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BACTERIAL AND FUNGAL MICROBIOME ANALYSIS OF STOOL, BLOOD, THROMBUS AND ANEURYSM WALL SAMPLES FROM ABDOMINAL AORTIC ANEURYSM PATIENTS

PhD thesis

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List of Abbreviations

16S rRNA	16S ribosomal Ribonucleic acid
AAA	Abdominal aortic aneurysm
DNA	Deoxyribonucleic acid
ITS	Internal Transcribed Spacer
LEfSe	Linear discriminant analysis effect size
NGS	Next generation sequencing
PCoA	Principal Coordinate analysis
PCR	Polymerase chain reaction
PPI	Peptidyl-prolyl isomerase
PIN 1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
RA	Rheumatoid arthritis
SCFAs	Short-chain fatty acids
SMC, SMCs	Smooth muscle cell, Smooth muscle cells

1. Introduction

An abdominal aortic aneurysm (AAA) constitutes a pathological condition in which the segment of the aorta located distal to the renal arteries exhibits progressive dilatation, resulting in an increase in vessel diameter by at least 150% relative to the adjacent non-diseased segment (1). Pathological changes within the vascular wall result from immune cell-mediated inflammatory processes and the consequent degradation of the medial layer (2, 3). The early asymptomatic phase of AAA frequently results in delayed diagnosis, often until the aneurysm is at risk of rupture. The recommended threshold for elective surgical intervention is an aneurysm diameter of ≥5 cm in women and ≥5.5 cm in men (4). Chronic aortic disease can progress to life-threatening consequences, including ruptured AAA with a mortality rate exceeding 80% (5). Major risk factors for the development of an AAA include smoking, male sex, a positive family history, advanced age, hypertension, hypercholesterolemia, obesity, and a range of cardiovascular and peripheral vascular diseases (6). These factors are prevalent within the general population, and the relevance of screening procedures in an individual can be elucidated solely through the knowledge of familial history. Numerous studies have established that the bacterial composition of the gut microbiome contributes to the advancement of risk factors including hypertension, metabolic disorders, and cardiovascular diseases (7-9). Recently, multiple studies have demonstrated a correlation between the composition of the gut microbiome and the development as well as progression of AAA (10-12). The diet influences the composition of the gut microbiome, thereby modulating immunological and inflammatory processes within the body. Consequently, further studies would be needed to explore the association between diet-induced changes in the gut microbiome and the progression of AAA (13). The transplantation of faecal microbiota from patients with AAA significantly elevates the incidence of aneurysm development, rupture, and mortality in AAA mouse models, whereas transplantation with microbiota from healthy human donors elicits an opposing protective effect (11). Gut or oral microbiota can modulate the inflammatory degradation of the vascular wall through both direct and indirect mechanisms. Indirectly, circulating microbial metabolites contribute to inflammatory and apoptotic pathways, while directly, translocated microbiota promote enhanced infiltration of inflammatory cells into the vascular tissue (14, 15). Studies analysing human samples from AAA patients or conducting experimental animal research

have predominantly focused on elucidating the relationship between the faecal microbiome and AAA (11, 14-20) although the role of the oral microbiome in the pathomechanism is also being extensively investigated (21, 22). The DNA (Deoxyribonucleic acid) of selected bacteria derived from the intestinal or oral microbiome is positively detectable in vascular, thrombus, or blood samples of patients AAA, indicating that bacteria may contribute to aneurysm progression through their direct presence (23-26). The DNA of the 11 enteral bacterial species investigated by Nakayama et al. was not consistently detected in all analyzed aneurysm and blood samples, and corresponding samples from the same patients did not always exhibit concordant bacterial presence (23). Our research group has previously demonstrated the presence of bacterial DNA in the walls of healthy blood vessels, detected through sequencing of the bacterial 16S rRNA (16S ribosomal Ribonucleic acid) V3-V4 region in arterial samples from healthy organ donors. The microbiome composition identified in this manner differs substantially from that observed in oral or faecal microbiomes (27). In the current study, we also determined the composition of the microbiome of AAA patients based on the bacterial 16S rRNA gene from vascular wall, thrombus, and faecal samples.

The fungal community in the gut accounts for less than 1% of the overall microbial population; however, it plays a crucial role in sustaining host health and regulating pathological conditions. The intestinal mycobiome affects nutrient absorption and metabolism via distinct metabolic pathways and modulates the host immune response (28, 29). Alongside the established correlations between gut bacterial microbiome composition and AAA-associated risk diseases, certain studies have also identified a link between the faecal mycobiome composition and these conditions (30-32). Yao et al. examined the role of gut fungal communities (mycobiota) in AAA pathogenesis. They reported marked dysbiosis in the gut mycobiomes of AAA patients relative to healthy controls, characterized by elevated abundances of *Candida* species and reduced levels of *Saccharomyces cerevisiae* (33). In this PhD thesis, building upon analyses of bacterial microbiome composition, we investigated the mycobiome profiles of vessel walls from both healthy individuals and those with aneurysms and we also determined the mycobiome profiles in thrombus, blood, and faecal samples from aneurysmal patients. For this study, we used a specific DNA region of the fungus ITS (Internal Transcribed Spacer).

2. Objectives

The main goal of this PhD thesis is to be the first to examine the bacterial and fungal microbiome characteristic of the vessel wall, thrombus, blood and stool samples of patients with aortic aneurysm.

1. Our primary goal is to determine whether the bacterial microbiome of the aortic aneurysmal vessel wall differs from the bacterial microbiome characteristic of the healthy vessel wall.

2. Second, to examine the characteristic bacterial microbiome of different samples from aneurysm patients, such as thrombus, blood, and stool, and whether bacteria present in the aneurysm vessel wall could originate from stool and travel there via the bloodstream.

3. Third, to examine whether we can assume a direct effect of microorganisms or an indirect role in protection or pathogenicity through their enzymatic activity based on the composition of the healthy and aneurysmal microbiome.

4. Fourthly, to compare the fungal microbiome characteristic of a healthy vascular wall with the mycobiota flora characteristic of an aneurysmal vascular wall.

5. Our next goal is to determine whether the fungal microbiome detected in different samples of aneurysm patients is also present in the stool and whether it reaches the vessel wall through the blood.

6. Finally, to identify certain types of fungi that may play a role in protecting healthy blood vessel walls or in the development and progression of aneurysms.

3. Methods

3.1. Sample collection

In this prospective PhD study, twenty-four patients diagnosed with abdominal aortic aneurysm who underwent surgical intervention between March 2022 and February 2023 at the Department of Vascular and Endovascular Surgery, Heart and Vascular Center, Semmelweis University were enrolled. The research was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Semmelweis University based on the Research Authorization Decisions (EIÜG) of the Hungarian National Center for Public Health (9882-8/2022 EÜIG, 9882-13/2022 EÜIG, 17273/5-2023 EIÜG). Twelve femoral arteries from human multi-organ donors were incorporated into the bacterial and fungal microbiome investigation as healthy negative controls (27). For participants in the aortic aneurysm group, administration of antibiotics, antifungals, or probiotics in the prior month constituted an exclusion criterion. Written informed consent was obtained from all participating patients. All individuals involved in the study provided explicit written consent for the publication of data derived from their personal test results. Data and inclusion criteria comprised age exceeding 18 years, legal capacity, presence of an abdominal aortic aneurysm warranting open surgical intervention, and thrombus within the aorta. The study's inclusion criterion was that the aneurysm be covered with a thrombus. However, we excluded three patients from our further comparative bacterial microbiome analysis, in whom aneurysm formation was presumably of infectious origin, as *Staphylococcus* sp., *Streptococcus* sp., and *Mycobacterium tuberculosis* were detected in high amounts in their samples. Samples of the aneurysmal wall and intraluminal thrombus were attentively collected to prevent contamination during open aortic repair surgery. Throughout the open abdominal surgery, rigorous aseptic and antiseptic protocols were meticulously adhered to minimize the risk of iatrogenic infection in the patient. Samples comprising approximately 3 g of intraluminal thrombus and aneurysm wall were harvested and transferred into sterile, DNase- and RNase-free, low-binding microfuge tubes (AM12450 Invitrogen Microfuge Tubes, Thermo Fisher Scientific, Waltham, MA, USA) to prevent any potential contamination. The samples were rinsed with sterile saline to remove blood, followed by immediate snap-freezing in liquid nitrogen. A minimum volume of 3 mL of whole blood

was obtained using citrate-containing VACUETTE collection tubes (Greiner Bio-One, Stonehouse, UK), following gentle tube inversion, the samples were immediately frozen at −80°C. Faecal samples were collected on the second preoperative day after patient admission, as well as intraoperatively following anesthesia via perianal swab. All samples were stored at −80°C until DNA extraction.

3.2. DNA extraction and purification

DNA isolation was carried out by NucleoSpin Blood, Mini kit (Macherey-Nagel, Allentown, PA, USA) from blood samples and by ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) from aneurysm, thrombus and stool samples according to the manufacturer's instructions. The aneurysm vessel wall and thrombus samples were previously subjected to enzymatic digestion with 20 µL of Proteinase K solution in 200 µL of lysis buffer (56°C, 3 h). Concentration of genomic DNA was measured using a Qubit2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

3.3. Bacterial microbiome analysis

Bacterial 16S rRNA is a ribosomal RNA that is located in the small subunit of the prokaryotic ribosome and is essential for protein synthesis. 16S rDNA contains nine hypervariable regions (V1-V9). Due to the low bacterial DNA content in blood vessel, thrombus, and blood samples, we performed nested polymerase chain reaction (PCR) to enhance sensitivity and specificity. From the amplified longer gene segment, we amplified the V3-V4 region in the second round, and this latter gene segments were subsequently used for sequencing, which are widely used in sequencing for bacterial identification **(Figure 1.)**.

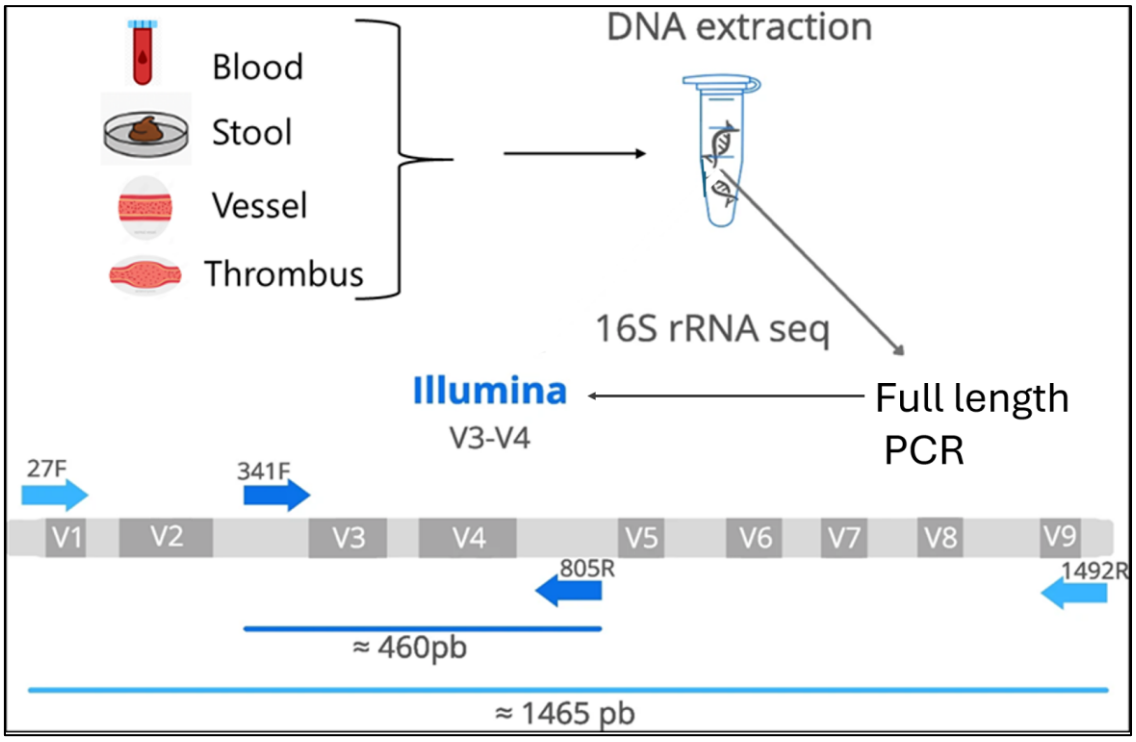


Figure 1. Schematic modified method of 16S rRNA gene analysis (34)

3.3.1. Nested PCR

Nearly full length bacterial 16S ribosomal DNA templates were amplified using the primers 27F/1492R were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium). These are the most widely used primer pair for species-level identification. Currently available primers can reveal the composition of the predominant bacteria in a sample through amplification and sequencing (35, 36). Preparation of 16S rDNA PCR: 2.5 µL DNA, 12.5 µL 2x KAPA HiFi HotStart Ready Mix (Roche, Basel, Switzerland), 5 µL primer 27F, 5 µL primer 1492R. We performed the following PCR protocol: 95°C for 3 min, (98°C for 20 sec, 57°C for 30 sec, 72°C for 1 min) for 25 cycles, and as final step 72°C for 5 min. PCR amplicons were purified by 20 µL SparQ PureMag beads (QuantaBio, a QIAGEN company, Beverly, MA USA). The first PCR amplification product is approximately 1500 bp.

3.3.2. 16S rRNA bacterial analysis

The larger fragment produced by the first round of PCR was used as the template for the second PCR. Amplification was performed using primers specified by the Illumina

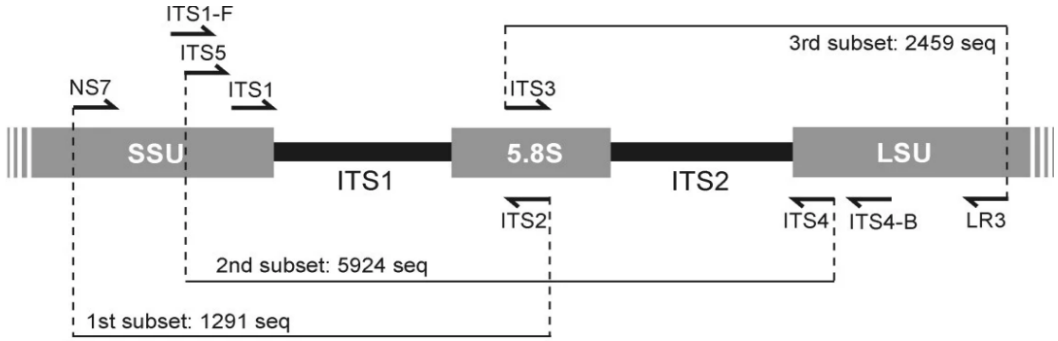
protocol (IDT, Leuven, Belgium), which target the V3-V4 region of the 16S rRNA gene. PCR amplification and DNA purification followed Illumina's standard protocol. Library quality was evaluated with a DNA 7500 Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar library pools were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) with the MiSeq Reagent Kit v3 (600-cycle paired-end) (37).

To minimize contamination and enhance study reliability, all procedures were conducted in duplicate. Extraction blanks, PCR negatives, and PCR positives (ZymoBIOMICS Microbial Community Standard, Zymo Research Corp., Irvine, CA, USA) were included in each run to assess reagent-derived DNA contamination. Raw sequencing data were downloaded from Illumina BaseSpace and analyzed via the CosmosID bioinformatics platform (38).

3.4. Fungal microbiome analysis

The ITS is a specific DNA region in fungi, widely employed as a universal barcode for fungal identification and phylogenetic classification owing to its high sequence variability and taxon-specific patterns; this region is localized between ribosomal RNA coding genes and plays a pivotal role in investigating mycotic biodiversity, ecology, and evolutionary dynamics **(Figure 2.)**.

(a)



(b)

Primer	Author	Primer sequence	Position
Forward primers			
NS7		GAGGCAATAACAGGTCTGTGATGC	1403-1426
ITS1-F		CTTGGTCATTTAGAGGAAGTAA	1723-1744
ITS5		GGAAGTAAAAGTCGTAACAAGG	1737-1758
ITS1		TCCGTAGGTGAACCTGCGG	1761-1779
ITS3		GCATCGATGAAGAACGCAGC	2024-2045
Reverse primers			
ITS2		GCTGCGTTCTTCATCGATGC	2024-2043
ITS4		TCCTCCGCTTATTGATATGC	2390-2409
ITS4-B		CAGGAGACTTGTACACGGTCCAG	2526-2548
LR3		CCGTGTTTCAAGACGGG	3029-3045

Figure 2. Schematic Structure of ITS region (a) and the forward and reverse primer sequences used for amplification of the ITS1 region, following the Illumina protocol recommendation (b) (39)

Amplification of the ITS region (specifically the ITS1 region using the ITS1-F and ITS2 primer pool recommended by the Illumina protocol) was performed with primers synthesized by IDT (Coralville, IA, USA). The standard Illumina fungal metagenomic protocol was modified to perform the ITS mycobiota analysis (40). The PCR was optimized using increased amount of purified DNA of 3.25 µL per reaction and using decreased volume of primers of 1.5 µL to minimize primer dimer formation. The total PCR reaction volume was 12.5 µL, of which 6.25 µL consisted of 2× KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland). The number of PCR cycles were increased to 30 for adequate amplification of the target regions. Non-specific products and primary dimers were removed in a two-step process. To this end, the sample was first brought to a volume of 50 µL with DNase- and RNase-free water, followed by sequential cleanup using 25 µL and 10 µL of beads, which confirmed the retention of the target amplicon with SparQ PureMag beads (QuantaBio, a QIAGEN company, Beverly, MA USA). To avoid contamination and to increase the reliability of the study, all analysis procedures

were done in duplicate. To evaluate the contribution of extraneous DNA from reagents, extraction negative controls and PCR negative controls were included in every run. Throughout the DNA isolation process, nuclease-free water was employed as a negative control, while the ZymoBIOMICS Microbial Community DNA Standard served as the positive control (Zymo Research Corp., Irvine, CA, USA). PCR product libraries were assessed using DNA 7500 Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (600 cy-cles PE). Raw sequencing data were retrieved from the Illumina BaseSpace and the data were analysed by the CosmosID bioinformatics platform (Cosmosid Inc., Germantown, ML, USA) (38).

3.5. Statistical analysis

The Wilcoxon Rank Sum test for Chao1 alpha diversity, PERMANOVA analysis for Bray-Curtis Principal Coordinate analysis (PCoA) beta diversity (applied to bacterial microbiome analysis), and PERMANOVA analysis for Jaccard PCoA beta diversity (applied to fungal microbiome analysis) were used for statistical testing between cohorts of samples, via the statistical analysis support of the CosmosID bioinformatics platform (38). Statistical significance was defined as a two-tailed p-value ≤ 0.05. Additionally, the CosmosID bioinformatics platform was employed for LEfSe (Linear discriminant analysis Effect Size) analysis to pinpoint differentially abundant features, including taxa and enzymatic pathways, that distinguish between the two cohorts (bacteria-specific application; CosmosID Inc., Germantown, MD, USA).

4. Results

4.1. Bacterial microbiome

Following 16S rRNA V3-V4 region sequencing of aneurysm vessel wall, thrombus, blood, and stool samples from 21 patients, we obtained adequate read counts across all samples to enable data comparison and analysis. The median read count per sample, irrespective of type, was 242,591 (IQR: 21,678). No detectable DNA was obtained from either the extraction negative controls or the PCR negative controls processed in parallel with the samples, indicating that neither DNA isolation nor PCR amplification yielded measurable amounts of DNA.

First, we compared the bacterial microbiome composition of the aneurysm wall with that of the healthy vascular wall previously characterized by our research group, significant differences were observed based on both alpha and beta diversity. The data presented in **Figure 3** shows that there is a significant difference between two groups based on the Chao1 alpha diversity **(Fig.3 A)** (p=0.0001) and Bray Curtis beta diversity (PCoA) **(Fig.3 B)** (p=0.001).

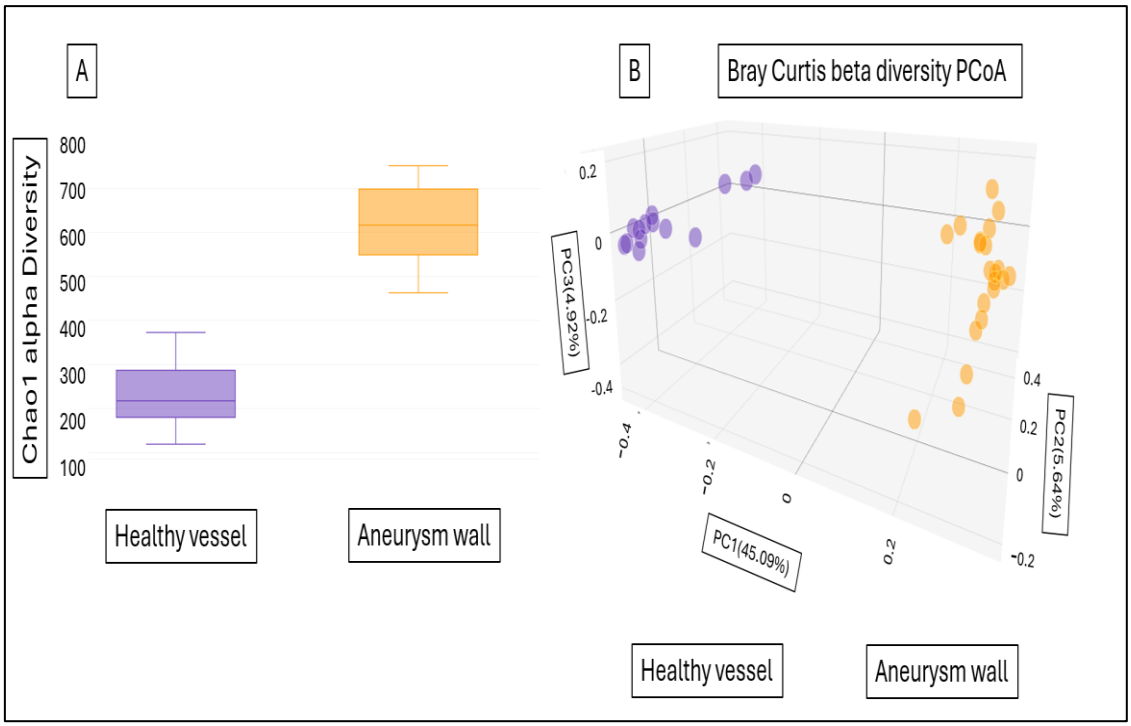


Figure 3. Chao1 alpha diversity **(A)** and Bray Curtis beta diversity PCoA **(B)** of healthy vessel and aneurysm wall microbiome. Purple: healthy vessel microbiome, yellow: aneurysm wall microbiome (41)

At the phylum level, the presence of the identical four taxa, *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria*, is characteristic of both vessel walls. However, there is the glaring discrepancy in the abundance of phyla between two groups. As opposed to the 34% abundance of *Proteobacteria* in the healthy vessel wall, this phylum was present in the aneurysm wall at 71%. The incidence of 29% *Firmicutes*, 10% *Bacteroidetes* and 15% *Actinobacteria* in the healthy vessel wall declined to 14%, 4% and 4% respectively in the aneurysm wall. The bacterial genus composition detected in healthy vascular wall samples did not show significant differences between samples; therefore, mean relative abundances were used to compare the vascular wall microbiome of patients. The dominant genera in the healthy vessel microbiome were *Pseudomonas* (from *Proteobacteria* phylum), *Staphylococcus* (from *Firmicutes* phylum) and *Corynebacterium* (from *Actinobacteria* phylum). However, the most common genera in the aneurysm vessel wall are *Acinetobacter*, *Burkholderia*, *Escherichia*, *Escherichia-Shigella* and *Sphingobium* from *Proteobacteria* phylum. From the *Firmicutes* phylum, the genus *Finegoldia* was present in greater amount, and the genus *Staphylococcus* in

lower amount in the aneurysm wall as opposed to the healthy vessel wall. The higher volume of *Corynebacterium* and *Propionibacterium* genera contributed to the higher *Actinobacteria* phylum abundance feature of the healthy vessel wall **(Figure 4)**.

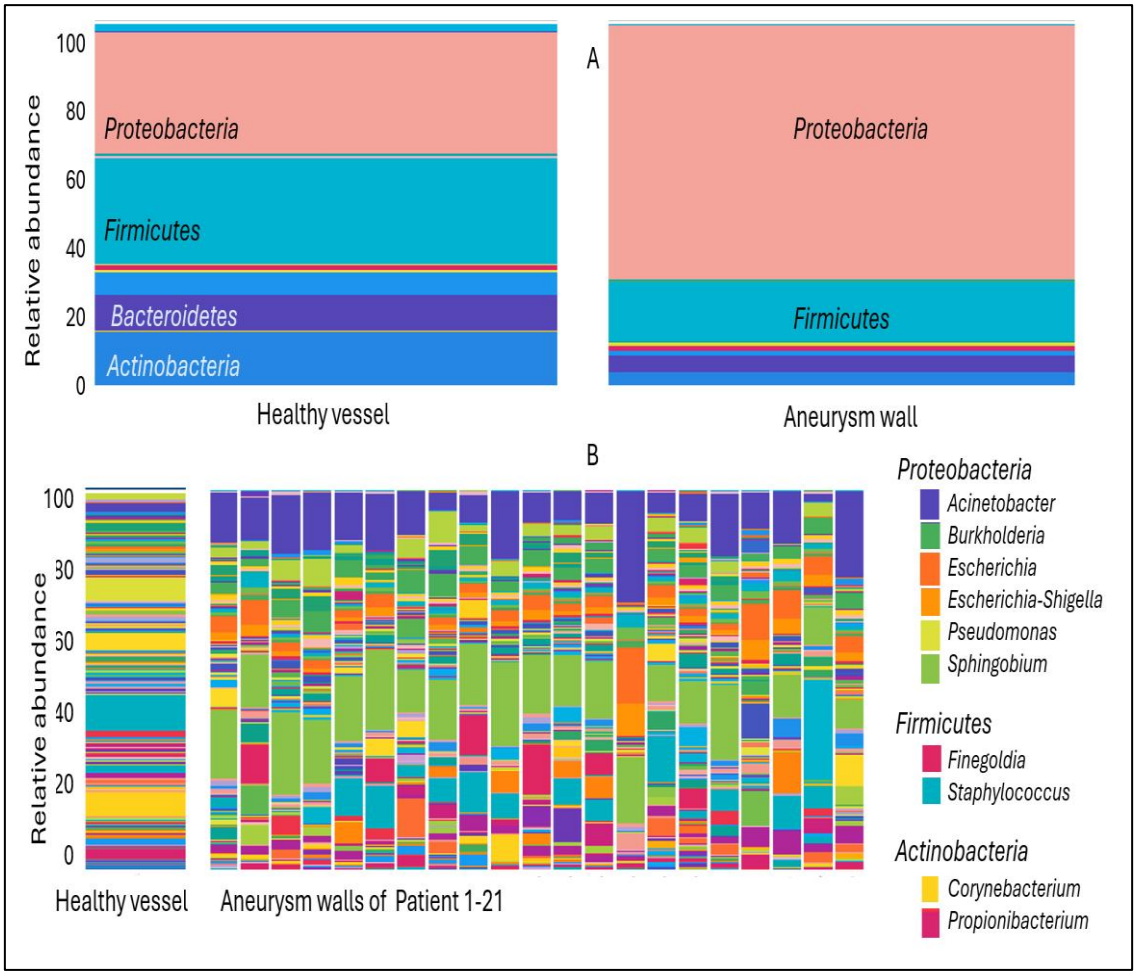


Figure 4. Relative taxon abundance of healthy and aneurysm vessel wall microbiome composition, A aggregated cochors at phylum level, B aggregated cochors of healthy vessel and individually indicated samples of AAA patients at genus level (41)

Comparing the bacterial microbiome composition of stool, blood, thrombus and aneurysm wall based on beta diversity, the PCoA diagram shows distinct groups of sample types **(Figure 5).** Significant difference can be shown between the beta diversity of the aneurysm wall and thrombus samples (p=0.014), on the other hand in all another groupings a discrepancy of p=0.001 can be calculated between the cohorts. The blood samples are situated between the feces and aneurysm-thrombus samples, pointing to their supposed intermediary role as shown in the 3D diagram.

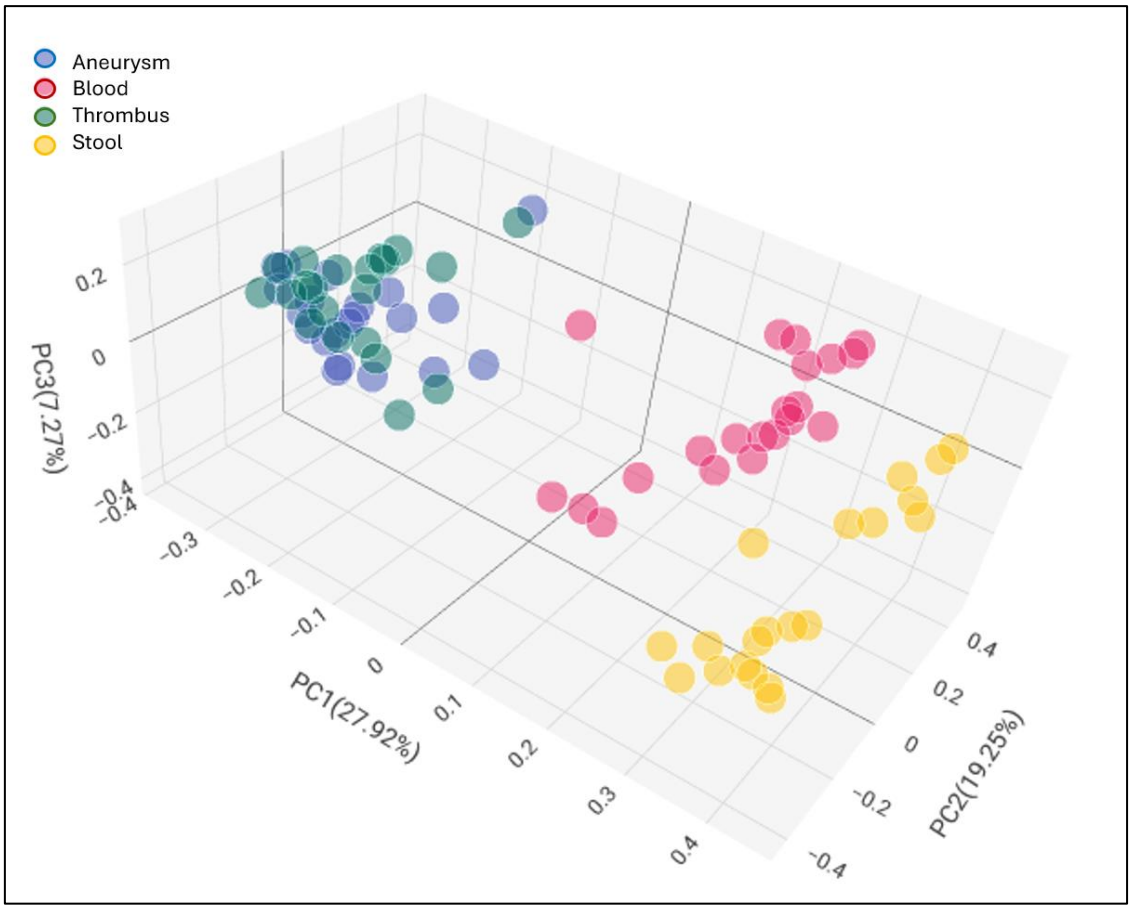


Figure 5. Bray Curtis beta diversity PCoA of stool (yellow), blood (red), thrombus (green) and aneurysm (blue) microbiome of AAA patients (41)

The results of the 25 most common bacterial genera characteristic of different sample types are presented in a heat map in **Figure 6**. These bacterial genera found in plenty of numbers in aneurysms and thrombus, such as *Sphingobium*, *Acinetobacter*, *Burkholderia* and *Aquabacterium* genera are found in very low numbers in stool and blood. The main genera detected in higher abundance in the faecal bacterial microbiome were *Bacteroides*, *Prevotella*, *Finegoldia*, *Peptoniphilus* and *Anaerococcus*, in contrast to blood, where they were not present in detectable quantities. In contrast, for instance, *Propionibacterium*, *Enhydrobacter* or *Moraxella* are not detectable in the feces microbiome, but are found in significantly higher numbers in the bloodstream. In addition, other genera, such as *Escherichia* and *Escherichia-Shigella* that occur in high abundance in the blood, may derived from the intestinal microbiome, considering these bacteria are also present in feces.

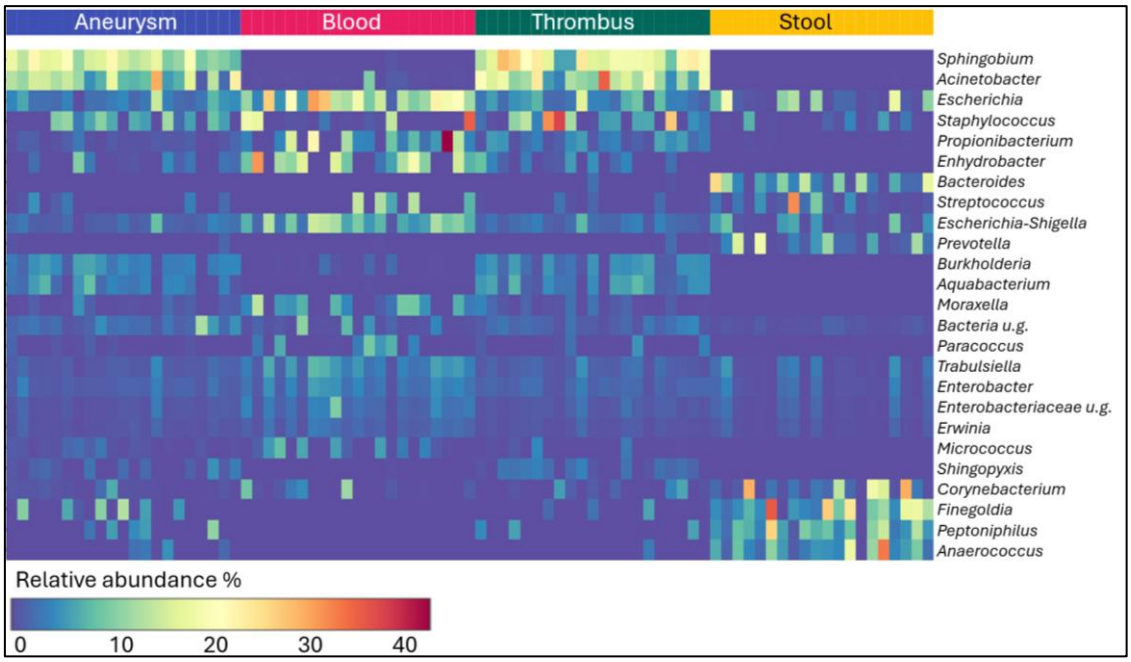


Figure 6. Heatmap about the most abundant 25 genera in aneurysm, blood, thrombus and stool samples (41)

The genera present in each sample type with notable different abundances were compared applying the LEfSe biomarker discovery analysis (**Figure 7**). Analysis of each sample type in relation to the stool sample, *Enhydrobacter* and *Cutibacterium* that are presented as newly appeared genera in the bloodstream, and these genera remain to occur significantly high amount in the thrombus and aneurysm vessel wall. Opposed to the feces, in the thrombus *Acinetobacter* and *Aquabacterium* were as new genera with significantly higher abundances, and the *Sphingobium* genus also appeared newly in the aneurysm wall. Contrasted with the bloodstream, *Sphingobium*, *Acinetobacter* and *Aquabacterium* were detected in significantly higher quantities in both the thrombus and the aneurysm vessel wall, nevertheless *Enhydrobacter* and *Cutibacterium* genera continued to have significantly higher abundance in the bloodstream.

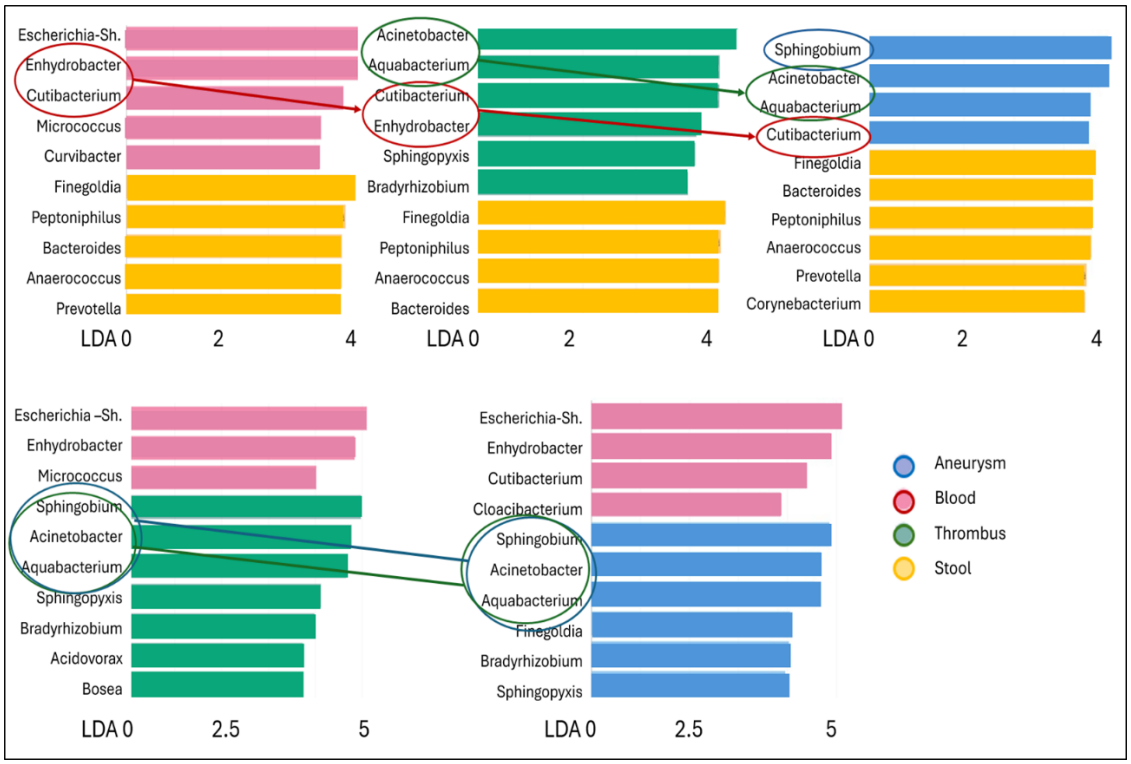


Figure 7. LEfSe bar chart visual representation of discriminative feature of genera abundances among the different sample types. LDA is the Linear Discriminant Analysis score (41)

The functional capabilities and abundance of the bacterial community of the microbiome were predicted and characterized based on amplicon content applying the PICRUST2 tool of CosmosID. The aneurysm and the healthy vessel wall differ significantly based on the expected enzyme activity of the bacteria present in the sample (**Figure 8**). The enzyme repertoire of bacteria presented in the healthy control vessel wall contains significantly more iron-chelate-transporting ATPase, polar-amino-acid-transporting ATPase, monosaccharide-transporting ATPase, N-acetylmuramoyl-L-alanine amidase and non-specific serine/threonine protein kinase, in contrast to the aneurysm microbiome enzyme set. However the pathological vascular wall contains in significantly larger quantities glutathione transferase, NADH:ubiquinone reductase, acetyl-CoA C-acetyltransferase, peptidylprolyl isomerase and enoyl-CoA hydratase in contrast with the healthy vessel wall.

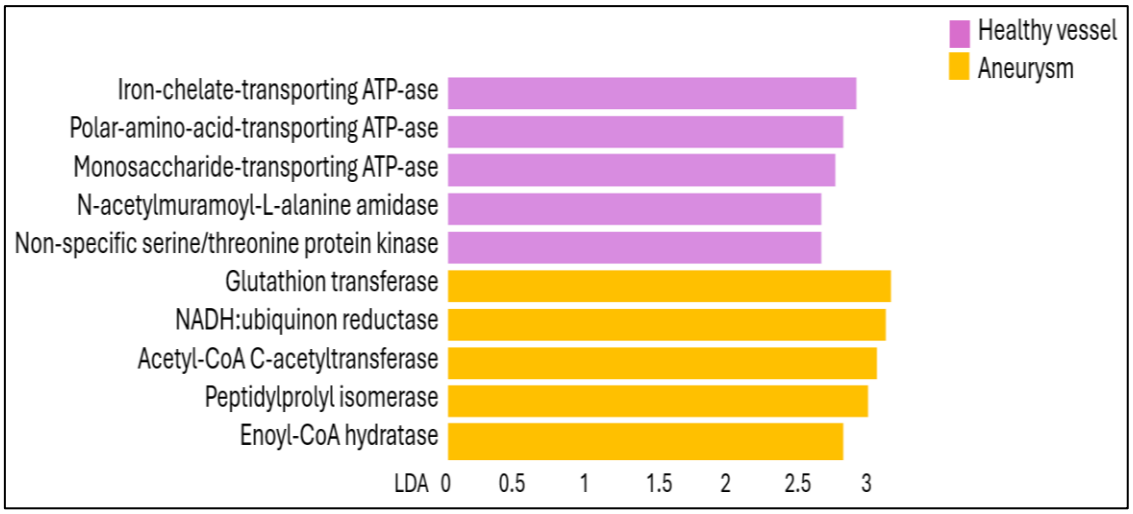


Figure 8. LEfSe bar chart representation of significantly different predicted enzyme activity among the microbiome compositions of healthy control and aneurysm vessel walls (41)

Data Availability

The datasets generated and analyzed during the current 16S rRNA study are available in the SRA repository: SRA/PRJNA1128157/www.ncbi.nlm.nih.gov (accessed on 26 June 2024).

4.2. Fungal microbiome

Library preparation and sequencing of the ITS region were successfully completed for all vessel wall, thrombus, blood, and stool samples from 24 patients with aortic aneurysms, as well as 12 healthy vessel wall samples included in the study. The average length of indexed ITS PCR amplicons was 724 bp, as determined by Agilent 2100 Bioanalyzer analysis. Per-sample read counts ranged from a minimum of 125,995 to a maximum of 463,713. The proportion of fungi identifiable at the genus level per sample ranged from 53% to 100%, with remaining reads assigned to the unidentified fungal genus category. Certain genus-level identifications were deemed unreliable due to implausibility, such as the detection of mushroom DNA (e.g., genera *Helotiales*, *Leotiomycetes*, and *Russula*) in blood, thrombus, and vascular wall samples. Consequently, these presumed misidentifications were reclassified as unidentifiable fungi in subsequent comparative analyses. Comparing the fungal microbiome composition of stool, blood, thrombus and aneurysm wall based on alpha and beta diversity gave different results **(Figure 9)**. The

data presented in **Figure 9 A** show that the diversity levels of different sample types were significantly different based on the Chao1 alpha, such as between blood and stool samples (p<0.001), between blood and thrombus samples (p=0.001), between blood and aneurysm samples (p=0.005) and between stool and aneurysm samples (p=0.034). In spite of the fact that the Jaccard beta diversity PCoA plot shows that the groups of different sample types overlap, for the samples analyzed by PERMANOVA statistical calculation, there is still a significant difference between the individual sample types, for example between blood-stool, blood-aneurysm, stool-thrombus and stool-aneurysm samples with the p value of 0.002, 0.004, 0.032 and 0.034, respectively **(Figure 9 B)**.

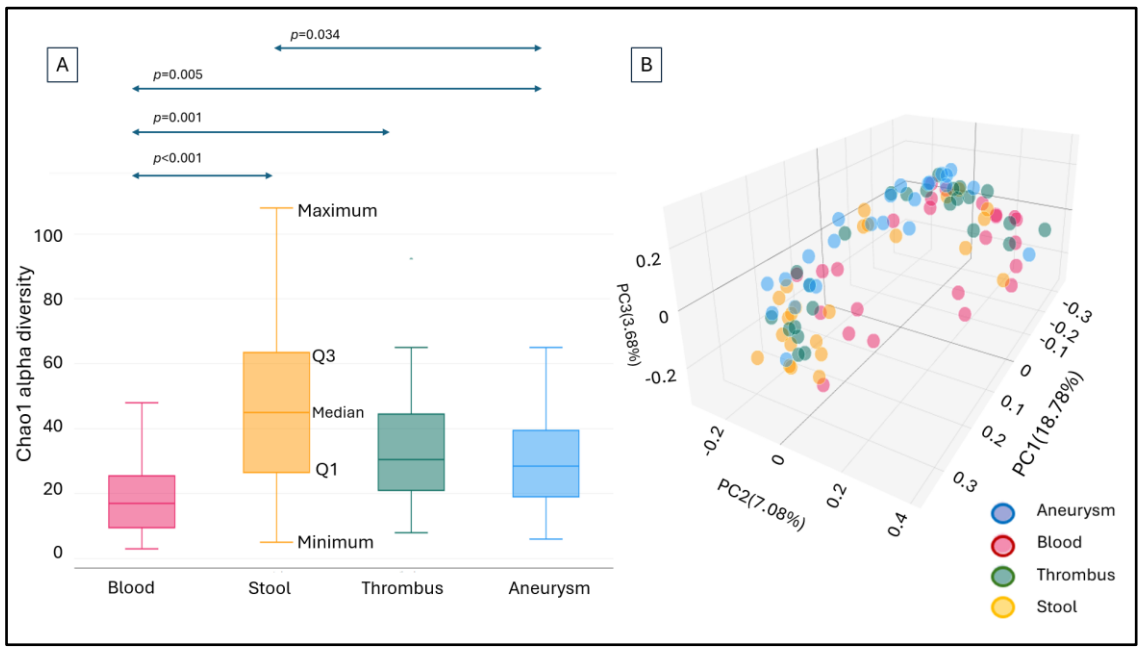


Figure 9. Mycobiome Chao1 alpha diversity (A) and Jaccard beta diversity PCoA (B) of blood, stool, thrombus and aneurysm wall samples of AAA patients: Boxplot displaying the data distribution with minimum, first quartile (Q1), median, third quartile (Q3), and maximum values, illustrating central tendency and variability, PC: principal coordinate (42)

We found a difference by summing the frequency of occurrence of fungal genera characteristic of the given sample type (blood, stool, thrombus, aneurysm), however this difference is not significant, because of the high standard deviation of each sample. **Figure 10** shows the average occurrence of the most common fungal genera per sample type.

Figure 10. Average abundance of the most common fungal genera in the different sample types (42)

No fungal genera can be found whose high occurring can be associated with a given sample type. Similarly, parallel examining of the four sample types per person did not confirm a personalized fungal microbiome composition. The comparison of individual patients' fungal genus abundance across different four sample types shows different groups (**Figure 11)**, there are patient who demonstrates similar distribution (P08, P20) and others whose samples are very different from each other (P04, P07). Several individuals whose aneurysm samples are characterized by high prevalence of *Malassezia* above 20%, which was associated with high abundance of *Malassezia* also in the stool (P5, P6, P12, P18) and in one case this was accompanied by high levels of *Malassezia* in the blood (P22). Contrary to the previous samples, there are some individuals (P14, P16, P21), in whom high *Malassezia* levels relate only in the aneurysmal sample and in patient P24, high abundance of *Malassezia* in the aneurysm presents also in the blood.

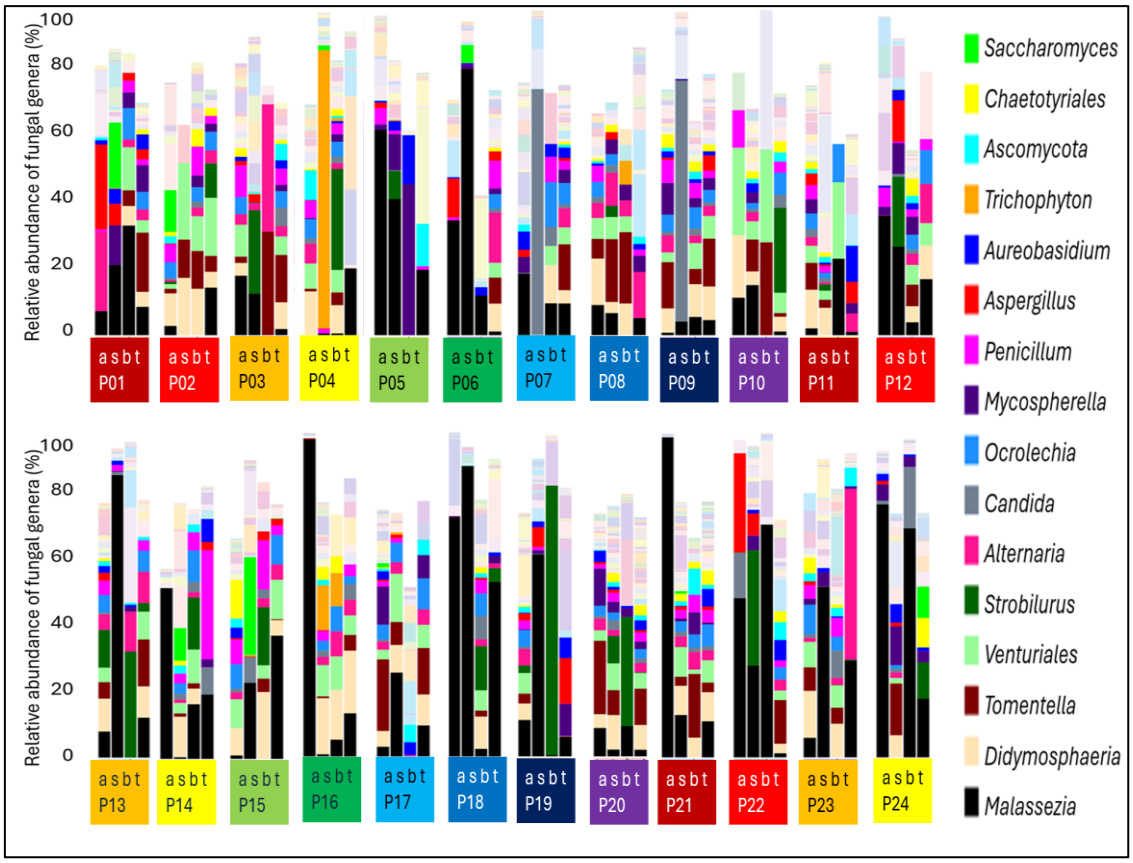


Figure 11. Stacked bar plot of fungal genus abundance for 4 sample types from each individual: a-aneurysm, b-blood, s-stool, t-thrombus (42)

Comparing the fungal microbiome composition of healthy and aneurysmal vessel walls, the glaring discrepancy was detected in both alpha diversity and beta diversity values. The Chao1 alpha diversity values presented in **Figure 12 A** showed that the value of the aneurysmal vessel wall mycobiome is significantly lower than the value of the healthy vessel wall mycobiome ($p \leq 0.001$). In **Figure 12 B** Jaccard beta diversity PCoA presents that healthy vessel wall samples and aneurysm samples are located into significantly separate groups (p=0.001).

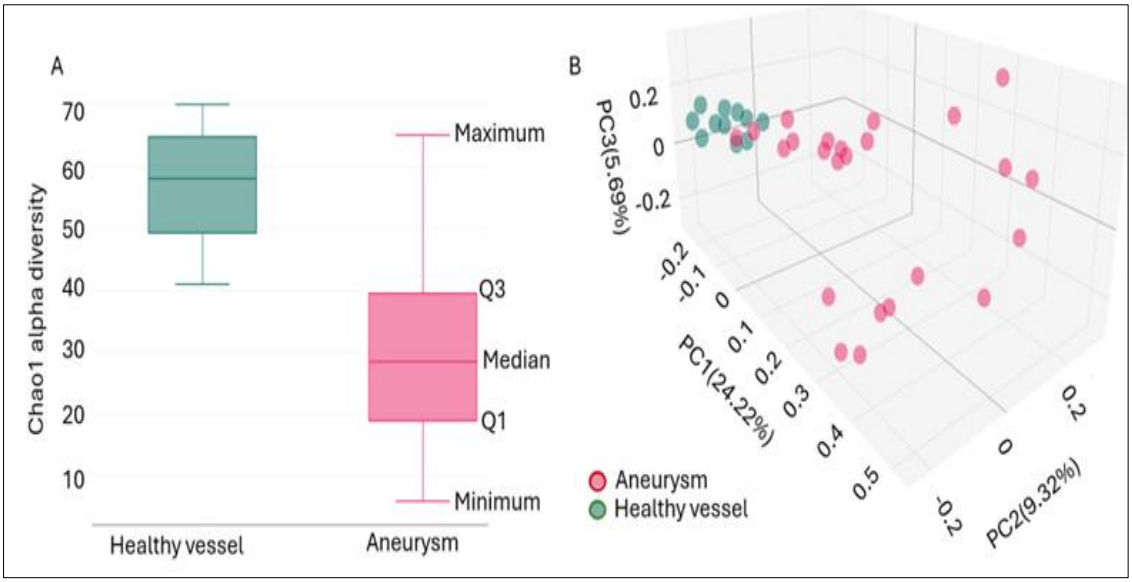


Figure 12. Mycobiome Chao1 alpha diversity **(A)** and Jaccard beta diversity PCoA **(B)** of healthy blood vessel wall and aneurysm wall samples: Boxplot displaying the data distribution with minimum, first quartile (Q1), median, third quartile (Q3), and maximum values, illustrating central tendency and variability, PC: principal coordinate (42)

The genera *Tomentella* (20.94%) and *Didymosphaeria* (13.36%) show up in high significant abundance in the control vessel wall as opposed to the pathological vascular tissue, which are predominantly characterized by the *Malassezia* genus (28.9%). A significant difference in the amount of *Venturiales* genus can also be detected in favor of the aneurysmal sample **(Table 1)**.

Table 1 Median abundance and significance values (marked with *) of the most common fungal genera in healthy and aneurysmal vessel wall, listed in descending order from highest frequency in the given sample type (42)

Genera	**Healthy vessel wall**	**Aneurysma wall**	***p* value**
Tomentella	20.94%	3.86%	0.01*
Didymosphaeria	13.36%	4.07%	0.09
Malassezia	1.92%	28.9%	0.01*
Venturiales	2.09%	6.37%	0.01*
Penicillium	3.55%	5.00%	0.44
Aspergillus	1.19%	3.64%	0.38
Alternaria	1.37%	2.34%	0.51

The mycobiome pattern of aneurysm wall samples can be classified into three different groups based on the quantity of *Malassezia* (**Figure 13**). In connection with the high prevalence of the genus *Malassezia* in the aneurysm sample, *Tomentella* and *Didymosphaeria* genera are not detected in the samples. The decrease in the frequency of the *Malassezia* genus correlates with the increase in the occurrence of the other two genera. However, even if the genus *Malassezia* is absent from the aneurysm mycobiome, *Tomentella* (7.8%) and *Didymosphaeria* (9.3%) still appear at a lower frequency compared to the healthy vessel wall (20.94% and 13.36%, respectively).

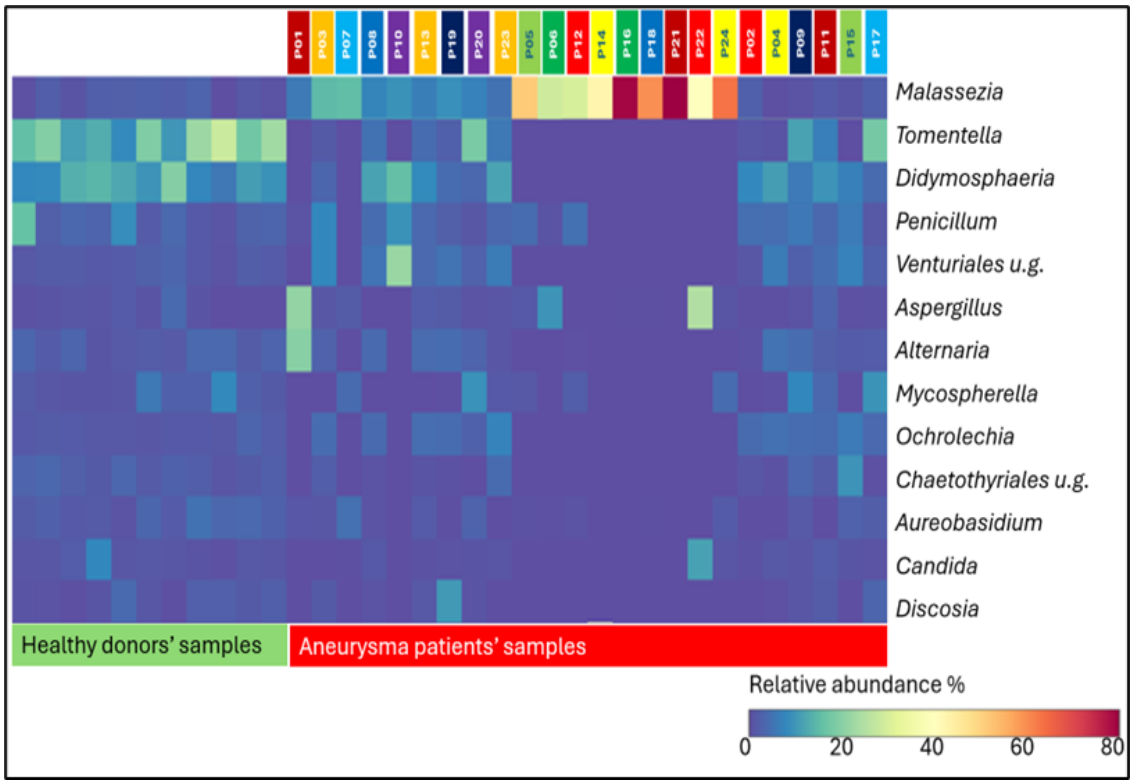


Figure 13. Heatmap representation of healthy and aortic aneurysm mycobiome composition (42)

Malassezia genus, which is barely detectable in the healthy vessel wall, was detected in the aneurysm mycobiome with an abundance of up to 80%. However, the frequency of the *Malassezia* genus, when examined individually, showed no correlation between the different sample types. The data presents a trend in **Figure 14** shows that patients with vascular walls with high *Malassezia* prevalence also more often have high amounts of the fungus in their stools, but there are exceptions. It cannot be verified that the *Malassezia* genus located in the aneurysm wall was transferred from the stool to the aneurysm wall via the bloodstream.

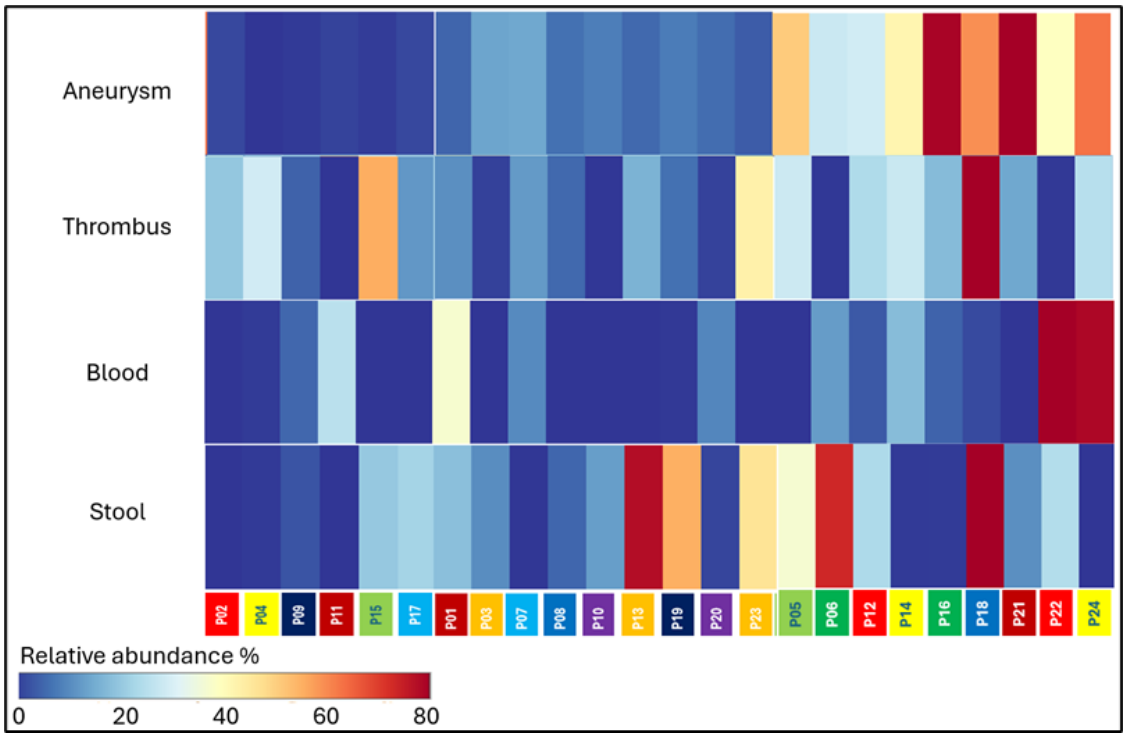


Figure 14. Heatmap about the *Malassezia* genus relative abundance in the aneurysm, thrombus, blood and stool in individual patients (42)

Data Availability

The datasets generated and analyzed during the current ITS study are available in the SRA repository: SRA/PRJNA1139113/www. ncbi. nlm. nih. gov (accessed on 22 October 2024).

5. Discussion

As far as we are aware, ours is the inaugural report of microbiome detection in the AAA vessel wall and its associated thrombus. By applying our established 16S rRNA sequencing protocol, which has proven effective in prior studies, we achieved robust microbiome profiling, with high read counts across all samples examined, from both blood and vessel walls (27, 43). As a previous similar research Nakayama et al. used reverse transcription-quantitative polymerase chain reaction to detect for DNA from 11 enteral bacterial species in aneurysm walls and peripheral blood from 30 patients with abdominal aortic aneurysm. These taxa were identified in 11 aneurysm samples and 19 blood samples. Confirmation of intestinal bacteria thereby demonstrated their translocation from the gut, through the bloodstream, to the vessel wall (23).

Studies utilizing diverse PCR techniques to identify odontopathogenic bacteria in AAA walls detected these microbes in proportions ranging from 0% to 86% (24-26).

In our prior research, we demonstrated that bacterial DNA is detectable in healthy vessel walls, originating from either the oral cavity, such as *Porphyromonas*, *Prevotella*, *Corynebacterium* or the gut in microbiome, namely *Akkermansia*, *Escherichia*, *Enterobacter*, *Ruminococcus* (27). In this study, we investigated the origins of bacterial taxa in AAA vessel walls, which differ from those in healthy vessel walls. Phylum-level taxon abundances in healthy versus AAA vessel walls revealed a shift characteristic of the faecal microbiome in AAA patients, as reported in a prior study (11), with an increased amount of *Proteobacteria* and a decrease in the abundance of *Bacteroidetes* and *Firmicutes*. Genera contributing to the increased abundance of *Proteobacteria* may also originate from the gut microbiome, such as *Escherichia* or *Escherichia-Shigella*. In the patients examined, these bacteria can reach the thrombus and vessel wall via the bloodstream following initial gut dysbiosis and bacterial translocation. Tian and colleagues' finding of *Proteobacteria* dominance in feces was confirmed in only 7 of the 21 patients examined. In the remaining patients, the dominance order aligned with Ito et al.: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (10). Among faecal microbiome bacteria in human samples, protective effects against AAA are attributed to *Bifidobacterium adolescentis* (10) and *Roseburia intestinalis* (11); in mouse models, such effects have been confirmed for *Akkermansia muciniphila* (16, 19). Among the previously

mentioned protective intestinal commensals associated with AAA, *A. muciniphila* was detected in the stool sample of only one of our patients, with a frequency of >1%. *Campylobacter gracilis*, a pathobiont (44), appeared in the stool samples of our patients with variable frequency (0.1–5.5%). In an animal model, a specific pathological subtype of *Faecalibacterium prausnitzii* was shown to promote AAA pathogenesis; strains of this species were present in our patients' faecal samples at 0.5–6.2% abundance (19). LEfSe analysis of bacterial translocation pathways to the thrombus or aneurysm wall revealed that genera highly abundant in feces like as *Finegoldia*, *Peptoniphilus*, *Bacteroides*, *Anaerococcus*, *Prevotella* did not appear in blood. Blood-associated *Enhydrobacter* and *Cutibacterium* genera are unlikely to derive from feces; the skin may serve as their entry portal (45, 46). Subsequently, these bacteria are transferred from the bloodstream to the thrombus and, to a lesser extent, to the aneurysm wall. However, the blood sample collected on the day of surgery did not contain the characteristic bacterial genera associated with the thrombus or aneurysm wall, namely *Sphingobium*, *Acinetobacter*, and *Aquabacterium*. We hypothesize that these bacteria may have been present earlier during the processes of aneurysm and thrombus formation, rather than originating from the bloodstream on the day of surgery. *Sphingobium*, *Acinetobacter*, and *Aquabacterium* are ubiquitously distributed across various environmental matrices, such as soil and water, and are common inhabitants of animal intestinal tracts (47, 48). Artificial products created by humans are not invariably associated with human-derived microbiomes; instead, they develop their own unique microbiomes, likely shaped by intense selective pressures imposed by the human environment (49). Microbiome analyses of everyday objects routinely subjected to cleaning, encompassing machines, coffee makers, dishwashers, saunas, refrigerators, and air conditioning systems, reveal substantial proportions of *Acinetobacter*, *Sphingobium*, *Aquabacterium*, and *Enhydrobacter* (50). It is unsurprising that these bacteria have been identified across various human body sites (including skin, oral cavity, ocular surface, duodenum, and blood) and implicated in diverse diseases (51-54). Patients with rheumatoid arthritis (RA) exhibit a heightened risk of aortic aneurysm development relative to the general population (55). Notably, our AAA-associated microbiome findings align with evidence that *Sphingobium* and *Aquabacterium* in periodontitis patients' saliva, along with host inflammatory mediator levels, serve as predictive biomarkers for RA (56).

In addition to bacterial DNA identified via 16S rRNA sequencing, the analyzed samples contained or had previously contained bacteria-specific enzymes. These enzymes exhibit activities that distinguish healthy vessel walls from aneurysms and act as effectors in various bacterial metabolic processes. The enzyme repertoire of bacteria presented in the healthy control vessel wall contains significantly more iron-chelate-transporting ATPase, polar-amino-acid-transporting ATPase, monosaccharide-transporting ATPase, N-acetylmuramoyl-L-alanine amidase, and non-specific serine/threonine protein kinase, in contrast to the aneurysm microbiome enzyme set. Such activities can provide protection against aneurysm development. However, the pathological vascular wall contains in significantly larger quantities glutathione transferase, NADH:ubiquinone reductase, acetyl-CoA C-acetyltransferase, peptidylprolyl isomerase, and enoyl-CoA hydratase in contrast with the healthy vessel wall. Such activities can promote aneurysm development. Local iron accumulation in the aortic wall contributes to AAA pathogenesis by promoting oxidative stress and inflammation (57). The complex process, in which activated T lymphocytes promote macrophage iron accumulation, lipid peroxidation, and migration to drive AAA formation, was inhibited in a mouse model using the iron chelator deferoxamine mesylate (58). Elevated iron-chelate transporting ATPase activity by bacteria in the healthy vessel wall may exert a protective effect against AAA formation analogous to that observed elsewhere. Plasma metabolomic analysis in AAA patients detected altered metabolism of key amino acids—alanine, aspartate, glutamate, arginine, and proline. While their precise role in AAA pathology is unknown, their concentrations correlated with aneurysm size (59). The polar-amino-acid-transporting ATPase enzyme, present in bacteria of healthy vessel walls, interacts with an extracytoplasmic substrate-binding protein to facilitate polar amino acid import, thereby conferring protection against AAA progression (60). AAA patients exhibit elevated peptidyl-prolyl isomerase (PPI) expression in vascular tissues, predominantly in smooth muscle cells (SMCs). Experiments in mice demonstrate that PPI deficiency reduces angiotensin II-induced abdominal aortic aneurysm formation (61) Human PPI PIN1 serves as a key regulator of smooth muscle cell (SMC) dysfunction and elastin degradation during AAA formation. Elevated bacterial PPI enzyme activity in aneurysmal vessel walls may exacerbate AAA pathogenesis.

Besides bacteria, fungi form integral elements of the human microbiome; the mycobiome is increasingly recognized as a key constituent of the human microbial ecosystem,

exerting substantial influence on health and disease states alike. However, mycobiome research is still in its infancy, and thus our knowledge in this area remains limited. Initially, it was essential to sequence the genomes of cultured fungi and establish databases, enabling the assignment of next-generation sequencing (NGS) metagenomic results to accurately identified fungal taxa. Currently, NGS-based amplicon sequencing targeting the ITS region represents the optimal method for assessing fungal diversity in microbial communities (62). Accurately resolving the mycobiome's community composition continues to pose significant bioinformatic challenges. Reliable identification of fungal taxa in the mycobiome is limited to the genus level at present. Moreover, current scientific understanding of gene sequences for fungi inhabiting the human body remains incomplete. Consequently, sequence identification per sample achieved only partial success (50–100%), with certain sequences remaining unidentified or incorrectly assigned (e.g., as mushrooms) due to the closest matches in existing databases.

The pronounced differences in alpha and beta diversity among stool, blood, thrombus, and vascular wall samples highlight the compartmentalization of fungal communities across body sites. This observation is consistent with previous studies showing distinct fungal assemblages in the gut, oral cavity, respiratory tract, skin, and urinary tract, driven by site-specific microenvironments and selective pressures (63). In obesity, a recognized risk factor for AAA (30), as well as in cohorts of patients with AAA, several studies have documented disease-associated alterations in the faecal mycobiome. Research indicates that *Candida* species are frequently enriched within the gut mycobiome of AAA patients, whereas the levels of beneficial yeasts like *Saccharomyces cerevisiae* are diminished (33, 64). Animal model studies have demonstrated the therapeutic promise of *S. cerevisiae* supplementation, which attenuated aneurysm progression by enhancing intestinal barrier function and mitigating systemic inflammation (33). In our AAA patient cohort, elevated *Candida* abundance was observed in stool samples from several individuals, with *Saccharomyces* detectable in only 3 of 24 cases. Nevertheless, the faecal mycobiome was predominantly characterized by the genus *Malassezia* across most patients. The precise mechanisms underlying fungal contributions to AAA pathogenesis remain hypothetical, potentially encompassing immune modulation, enzymatic effects, or interplay with bacterial communities. For example, *Candida* species are recognized for inducing inflammation via T-helper 17 cell responses, which may aggravate vascular damage (65,

66). While most fungi do not autonomously synthesize butyrate, they engage in symbiotic interactions with gut bacteria that modulate the production of short-chain fatty acids (SCFAs), including butyrate. Beneficial fungi, such as *Saccharomyces cerevisiae*, can elevate butyrate concentrations, thereby indirectly promoting intestinal homeostasis and potentially attenuating aneurysm progression (33). Our findings, which reveal elevated levels of *Candida* coupled with diminished abundance of *Saccharomyces* in faecal samples, corroborate the hypothesis that fungal dysbiosis contributes to AAA pathogenesis via intricate host-microbe interactions. In accordance with the principle that alterations in faecal bacterial composition can be partially reflected in the blood, thrombus, and AAA vessel wall microbiome (41), we hypothesized the presence of faecal mycobiome constituents in the blood and vessel wall. The mycobiome profiles across sample types (gut, blood, thrombus, and AAA vessel wall) from all examined individuals showed no significant differences in fungal genus abundances, with no specific fungal taxon exhibiting elevated prevalence in any particular sample type. Individual-level analyses of these mycobiome compositions revealed inconsistent abundances across personal samples. Translocation of fungal genera from feces via the bloodstream to the thrombus or AAA vessel wall could not be substantiated. Additional investigations are warranted to elucidate whether the mycobiome in the aneurysmal wall derives from the oral cavity, urinary tract, or skin. Numerous studies have explored the pivotal role of the human mycobiome in health and disease within the gut, oral cavity, urinary tract, and skin (67), however, the existence of a mycobiome in human blood or vascular walls as a constituent of the normal microbiome remains poorly documented and acknowledged. We utilized preserved DNA samples, previously isolated from the femoral arteries of healthy organ donors in our prior research (27), to investigate the composition of the healthy mycobiome within the vessel wall. Our investigation uncovered marked disparities in mycobiome composition between healthy and aneurysmal vessel walls, as indicated by divergences in alpha and beta diversity measures. The genera *Tomentella* and *Didymosphaeria* predominated in the healthy vascular mycobiome, exhibiting mean abundances of 20.94% and 13.36%, respectively. Conversely, the aneurysmal mycobiome was dominated by *Malassezia*, comprising 28.9% of its profile. The *Venturiales* genus appeared in both healthy (2.09%) and AAA (6.37%) samples at low median levels, yet showed statistically significant differences between groups. Although *Venturiales* are predominantly recognized for plant-pathogenic species, such as those in the genus *Venturia* that inflict substantial damage on fruit crops, certain taxa within this

order also pose risks to human health (68). Although uncommon, infections by melanized fungi within *Venturiales* can prove severe, spanning from mild cutaneous conditions to systemic or disseminated disease, especially in immunocompromised patients (69, 70). *Tomentella*, a genus in the fungal kingdom, is chiefly encountered in soil and decaying organic substrates. Recent investigations, however, have detected *Tomentella* within human microbiomes, including the oral cavity, gastrointestinal tract, and brain tumor tissues (71-73). Comprehensive investigations into *Tomentella*'s role in human health remain scarce, with its precise functions and interactions in the human body largely unexplored. Existing findings on its beneficial or pathogenic potential are inconsistent; for instance, elevated *Tomentella* abundance has been documented in the faecal microbiota of patients with RA and undifferentiated connective tissue disease, as well as in blood and tumor tissues from individuals with brain tumors. In contrast, animal models indicate that *Tomentella* ranks among the few fungal genera capable of producing butyrate and acetate—SCFAs essential for intestinal health and immune modulation (74, 75). The genus *Didymosphaeria* is predominantly linked to plants, manifesting as a saprotroph, endophyte, or pathogen. Despite increasing focus on fungal diversity in human and animal microbiomes, no reports exist of specific *Didymosphaeria* infections in mammals. Scientific literature has documented increased levels of *Didymosphaeria* in the mammalian mycobiome, notably within the gut microbiome of bats and human brain tumors (73, 76). The genus *Malassezia* represents a dominant element of the human mycobiome, especially on the skin, where it constitutes the primary fungal colonizer. It occurs in nearly every individual and exerts a multifaceted influence on dermal health, occasionally promoting disorders such as atopic dermatitis through antigenic sensitization (77). The *Malassezia* genus is acknowledged as a key component in both the oral mycobiome and the faecal mycobiome (71). Elevated intestinal levels of *Malassezia* have been associated with various pathological states, including inflammatory bowel disease, colorectal cancer, and pancreatic cancer (78). *Malassezia* species have been linked to intestinal inflammation and exhibit heightened abundance in patients with AAA, indicating that their potential modulation of systemic inflammation may indirectly compromise vascular integrity (33). There is no direct evidence associating *Malassezia* with mycotic aneurysms, which are predominantly caused by fungal pathogens such as *Aspergillus*, *Candida*, *Mucor*, or *Penicillium* that invade vascular walls. These infections typically arise in the context of systemic illnesses and immunosuppression (79, 80). In certain AAA vessel wall samples from our study, the genus *Malassezia* exhibited

remarkably high abundance, with its levels rising in inverse correlation to those of *Tomentella* and *Didymosphaeria*. We cannot confirm or rule out a gut-blood-vascular wall mycobiome axis or the translocation of *Malassezia* or *Tomentella* from the intestine to the vascular wall. Instead, the primary colonization sites for these fungal taxa might involve other human body regions, including the nasal or oral cavity, urinary tract, genitals, or skin. Nonetheless, our mycobiome abundance profiles in pathological versus healthy vascular walls align with prior findings highlighting *Tomentella*'s anti-inflammatory effects and *Malassezia*'s pro-inflammatory characteristics.

A key limitation of this study lies in the incomplete and erroneous identification of sequences, likely attributable to the inadequacy of current databases in comprehensively detecting all human-associated fungal DNA. Abdominal aortic aneurysm primarily impacts older males, whereas the arterial tissue samples employed as healthy negative controls were derived from young, multiorgan-eligible donors. As a result, the study cohort exhibited limited age diversity and substantial disparities from the healthy arterial wall microbiome control group. Observed variations in mycobiome composition may partly stem from the age and health status differences between the two groups. Procuring age-matched healthy aortic tissue remains highly challenging owing to ethical and logistical barriers, as such samples are seldom accessible outside of organ donors or autopsies—who typically diverge in age from aneurysm patients. This constraint is prevalent in aortic disease investigations and has been acknowledged in prior research (81, 82). Stool and blood samples suitable for microbiome analysis from arterial wall-negative controls were not obtainable. Additionally, the study omitted evaluation of comorbidities, ongoing medication use, or other clinical variables in AAA patients. Analysis of these elements in an expanded cohort could deliver sharper and more reliable understanding of disease-mycobiome links across compartments.

6. Conclusion

This PhD thesis firstly investigated the bacterial and fungal microbiome in patients with AAA, sampling the aneurysm wall, blood, thrombus and stool.

1. We confirmed a significant difference between the bacterial composition of the aneurysm wall and healthy vessel wall. Bacteria genera most characteristic of aneurysms were *Acinetobacter*, *Aquabacterium,* and *Sphingobium* species.

2. The AAA specific species could originate from the environment via the skin or oral cavity rather than being primarily derived from a dysbiotic gut microbiome.

3. Furthermore, the study found that bacterial enzyme activity in the aneurysm wall promotes AAA progression, while activity in the healthy vessel wall can inhibit it.

4. This analysis of fungal communities in four distinct anatomical locations (feces, blood, thrombus, and aneurysm wall) in AAA patients could not confirm a direct gut-blood-vessel wall axis for fungal translocation. Fungal genera identified in the vessel wall may have migrated from sources other than the gut.

5. Regarding the fungal microbiome, we also demonstrated a significant difference between the fungal composition of the aneurysm wall and the healthy vessel wall. A comparison of healthy and aneurysmal vessel walls revealed a higher abundance of the genus Tomentella, Didymosphera in healthy tissue, while Malassezia and Venturiales were more prevalent in aneurysms.

6. The results suggest a possible association between *Tomentella* and *Malassezia* and aneurysm progression, with *Malassezia* being pro-inflammatory and *Tomentella* being anti-inflammatory.

7. Summary

Abdominal aortic aneurysm is a life-threatening vascular condition characterized by pathological dilation and inflammatory degeneration of the vascular wall. Several studies have demonstrated that, besides established risk factors including smoking, hypertension, obesity, and cardiovascular disease, microbial factors also play a significant role in the pathogenesis and progression of AAA. Previous research has confirmed that there is a connection between the composition of the gut, but also the oral microbiome, and the development and progression of AAA. In this PhD thesis, we analysed the bacterial and fungal microbiome composition of stool, blood, thrombus and damaged vessel wall samples collected during surgery from AAA patients using bacterial 16S rRNA and fungal ITS sequencing. As a negative control for the blood vessel wall microbiome comparisons, we used human femoral arteries collected from healthy donors. The primary objective of our study was to investigate potential differences in the microbiome composition of aneurysmal versus healthy vessel walls, focusing on both bacterial and fungal communities. Secondly, to investigate whether the microorganisms detected in thrombus and aneurysm may originate from stool and be transported to these sites via the bloodstream. Thirdly, based on the compositional differences between healthy and aneurysmal microbiomes, it can be hypothesized whether microorganisms exert a direct effect or play an indirect role through their enzymatic activity in protection or pathogenicity. We demonstrated the glaring discrepancy in the bacterial and fungal microbiome composition between healthy and aneurysmal vascular walls. Upon individual analysis of the four sample types, we were unable to confirm or exclude the existence of the gut-vascular wall axis; however, it is hypothesized that the microbiome detected in the vascular wall may originate from anatomical sites other than the gut, such as the oral cavity, skin, or urinary tract. Examination of the enzymatic activities of bacteria within the microbiome revealed that iron chelate-transporting ATPase and polar amino acid-transporting ATPase exert protective effects in the healthy vascular wall. Furthermore, bacterial peptidylprolyl isomerase activity detected in aneurysmal tissue contributes to the exacerbation of aneurysm development. By examining the differences in the fungal genera present in the microbiome in aneurysmal and healthy vessel walls, we also supported the pro-inflammatory effect of *Malassezia* and the anti-inflammatory effect of *Tomentella*.

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9. Bibliography of the candidate's publications

1. Publications related of the topic of the thesis

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2. Further publications

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