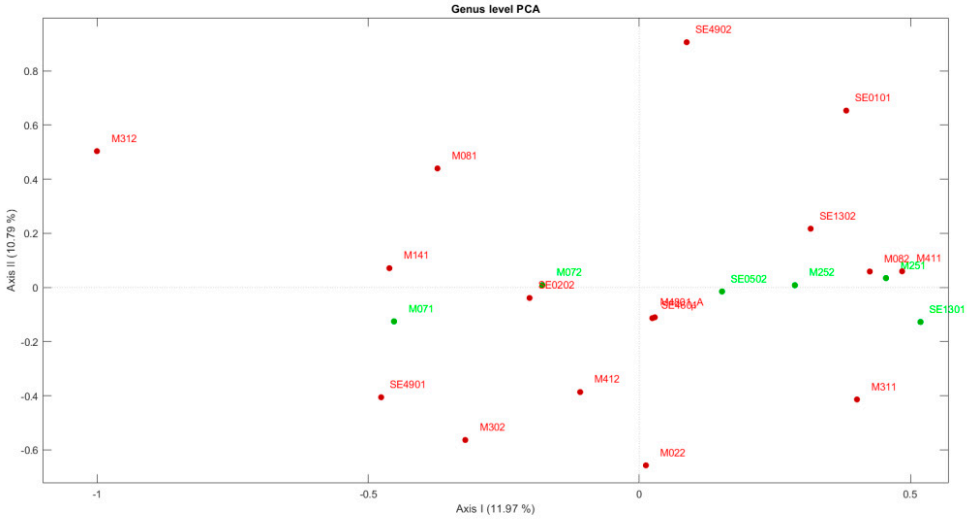

Figure 12. The results of PCA at the genus level. Green: OSA − atherosclerosis. Red: OSA + atherosclerosis.

No visible clustering was seen in PCA space. No descriptor variables (bacterial groups) were observed with outstanding importance.

Figures 13–16 show taxonomic categories with more than 1% relative abundance on average through the samples.

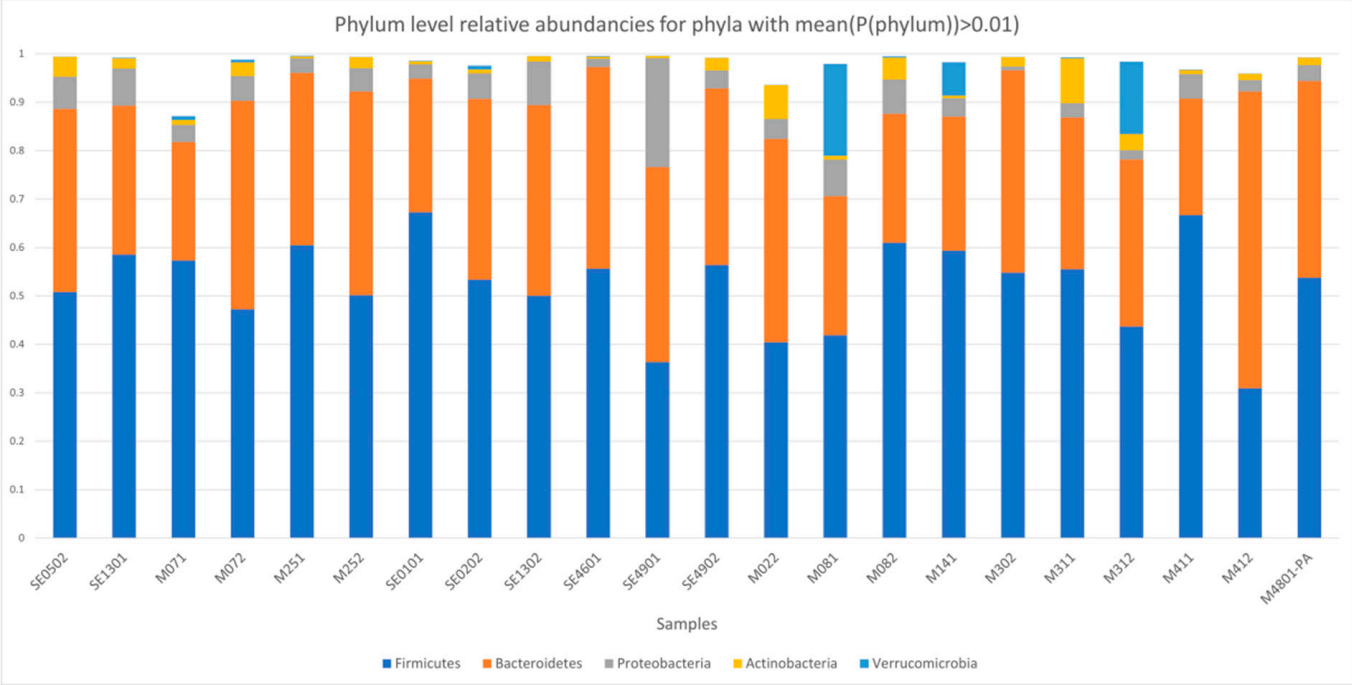

Figure 13. The relative abundances of bacteria at the phylum level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group.

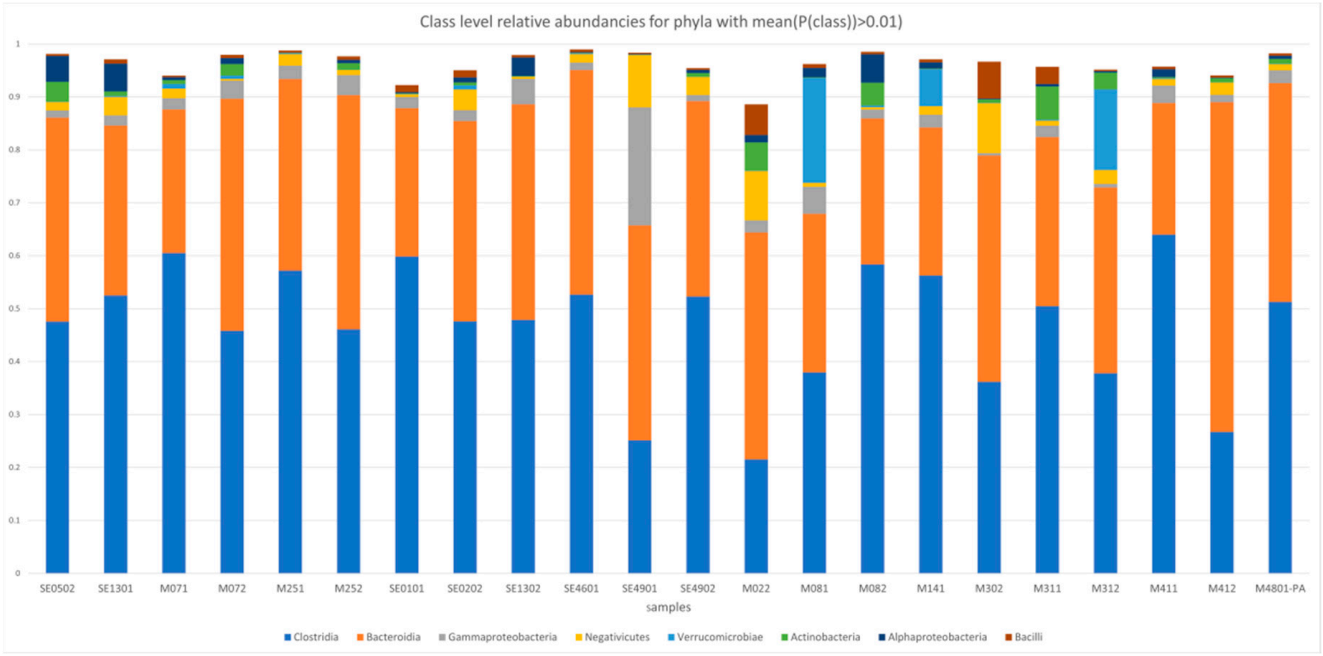

Figure 14. The relative abundances of bacteria at the class level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group.

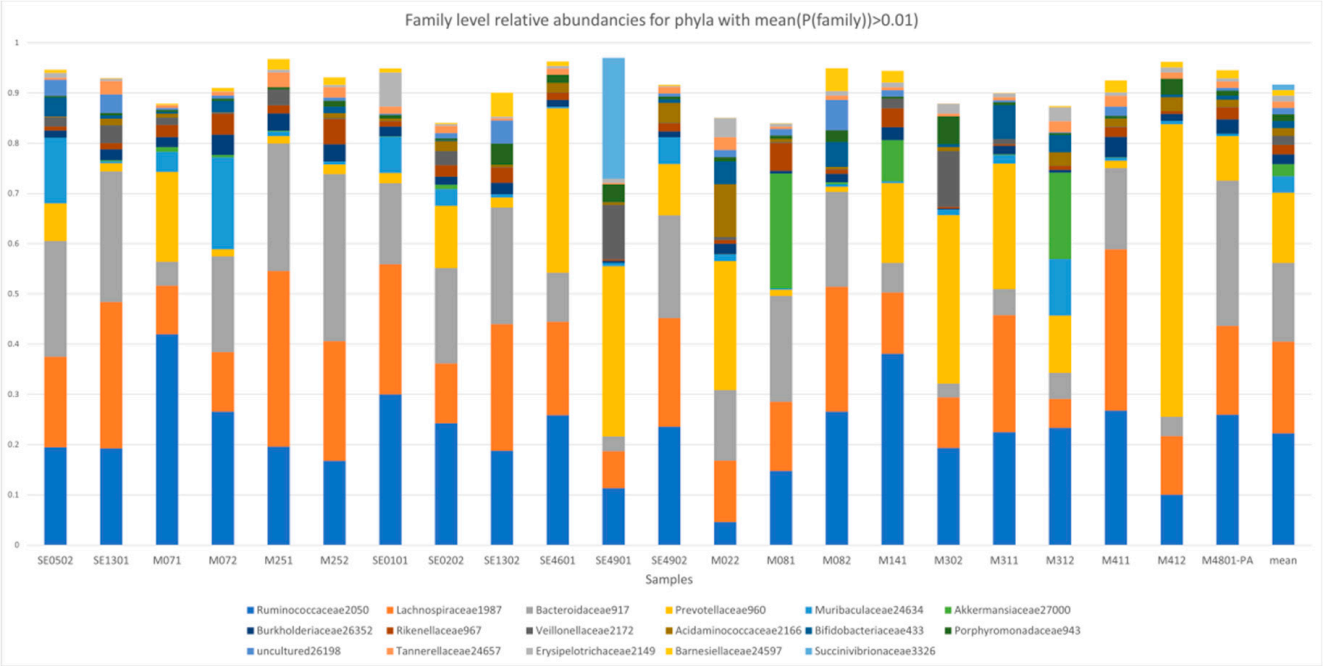

Figure 15. The relative abundances of bacteria at the family level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group.

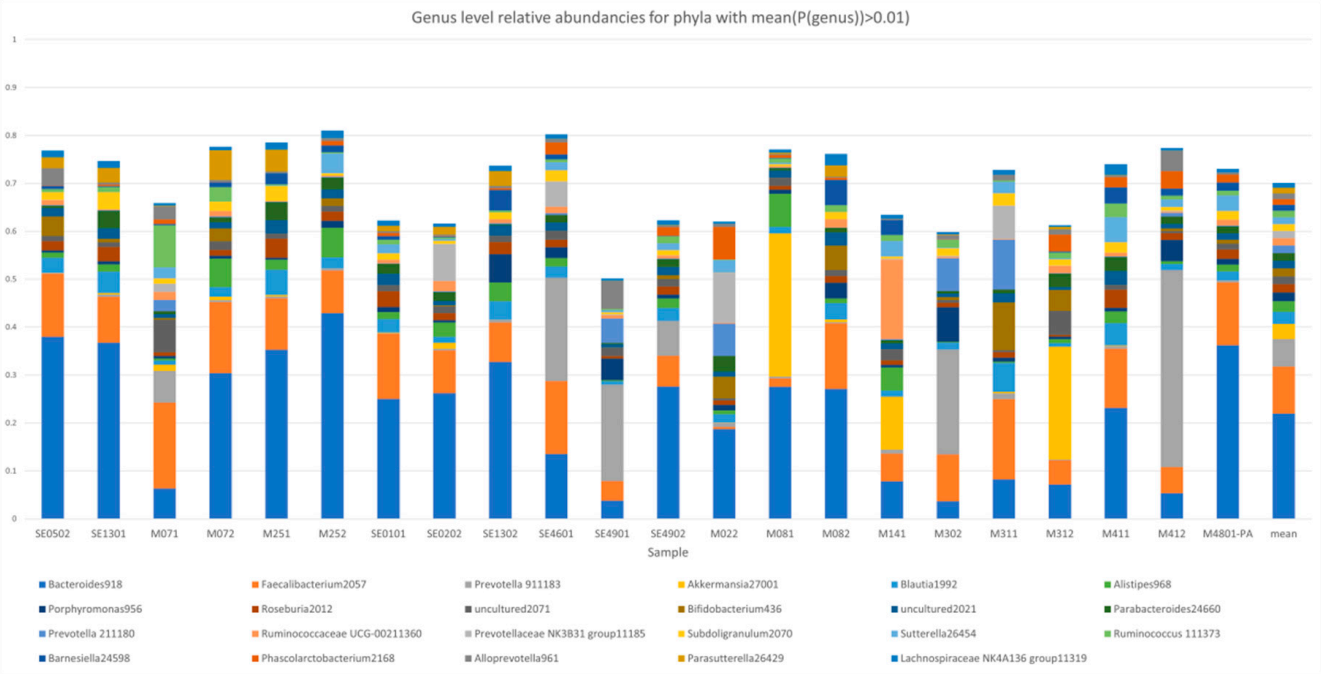

Figure 16. The relative abundances of bacteria at the genus level between the two groups. The first 6 columns are the subjects of the OSA − atherosclerosis group, and the next 16 columns are the subjects of the OSA + atherosclerosis group.

Next, we examined by permutation tests whether there was a difference between the two samples for each microbe, using the difference between the median fractional reads of the microbes in the atherosclerotic and non-atherosclerotic samples. Since many tests were performed, we corrected the possibility of error from the multiple hypothesis test using the Benjamini–Hochberg procedure, using $p < 0.1$ more acceptable p-values. In atherosclerotic patients, we found considerably reduced *Peptostreptococcaceae* levels (Table 3 and Figure 17).

Table 3. The result of the permutation test for the *Peptostreptococcaceae* family.

	Median (Non-Atherosclerotic)	Median (Atherosclerotic)	ΔMedian	*p*-Value
Peptostreptococcaceae	0.002360	0.000950	0.00141	<0.00001

Without correction, where $p < 0.05$ for the permutation test, in the two groups the candidate microbes at the genus level were *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella* in the non-atherosclerotic group; and *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae* in the atherosclerotic group (Figure 18).

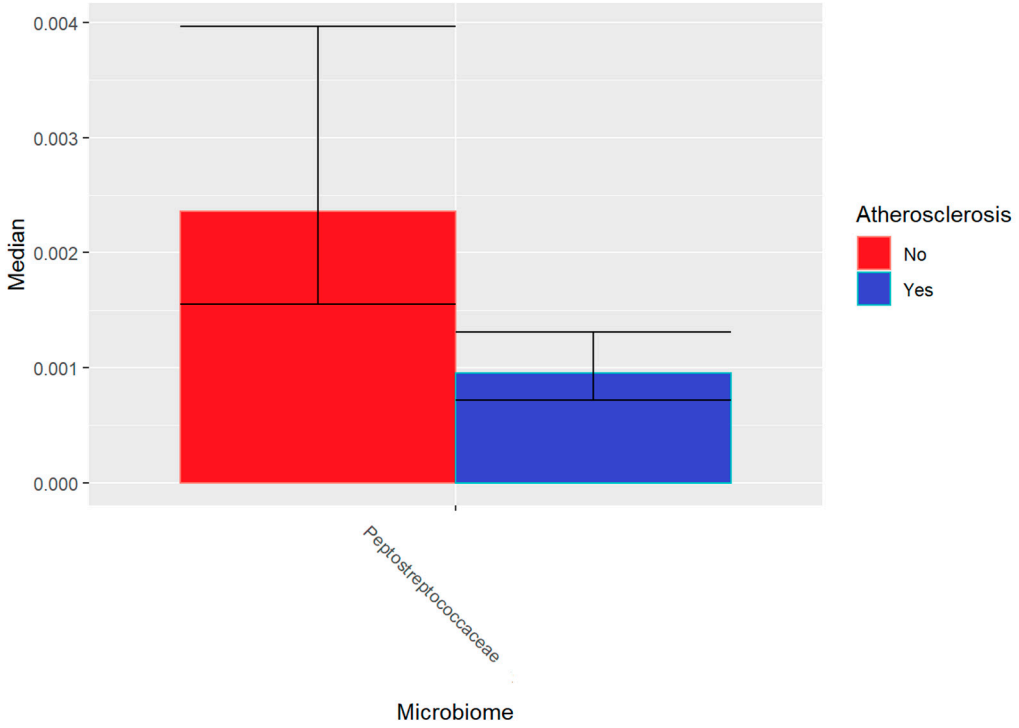

Figure 17. The median and the interquartile range of the fractions of *Peptostreptococcaceae*.

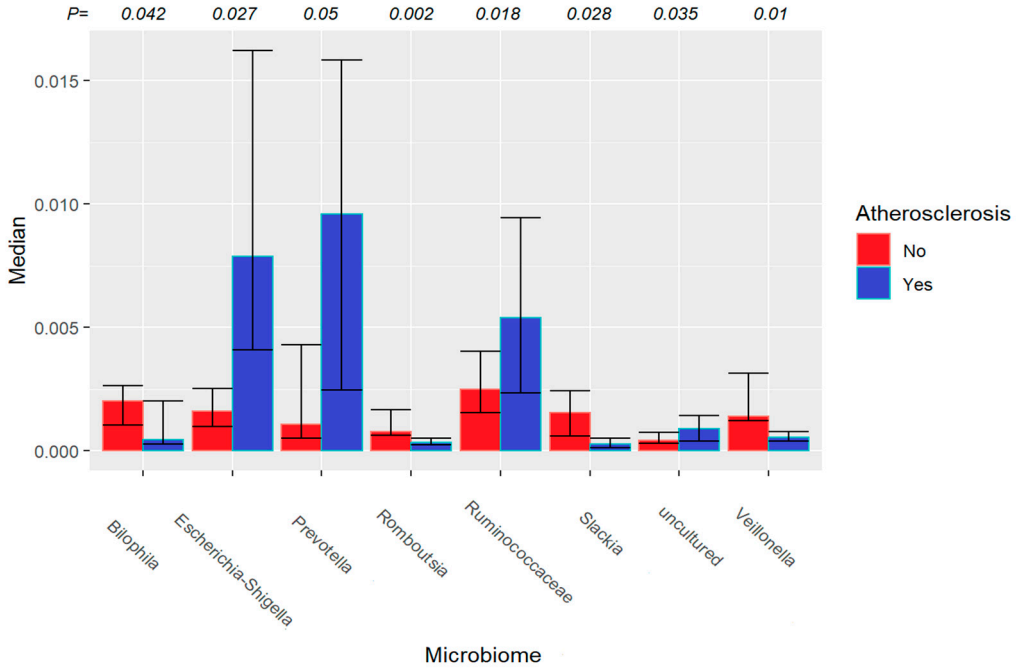

Figure 18. The candidate microbes at the genus taxonomic level without correction in the two groups.

4. Discussion

As far as we are aware, this is the first pilot study to analyze the association between the intestinal microbiome and atherosclerosis in adult patients with OSA, with and without atherosclerosis. LEfSe, alpha and beta diversity, PCA analyses as well as plots show relative abundances were made for a detailed analysis of the differences between the two groups. Furthermore, we examined the correlation between the alpha diversity and the maximal CCA IMT, as well as the difference between the atherosclerotic and non-atherosclerotic samples for each microbe at genus taxonomic level.

With LEfSe analysis at 4 taxonomic levels, we found in total 15 taxa significantly more abundant in the non-atherosclerotic group and 10 taxa in the atherosclerotic group. With alpha diversity analyses at phylum and genus taxonomic levels, we did not find a significant difference between the two groups. With beta diversity analyses at 4 taxonomic levels, we did not find visible clustering in the PCoA space. With PCAs at 4 taxonomic levels, we did not find visible clustering in PCA space. We discovered that higher maximal CCA IMT is significantly correlated with lower phylum-level diversity. This preliminary finding is consistent with another study [5], in which Kashtanova et al. examined 92 adult Caucasian people from the Moscow region with no clinical cardiovascular or other chronic diseases and found a higher IMT within the cluster of lower alpha diversity after carotid ultrasound examinations and stool sample 16S rRNA sequencing. It should be noted that the presence of OSA was not investigated. They also reported links between certain bacteria and various metabolic statuses, such as a higher proportion of *Prevotella*—which is included in our results as one of the candidate microbes in the atherosclerotic group—being associated with general and abdominal obesity. Although there was no significant difference in alpha diversity between groups with high and normal IMT in our previous study [4], this can be attributed to the smaller sample size compared to the Moscow study. Gut dysbiosis has been linked to many risk factors for atherosclerosis, including diabetes [29,30], obesity [30,31], and smoking [32]; whereas the normal microbiota (commensal bacteria) has been shown to possibly protect against the development of atherosclerosis [33]. Aside from various microbial metabolites, one possible pathological process is the dysbiosis-associated leaky gut-induced immune response by the host. Dysbiosis leads to the translocation of lipopolysaccharide (LPS) to the bloodstream by compromising the gut barrier, LPS can activate Toll-like receptor 4 signaling and decrease reverse cholesterol transport (RCT) to exacerbate atherosclerosis [34–36]. The relationship between the gut microbiome and disease is a complex process and is still under investigation, where our preliminary findings support the importance of dysbiosis in the development of atherosclerosis.

We discovered a significantly lower level of *Peptostreptococcaceae* in atherosclerotic subjects, which contradicts previous research. Using computer-assisted image processing to calculate the amount of atherosclerosis and 16S rRNA sequencing for microbiome profiling, Xue et al. tested whether intermittent hypoxia (IH) or intermittent hypercapnia (IC) changes the metabolome and microbiota to cause a pro-atherosclerotic condition after 4-week IH or IC conditions. They found that *Peptostreptococcaceae* increased in relative abundance two weeks before atherosclerosis appeared, demonstrating that *Peptostreptococcaceae* contributes to the development of IH- and IC-induced atherosclerosis in Apolipoprotein E knock-out mice (ApoE$-/-$) [17]. 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 [37]. Koeth et al. [30,38,39] discovered that an omnivorous diet and trimethylamine N-oxide (TMAO) production, which is important in the progression of atherosclerosis, are positively correlated with the abundance of the *Peptostreptococcaceae* family in humans (30 omnivore and 23 vegetarian or vegan healthy adults). However, in the first two studies, specific groups of mice were studied, whereas in the third report, the authors focused on diet and microbiome metabolites. Furthermore, mice were only exposed to IH or IC in the first case. Therefore, additional research is required to uncover the differences in the results.

We reported the candidate microbes at the genus level in the non-atherosclerotic group: *Bilophila*, *Romboutsia*, *Slackia*, and *Veillonella*; and in the atherosclerotic group: *Escherichia-Shigella*, *Prevotella*, and *Ruminococcaceae*. *Prevotella* and *Ruminococcaceae* should be highlighted from the list because they have also been implicated in other studies on a similar topic. In the study of Ko et al., a total of 113 subjects were involved, diagnosed by polysomnography and examined with 16S rRNA pyrosequencing [11]. As a result, in OSA patients three enterotypes, *Bacteroides*, *Ruminococcus*, and *Prevotella*, were recognized, and a connection between the *Prevotella* enterotype and dramatically altered arousal-related

metrics or sleep stages was found. 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 [10]. Furthermore, Xue et al. reported increased relative abundances in pro-atherosclerotic *Ruminococcaceae*, *Coriobacteriaceae*, and *Lachnospiraceae*; and decreased relative abundances in anti-atherosclerotic *Erysipelotrichaceae* in IH-conditioned ApoE-/- mice [17]. The third prominent microbes of the atherosclerotic group, *Escherichia-Shigella,* belongs to the *Enterobacteriaceae* family. Jie et al. aimed to extensively investigate the intestinal microbiome's composition in connection to cardiovascular diseases with a total of 405 participants (218 atherosclerotic cardiovascular disease (ACVD) patients and 187 healthy controls, not checking for OSA status), using shotgun sequencing and found a higher proportion of *Enterobacteriaceae* and *Streptococcus* spp. in ACVD patients compared to the control group [40]. It should be noted that this latter study is the only one of those mentioned that performed shotgun sequencing, as opposed to the others, which performed 16S rRNA sequencing.

In order to understand the pathological role of certain bacteria in the development of atherosclerosis, we must examine the processes induced by them Trimethylamine can be produced in the gut by high animal protein/L-carnitine diets, which can then be converted to TMAO in the liver by the action of Flavin monooxygenase 3. By increasing foam cell formation, decreasing RCT, and exerting pro-thrombotic effects, TMAO may contribute to atherosclerosis [36]. While enriched Prevotella proportions showed higher plasma TMAO levels [30], another study found that the relative abundance of TMAO was positively correlated with *Prevotella copri* [41]. *Veillonella*, one of our candidate microbes in the non-atherosclerotic group, is one of the propionate-producing bacteria [42]. Short-chain fatty acids (SCFAs) are the major end products of gut microbial enzymatic conversion of dietary complex carbohydrate to monosaccharides. Acetate, propionate, and butyrate are the major SCFAs produced during this process, accounting for 90% of the total SCFA produced by gut bacteria [43]. They are thought to have a beneficial effect on atherosclerosis by protecting the gut barrier and lowering overall systemic inflammation [36]. Moreover, propionate may reduce lipogenesis, serum cholesterol levels, and carcinogenesis in other tissues [44]. The aforementioned prominent species may become unique targets with further investigations.

The first of our limitations is the rather small sample size of the study; type II error may have rendered certain differences insignificant. Second, discordant twin examination 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 sample with the non-atherosclerotic sample as if they were independent subjects; however, this may also partially influence our results due to the effect of genetics. Third, the quality of the incoming sample due to postal delivery and possibly partially different individual sampling techniques, despite that, participants got detailed sampling instructions. All samples received that were not of adequate quality were repeated separately. Forth, similar to most studies of this nature and topic, we used 16S rRNA sequencing, primarily due to limited resources, instead of the much more expensive shotgun sequencing, which would have enabled a more accurate and reliable taxonomic classification. Fifth, we lack data on diet and exercise which could have a significant impact on our findings. Sixth, since we used self-reported questionnaires, we did not become aware of any untrue or suppressed data and conditions. At the same time, the questionnaires did not include overly intimate or intrusive questions according to the general values; furthermore, for each question, we gave the respondent the opportunity to refuse the given answer, thus avoiding distortions resulting from false data as much as possible. Lastly, since none of the patients had CPAP, it is impossible to determine how the therapy affected the intestinal bacteria or whether it influenced atherosclerosis. In order to comprehend the impact of atherosclerosis on the gut microbiome in OSA patients, we trust that our preliminary findings will provide the basis for additional extensive research.

5. Summary and Conclusions

In summary, an increased maximal CCA IMT is significantly associated with decreased phylum-level diversity. *Peptostreptococcaceae* levels were found to be significantly lower in atherosclerotic subjects. Other candidate microbes that appeared at the genus level in the two groups were *Bilophila*, *Escherichia-Shigella*, *Prevotella*, *Romboutsia*, *Ruminococcaceae*, *Slackia*, and *Veillonella*, all of which may play important roles.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting reported results can be found by the authors at request.

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References

1. Sánchez-De-La-Torre, M.; Campos-Rodriguez, F.; Barbé, F. Obstructive sleep apnoea and cardiovascular disease. *Lancet Respir. Med.* **2013**, *1*, 61–72. [CrossRef]
2. McEvoy, R.D.; Antic, N.A.; Heeley, E.; Luo, Y.; Ou, Q.; Zhang, X.; Mediano, O.; Chen, R.; Drager, L.F.; Liu, Z.; et al. CPAP for Prevention of Cardiovascular Events in Obstructive Sleep Apnea. *N. Engl. J. Med.* **2016**, *375*, 919–931. [CrossRef]
3. Trøseid, M.; Andersen, G.Ø.; Broch, K.; Hov, J.R. The gut microbiome in coronary artery disease and heart failure: Current knowledge and future directions. *EBioMedicine* **2020**, *52*, 102649. [CrossRef] [PubMed]
4. Szabo, H.; Hernyes, A.; Piroska, M.; Ligeti, B.; Fussy, P.; Zoldi, L.; Galyasz, S.; Makra, N.; Szabo, D.; Tarnoki, A.; et al. Association between Gut Microbial Diversity and Carotid Intima-Media Thickness. *Medicina* **2021**, *57*, 195. [CrossRef]
5. Kashtanova, D.A.; Tkacheva, O.N.; Doudinskaya, E.N.; Strazhesko, I.D.; Kotovskaya, Y.V.; Popenko, A.S.; Tyakht, A.V.; Alexeev, D.G. Gut Microbiota in Patients with Different Metabolic Statuses: Moscow Study. *Microorganisms* **2018**, *6*, 98. [CrossRef]
6. Li, J.; Lin, S.; Vanhoutte, P.M.; Woo, C.W.; Xu, A. *Akkermansia Muciniphila* Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in *Apoe* $^{-/-}$ Mice. *Circulation* **2016**, *133*, 2434–2446. [CrossRef] [PubMed]
7. Karlsson, F.; Fåk, F.; Nookaew, I.; Tremaroli, V.; Fagerberg, B.; Petranovic, D.; Bäckhed, F.; Nielsen, J. Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat. Commun.* **2012**, *3*, 1245. [CrossRef]

8. Albenberg, L.; Esipova, T.V.; Judge, C.P.; Bittinger, K.; Chen, J.; Laughlin, A.; Grunberg, S.; Baldassano, R.N.; Lewis, J.D.; Li, H.; et al. Correlation Between Intraluminal Oxygen Gradient and Radial Partitioning of Intestinal Microbiota. *Gastroenterology* **2014**, *147*, 1055–1063. [CrossRef]
9. Bikov, A.; Szabo, H.; Piroska, M.; Kunos, L.; Szily, M.; Ligeti, B.; Makra, N.; Szabo, D.; Tarnoki, D.L.; Tarnoki, A.D. Gut Microbiome in Patients with Obstructive Sleep Apnoea. *Appl. Sci.* **2022**, *12*, 2007. [CrossRef]
10. Ko, C.-Y.; Liu, Q.-Q.; Su, H.-Z.; Zhang, H.-P.; Fan, J.-M.; Yang, J.-H.; Hu, A.-K.; Liu, Y.-Q.; Chou, D.; Zeng, Y.-M. Gut microbiota in obstructive sleep apnea–hypopnea syndrome: Disease-related dysbiosis and metabolic comorbidities. *Clin. Sci.* **2019**, *133*, 905–917. [CrossRef]
11. Ko, C.; Fan, J.; Hu, A.; Su, H.; Yang, J.; Huang, L.; Yan, F.; Zhang, H.; Zeng, Y. Disruption of sleep architecture in *Prevotella* enterotype of patients with obstructive sleep apnea-hypopnea syndrome. *Brain Behav.* **2019**, *9*, e01287. [CrossRef]
12. Collado, M.C.; Katila, M.K.; Vuorela, N.M.; Saarenpää-Heikkilä, O.; Salminen, S.; Isolauri, E. Dysbiosis in Snoring Children: An Interlink to Comorbidities? *J. Pediatr. Gastroenterol. Nutr.* **2019**, *68*, 272–277. [CrossRef]
13. Valentini, F.; Evangelisti, M.; Arpinelli, M.; Di Nardo, G.; Borro, M.; Simmaco, M.; Villa, M.P. Gut microbiota composition in children with obstructive sleep apnoea syndrome: A pilot study. *Sleep Med.* **2020**, *76*, 140–147. [CrossRef]
14. Hu, C.; Wang, P.; Yang, Y.; Li, J.; Jiao, X.; Yu, H.; Wei, Y.; Li, J.; Qin, Y. Chronic Intermittent Hypoxia Participates in the Pathogenesis of Atherosclerosis and Perturbs the Formation of Intestinal Microbiota. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 560201. [CrossRef]
15. Tripathi, A.; Melnik, A.V.; Xue, J.; Poulsen, O.; Meehan, M.J.; Humphrey, G.; Jiang, L.; Ackermann, G.; McDonald, D.; Zhou, D.; et al. Intermittent Hypoxia and Hypercapnia, a Hallmark of Obstructive Sleep Apnea, Alters the Gut Microbiome and Metabolome. *mSystems* **2018**, *3*, e00020-18. [CrossRef]
16. Wang, F.; Zou, J.; Xu, H.; Huang, W.; Zhang, X.; Wei, Z.; Li, X.; Liu, Y.; Zou, J.; Liu, F.; et al. Effects of Chronic Intermittent Hypoxia and Chronic Sleep Fragmentation on Gut Microbiome, Serum Metabolome, Liver and Adipose Tissue Morphology. *Front. Endocrinol.* **2022**, *13*, 820939. [CrossRef]
17. Xue, J.; Allaband, C.; Zhou, D.; Poulsen, O.; Martino, C.; Jiang, L.; Tripathi, A.; Elijah, E.; Dorrestein, P.C.; Knight, R.; et al. Influence of Intermittent Hypoxia/Hypercapnia on Atherosclerosis, Gut Microbiome, and Metabolome. *Front. Physiol.* **2021**, *12*, 663950. [CrossRef]
18. Szily, M.; Tarnoki, A.D.; Tarnoki, D.L.; Kovacs, D.T.; Forgo, B.; Lee, J.; Kim, E.; Sung, J.; Kunos, L.; Meszaros, M.; et al. Genetic influences on the onset of obstructive sleep apnoea and daytime sleepiness: A twin study. *Respir. Res.* **2019**, *20*, 125. [CrossRef]
19. Littvay, L.; Métneki, J.; Tarnoki, A.D.; Tarnoki, D.L. The Hungarian Twin Registry. *Twin Res. Hum. Genet.* **2012**, *16*, 185–189. [CrossRef]
20. Tarnoki, A.D.; Tarnoki, D.L.; Forgo, B.; Szabo, H.; Melicher, D.; Metneki, J.; Littvay, L. The Hungarian Twin Registry Update: Turning From a Voluntary to a Population-Based Registry. *Twin Res. Hum. Genet.* **2019**, *22*, 561–566. [CrossRef]
21. Mansour, B.; Monyók, Á.; Makra, N.; Gajdács, M.; Vadnay, I.; Ligeti, B.; Juhász, J.; Szabó, D.; Ostorházi, E. Bladder cancer-related microbiota: Examining differences in urine and tissue samples. *Sci. Rep.* **2020**, *10*, 11042. [CrossRef] [PubMed]
22. Ewels, P.; Magnusson, M.; Lundin, S.; Käller, M. MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **2016**, *32*, 3047–3048. [CrossRef]
23. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef] [PubMed]
24. Lu, J.; Salzberg, S.L. Ultrafast and accurate 16S rRNA microbial community analysis using Kraken 2. *Microbiome* **2020**, *8*, 124. [CrossRef]
25. Wood, D.E.; Salzberg, S.L. Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **2014**, *15*, R46. [CrossRef]
26. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
27. Breitwieser, F.P.; Lu, J.; Salzberg, S.L. A review of methods and databases for metagenomic classification and assembly. *Briefings Bioinform.* **2017**, *20*, 1125–1136. [CrossRef]
28. Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60. [CrossRef]
29. Wang, Z.; Klipfell, E.; Bennett, B.J.; Koeth, R.; Levison, B.S.; DuGar, B.; Feldstein, A.E.; Britt, E.B.; Fu, X.; Chung, Y.-M.; et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. *Nature* **2011**, *472*, 57–63. [CrossRef]
30. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* **2013**, *19*, 576–585. [CrossRef]
31. Koeth, R.A.; Levison, B.S.; Culley, M.K.; Buffa, J.A.; Wang, Z.; Gregory, J.C.; Org, E.; Wu, Y.; Li, L.; Smith, J.D.; et al. γ-Butyrobetaine Is a Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO. *Cell Metab.* **2014**, *20*, 799–812. [CrossRef] [PubMed]
32. 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.* **2017**, *8*, 139. [CrossRef] [PubMed]
33. Stepankova, R.; Tonar, Z.; Bartova, J.; Nedorost, L.; Rossman, P.; Poledne, R.; Schwarzer, M.; Tlaskalova-Hogenova, H. Absence of Microbiota (Germ-Free Conditions) Accelerates the Atherosclerosis in ApoE-Deficient Mice Fed Standard Low Cholesterol Diet. *J. Atheroscler. Thromb.* **2010**, *17*, 796–804. [CrossRef] [PubMed]

34. Desai, M.S.; Seekatz, A.M.; Koropatkin, N.M.; Kamada, N.; Hickey, C.A.; Wolter, M.; Pudlo, N.A.; Kitamoto, S.; Terrapon, N.; Muller, A.; et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell* **2016**, *167*, 1339–1353.e21. [CrossRef]
35. Ma, J.; Li, H. The Role of Gut Microbiota in Atherosclerosis and Hypertension. *Front. Pharmacol.* **2018**, *9*, 1082. [CrossRef]
36. Anto, L.; Blesso, C.N. Interplay between diet, the gut microbiome, and atherosclerosis: Role of dysbiosis and microbial metabolites on inflammation and disordered lipid metabolism. *J. Nutr. Biochem.* **2022**, *105*, 108991. [CrossRef]
37. Kramer, C.D.; Simas, A.M.; He, X.; Ingalls, R.R.; Weinberg, E.O.; Genco, C.A. Distinct roles for dietary lipids and Porphyromonas gingivalis infection on atherosclerosis progression and the gut microbiota. *Anaerobe* **2017**, *45*, 19–30. [CrossRef]
38. Wilson, A.; McLean, C.; Kim, R.B. Trimethylamine-N-oxide: A link between the gut microbiome, bile acid metabolism, and atherosclerosis. *Curr. Opin. Lipidol.* **2016**, *27*, 148–154. [CrossRef]
39. Zhu, Y.; Li, Q.; Jiang, H. Gut microbiota in atherosclerosis: Focus on trimethylamine N-oxide. *APMIS* **2020**, *128*, 353–366. [CrossRef]
40. Jie, Z.; Xia, H.; Zhong, S.-L.; Feng, Q.; Li, S.; Liang, S.; Zhong, H.; Liu, Z.; Gao, Y.; Zhao, H.; et al. The gut microbiome in atherosclerotic cardiovascular disease. *Nat. Commun.* **2017**, *8*, 845. [CrossRef]
41. Tan, S.C.; Chong, C.W.; Yap, I.K.S.; Thong, K.L.; Teh, C.S.J. Comparative assessment of faecal microbial composition and metabonome of swine, farmers and human control. *Sci. Rep.* **2020**, *10*, 8997. [CrossRef] [PubMed]
42. Louis, P.; Flint, H.J. Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* **2017**, *19*, 29–41. [CrossRef] [PubMed]
43. Hill, M.J. Bacterial fermentation of complex carbohydrate in the human colon. *Eur. J. cancer Prev.* **1995**, *4*, 353–358. [CrossRef] [PubMed]
44. Hosseini, E.; Grootaert, C.; Verstraete, W.; Van De Wiele, T. Propionate as a health-promoting microbial metabolite in the human gut. *Nutr. Rev.* **2011**, *69*, 245–258. [CrossRef] [PubMed]