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Interaction of Gut Microbiota-Derived Metabolites With Inflammatory Markers and Cellular Rejection in Patients With Advanced Heart Failure Treated With Heart Transplantation

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Gut microbiota–derived metabolites (GMDMs) are involved in the pathophysiology of heart failure (HF) and may play an important role in patients after orthotopic heart transplantation (Htx). This study aimed to evaluate key GMDMs and their relationship with inflammation and the occurrence of acute cellular rejection (ACR) in patients undergoing Htx.

Material/Methods: This was an observational study that enrolled consecutive patients qualified for Htx between June 2022 and November 2023. The study included 15 healthy volunteers, 20 patients undergoing non-Htx cardiac surgery, and 32 consecutive patients undergoing Htx. The GMDMs trimethylamine oxide (TMAO), indoxyl sulfate, and p-cresol sulfate were evaluated in the serum before the procedure and at 1, 3, and 6 months after procedure in the study and control groups. ACR was diagnosed using endomyocardial biopsies.

Results: The study group consisted of 32 patients qualified for Htx (78% male, 52 ± 11 years old). Baseline levels of TMAO (0.77 [0.52-1.45] ug/mL) and p-cresol sulfate (9.90 [4.03-18.21] ug/mL) were significantly higher than in the healthy control group (0.28 [0.15-0.51] ug/mL and 4.45 [0.79-8.35] ug/mL, respectively; all $P < 0.05$). Significant correlations were found between TMAO and endothelin (ET-1), as well as with procalcitonin, at 1 and 6 months after Htx. The study group did not show significant changes in metabolites in the follow-up period. Despite GMDMs being higher in the ACR group, the differences did not reach statistical significance.

Conclusions: TMAO and p-cresol sulfate were increased in patients with advanced HF. Moreover, TMAO correlated with ET-1 and procalcitonin; however, there was no association between GMDMs and ACR incidence.
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Introduction

The prevalence of heart failure (HF) is increasing, and approximately 10% of patients diagnosed with HF may develop advanced HF with the need for orthotopic heart transplantation (Htx) or mechanical circulatory support [1-4]. Previous studies have shown the important involvement of the gut microbiota in the development of cardiovascular diseases, including HF [5-7]. Disturbances of the homeostasis of the gastrointestinal microbiota, together with intestinal barrier dysfunction, lead to the translocation of bacteria and their metabolites to the circulation. Bacterial endotoxemia, which aggravates inflammation and oxidative stress, also affects the bioavailability of drugs, including immunosuppressive therapy [8].

The first of the active metabolites is trimethylamine oxide (TMAO), which is converted from trimethylamine (TMA) [9]. Previous studies have shown that its increased concentrations are associated with a higher risk of myocardial infarction, stroke, and HF [10]. Hence, TMAO was suggested as a potential therapeutic target in patients with HF [11]. Other examples of gut microbiota–derived metabolites affecting the cardiovascular system are uremic toxins, namely indoxyl sulfate and p-cresol sulfate. Indoxyl sulfate appears in the liver as a product of processing indol as a result of tryptophan degradation by the gut microbiota [12]. Similarly, p-cresol sulfate is derived from tyrosine by the gut microbiota and converted to p-cresyl sulfate by liver sulfotransferase [13]. These substances, through pro-inflammatory and pro-atherosclerotic mechanisms, are associated with a higher risk of cardiovascular mortality in patients with kidney dysfunction [14]. Finally, cluster of differentiation 14 (CD14) and endothelin-1 (ET-1) are additional biomarkers involved in the vascular-immune axis, which are not widely used in practice [15-17]. More importantly to HF, however, CD14 and ET-1 may be evaluated in terms of the role of GMDMs in inflammation, with their involvement suggested in myocardial dysfunction [15-18]. The multifaceted role of CD14 includes the recognition of bacterial lipopolysaccharides, maintenance of intestinal integrity, and release of pro-inflammatory cytokines [16]. Cytokines stimulate the endothelium to produce ET-1, which affects vasoconstriction and vascular integrity [17]. Importantly, these cytokines are also released during acute cellular rejection (ACR) [15]. ACR is the most common cause of graft dysfunction of the transplanted organ, occurring in the first months after transplantation [19]. Immunoinflammatory activation caused by dysbiosis may affect the process of rejection of the transplanted heart [20].

More generally, the importance of GMDMs, including TMA/TMAO, indoxyl sulfate, and p-cresol sulfate, in patients undergoing Htx is not well understood. To date, only the carnitine-γ-butyrobetaine-TMAO pathway has been evaluated in a study regarding ACR after Htx [21]. Problematically, solely an increase in carnitine-γ and butyrobetaine was associated with a higher incidence of ACR. Because the results of the aforementioned study were not confirmatory, we assessed the TMAO's role in a different cohort. The primary question of this study was to evaluate whether baseline GMDMs levels differ between patients with advanced HF requiring Htx and controls. The secondary aim was to characterize longitudinal metabolite changes after Htx. The exploratory aims were to assess the association between GMDMs and inflammatory, vascular-immune activation biomarkers after Htx and the association between GMDM levels and the occurrence of ACR. Metabolite levels were measured before and after transplantation at predetermined follow-up time points, while the ACR was assessed based on endomyocardial biopsies.

Material and Methods

The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of Wrocław Medical University (KB 678/2022, 03.10.2026).

Study Population

Patients with advanced HF, defined according to the European Society of Cardiology Guidelines 2021, who were eligible for Htx, ≥18 years old, and consented to participate in the study were included [2]. There were 2 control groups: first, healthy volunteers who underwent assessment of GMDMs at baseline; and second, patients undergoing cardiac surgery with extracorporeal circulation for indications other than Htx who underwent assessment of GMDMs at 4 timepoints: at baseline and at 1, 3, and 6 months after the procedure. The objective of this analysis was to evaluate differences in baseline GMDMs between healthy controls, the non-Htx cardiac surgery group, and the study group. GMDM measurements at 1, 3, and 6 months after the procedure were compared between the study group and non-Htx cardiac surgery group to account for the effect of extracorporeal circulation on metabolite levels.

Study Procedures

For the study group, serum samples were tested for TMAO, indoxyl sulfate, and p-cresol sulfate, along with the systemic inflammatory markers C-reactive protein (CRP), leukocytes (WBC), procalcitonin and markers of vascular-immune activation, CD14 and ET-1. Metabolites and the aforementioned markers were acquired 4 times in the study group: at baseline (pre-Htx), and 1, 3, and 6 months after Htx (**Figure 1**). According to the local guidelines, there were approximately 9 endomyocardial biopsies within 6 months after Htx. Biopsies were performed as routine post-transplant clinical surveillance and scheduled 1, 2, 3, 4, 6, and 8 weeks and 3, 4, and 6 months after Htx.

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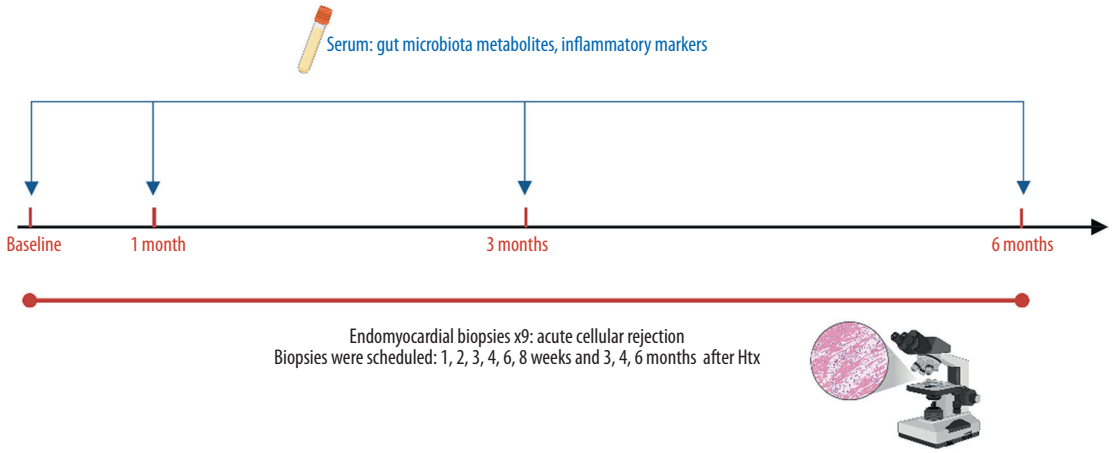


Figure 1. Study design. Samples were tested for trimethylamine oxide (TMAO), indoxyl sulfate, and p-cresol sulfate, along with systemic inflammatory markers: C-reactive protein (CRP), leukocytes (WBC), procalcitonin (Pct), and markers of vascular-immune activation: CD14 and ET-1 four times in the study group: baseline (pre-Htx), and 1, 3, and 6 months after heart transplantation (Htx). Within 6 months after Htx, 9 endomyocardial biopsies were scheduled.

The occurrence of ACR in biopsies was determined based on the International Society for Heart and Lung Transplantation classification (no rejection, grade 0R; mild rejection, grade 1R; moderate rejection, grade 2R; severe rejection, grade 3R) from 2005, and grades 2R and 3R were treated as rejection [22]. To assess the relationship of GMDMs with subsequent clinical outcomes, metabolite concentrations were measured at the beginning of each observational interval (baseline-1 month, 1-3 months, 3-6 months). Delayed biopsies were classified in the corresponding observational interval according to the actual date on which the biopsy was performed. Deaths, delayed or no endomyocardial biopsies available, and missing measurements censored observations and reduced the number of patients evaluated in the prespecified time intervals regarding ACR. We conducted exploratory temporal association testing to explore whether metabolite levels at the beginning of each interval differed depending on subsequent ACR occurrence.

Laboratory Tests

Blood was taken in heparinized tubes and processed to obtain plasma by spinning at 1500 × g for 10 minutes. Plasma was frozen at -80 °C until analysis was performed. The measurement of TMAO, indoxyl sulfate, and p-cresol sulfate concentrations was performed using the liquid chromatography–mass spectrometry (LC-MS) method developed in the Laboratory of Elemental Analysis and Structural Research of Wrocław Medical University [23,24]. Reagents were as follows: reference standards: TMAO (Merck, USA), p-cresol sulfate potassium salt (Cayman Chemical Company, USA), indoxyl sulfate potassium salt (Sigma-Aldrich, USA), labeled internal standards d9-TMAO (Cambridge Isotope Laboratories, USA), indoxyl sulfate potassium salt ($^{13}C_6$, 99%) CP 95% (Cambridge Isotope Laboratories, USA), *p*-Cresol sulfate potassium salt (D7, 98%; Cambridge Isotope Laboratories, USA). Eluents and additives to chromatography phases were as follows: acetonitrile hypergrade for LC-MS LiChrosolv (Merk, Germany), water hypergrade for LC-MS LiChrosolv (Merk, Germany), and ammonium bicarbonate (Sigma-Aldrich, USA). Analytical chromatography was performed using an ACQUITY Ultra Performance Liquid Chromatography (UPLC) Bridged Ethyl Hybrid (BEH) Amide column (130Å, 1.7 µm, 2.1 × 100 mm) coupled with an ACQUITY UPLC BEH Amide VanGuard precolumn (130Å, 1.7 µm, 2.1 × 5 mm). The chromatographic system utilized was the Dionex Ultimate 3000 (Thermo Scientific, USA), equipped with a cooled autosampler and column oven set at 45 °C and 4 °C, respectively. Mass spectrometric analysis was conducted using a Compact Quadrupole-TOF MS (Bruker Daltonics, Germany) with an electrospray ionization source. Data collection was performed using HyStar software version 3.2 (Bruker Daltonics, Germany). Spectra of compounds were analyzed using DataAnalysis software version 4.2 (Bruker Daltonics, Germany). The quantitative analysis was performed using QuantAnalysis version 2.2 (Bruker Daltonics, Germany).

Sample Preparation

Heparinized plasma samples were defrosted, centrifuged at 1500 × g for 10 minutes, and 100 µL of each sample was aliquoted into Eppendorf tubes. Subsequently, 400 µL of ice-cold methanol containing internal standard mix (d9-TMAO, $^{13}C_6$-indoxyl sulfate and d7-cresol sulfate, 1 mg/mL) was added. The samples were vortexed at 4 °C for 5 minutes and incubated at 4 °C for 30 minutes to precipitate proteins. Following incubation, samples were centrifuged at 14 000 × g for 10 minutes,

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and supernatants were transferred to clean tubes. Each supernatant was analyzed in 2 separate LC-MS injections using different chromatographic columns: one for TMAO and the other for indoxyl sulfate and p-cresol sulfate.

Calibration Curve

A standard stock solution of TMAO, indoxyl sulfate, and p-cresol sulfate was prepared in methanol at a concentration of 1 mg/mL. A mixed standard solution was made by pooling 100 µL of each individual stock solution, followed by serial dilutions. Calibration curves ranging from 0.1 to 100 µg/mL were constructed using charcoal-treated human plasma, following the same procedure as for the experimental samples.

Conditions of LC-MS/MS Analysis

TMAO was measured in positive ion mode as described previously [25]. Indoxyl sulfate and p-cresol sulfate were measured as follows: 5 µL of the sample was injected into the LC-MS system (Ultimate 3000 Dionex, Compact Quadrupole, TOF MS Bruker Daltonics). Analytical chromatography was conducted on ACQUITY UPLC BEH Amide (130 Å, 1.7 µm, 2.1 × 100 mm) with precolumn ACQUITY UPLC BEH Amide VanGuard (130 Å, 1.7 µm, 2.1 × 5 mm). The column and autosampler temperatures were set at 45 °C and 4 °C, respectively. Mass spectrometry (MS) analysis was conducted using the electrospray ion source operated in negative ion mode (ES-). The total run time of the method was 12 minutes with a flow rate of 400 µL min-1 and 95% (10 mM NH_4HCO_3, pH = 9): 5% acetonitrile was used as mobile phase A and 95% acetonitrile: 5% (10 mM NH_4HCO_3, pH = 9) as mobile phase B. The applied gradient was as follows: 99% B for 0 to 1 minutes, 99% to 30% B for 1 to 7 minutes, 30% to 99% B for 7 to 7.5 minutes, 99% B for 7.5 to 12 minutes. Data were acquired from m/z 70 to 1000 in auto MS/MS mode with collision energy in the range 25 to 35 eV. The spray voltage and the desolvation temperature were set at 4.5 kV and 210 °C, respectively. The pressure of nitrogen used as the nebulizer gas was set to 2.0 Bar. Drying gas flow rate was set at 8 L min-1.

Analytical Quality Control and Assay Performance

Analyte-specific stable-isotope-labeled internal standards were added before protein precipitation to control for variability during sample preparation and instrumental analysis. Matrix-matched calibration curves and quality-control samples were included in each analytical batch. The coefficients of determination (R^2) were 0.9997 for TMAO, 0.999957 for indoxyl sulfate, and 0.999651 for p-cresyl sulfate. The limits of detection, calculated as 3.3 σ/S, where σ represents the standard deviation of the response and S the slope of the calibration curve, were 0.0177 µg/mL (0.236 µM) for TMAO, 0.0217 µg/mL (0.102 µM) for indoxyl sulfate, and 0.0354 µg/mL (0.188 µM) for p-cresyl sulfate. The corresponding limits of quantification were 0.0532 µg/mL (0.708 µM), 0.15 µg/mL (0.704 µM), and 0.17 µg/mL (0.903 µM), respectively. The intra-assay and inter-assay coefficients of variation did not exceed 8% at the evaluated concentration levels, and analytical accuracy ranged from 95% to 105%. No clinically validated reference intervals for TMAO, indoxyl sulfate, or p-cresyl sulfate were applied in this study. Concentrations measured in the healthy control group were used as descriptive comparator values and should not be interpreted as clinical reference intervals.

Statistical Analysis

Categorical variables are presented as n (%). Continuous variables are described by mean with standard deviation or medians with lower and upper quartiles, depending on the distribution. The differences between study and both control groups were tested using the *t* test or Mann-Whitney, where appropriate. Variables measured at multiple time points were assessed using the Friedman ANOVA for repeated measures or Wilcoxon signed-rank tests for incomplete longlitudinal measurements. A value of $P < 0.05$ was considered statistically significant. Associations between variables were assessed using Pearson or Spearman's correlation coefficients, depending on data distribution. Correlations between GMDMs and markers of systemic inflammation (CRP, WBC, procalcitonin), as well as biomarkers of the immune- vascular axis (CD14, ET-1), were analyzed as 2 separate hypothesis families. Missing data were handled using pairwise deletion. Bonferroni correction was applied within each hypothesis family. Statistical analysis was performed using the STATISTICA 13 data analysis software system (StatSoft, Inc).

Results

Study Population

The study group consisted of 32 consecutive patients, 78% of whom were male, with advanced HF who were eligible for Htx. The control group undergoing non-Htx cardiac surgery consisted of 20 patients, 90% of whom were male. Baseline clinical characteristics with treatment and laboratory values are shown in **Table 1**. Patients from the study group were significantly younger (52 ± 11 years old vs 59 ± 10 years) with a lower mean BMI (26 ± 4 vs 30 ± 4). The study group less often had ischemic etiology of HF [9 (28%) vs 9 (45%)], whereas higher prevalence of atrial fibrillation was observed among patients undergoing Htx [20 (63%) vs 6 (30%)]. The Htx group had lower use of insulin [0 (0%) vs 3 (15%)], statins [16 (50%) vs 16 (80%)] and aspirin [7 (22%) vs 12 (60%)], while treatment with sodium-glucose cotransporter 2 inhibitors and antibiotics was more common

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Table 1. Patients' baseline characteristics.

Parameter	All patients (n = 52)	Htx (n = 32)	Non Htx (n = 20)	*P* value
Age, years (SD)	55 ± 11	52 ± 11	59 ± 10	**0.03**
BMI, kg/m^2 (SD)	27 ± 5	26 ± 4	30 ± 4	**0.005**
Sex, male, n (%)	43 (83)	25 (78)	18 (90)	0.27
Comorbidities				
Heart failure, n (%)	52 (100)	32 (100)	20 (100)	1.00
Ischemic HF etiology, n (%)	18 (35)	9 (28)	9 (45)	0.04
CABG/PCI, n (%)	15 (29)	10 (31)	5 (25)	0.63
Hypertension, n (%)	27 (52)	17 (53)	10 (50)	0.83
Hyperlipidemia, n (%)	26 (50)	15 (47)	11 (55)	0.57
Atrial fibrillation, n (%)	26 (50)	20 (63)	6 (30)	0.02
Stroke/TIA, n (%)	5 (10)	1 (3)	4 (20)	0.05
Asthma/COPD, n (%)	2 (4)	2 (6)	0 (0)	0.24
Diabetes mellitus, n (%)	15 (29)	11 (34)	4 (20)	0.31
Chronic kidney disease, n (%)	21 (40)	12 (38)	9 (45)	0.59
Thyroid disease, n (%)	13 (25)	10 (31)	3 (15)	0.19
Treatment at baseline				
ACEI/ARB/ARNI, n (%)	36 (69)	23 (72)	13 (65)	0.60
Loop diuretics, n (%)	42 (81)	28 (88)	14 (70)	0.12
Beta-blockers, n (%)	45 (87)	29 (91)	16 (80)	0.27
MRA, n (%)	34 (65)	23 (72)	11 (55)	0.21
SGLT-2, n (%)	36 (69)	26 (81)	10 (50)	**0.02**
Insulin, n (%)	3 (6)	0 (0)	3 (15)	**0.02**
Statin, n (%)	32 (62)	16 (50)	16 (80)	0.01
Aspirin, n (%)	19 (37)	7 (22)	12 (60)	0.005
Proton pump inhibitors, n (%)	30 (58)	20 (63)	10 (50)	0.41
Antibiotics, n (%)	13 (25)	11 (34)	2 (10)	**0.05**
Standard laboratory parameters at baseline				
White blood cells, k/µL(IQR)	7.55 (6.23-9.63)	8.11 (6.28-10.63)	6.81 (5.93-8.86)	0.29
C-reactive protein, mg/L (IQR)	5.97 (2.18-16.35)	6.15 (1.76-22.25)	4.47 (2.18-16.35)	0.87
Procalcitonine, ng/L (IQR)	0.06 (0.03-0.18)	0.06 (0.03-0.18)	0.05 (0.02-0.2)	0.66
Hemoglobin, g/dL (IQR)	13.2 (11.6-15.3)	13.2 (11.5-15.4)	12.65 (11.8-15.15)	0.58
Bilirubin, mg/dL (IQR)	0.95 (0.80-1.9)	1.25 (0.9-2.3)	0.8 (0.6-0.9)	**0.0004**

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Table 1 continued. Patients' baseline characteristics.

Parameter	All patients (n = 52)	Htx (n = 32)	Non Htx (n = 20)	*P* value
ALAT, IU/L (IQR)	29 (18-39)	28 (17-40)	32.5 (23-38.5)	0.39
Aspat, IU/L (IQR)	29 (22-46)	30 (22-48)	28.5 (22-37)	0.59
Albumin, g/dL (IQR)	4.0 (3.2-4.1)	4.05 (3.6-4.15)	3.8 (2.9-4.1)	0.48
Creatinine, mg/dL (IQR)	1.02 (0.83-1.42)	1.16 (0.88-1.45)	0.91 (0.79-1.39)	0.11
Sodium, mmol/L (IQR)	138 (137-141)	139 (137-142)	137.5 (137-139.5)	0.16
Potassium, mmol/L (IQR)	4.2 (3.9-4.5)	4.2 (3.8-4.6)	4.15 (3.9-4.4)	0.70
Troponin, ng/L (IQR)	33.6 (11.1-412.9)	54.4 (8.6-1723.5)	19.9 (11.4-109.1)	0.54
NTproBNP, pg/mL (IQR)	1942.3 (637-3408)	1558 (640-3806)	2371.1 (502.6-2576.5)	0.79
CD14, mg/mL (IQR)	0.79 (0.44-1.56)	1.11 (0.49-1.65)	0.77 (0.39-2.08)	0.38
ET-1, pq/mL (IQR)	2.00 (1.36-3.17)	2.25 (1.51-3.57)	1.98 (1.59-3.02)	0.85

Values are expressed as mean ± standard deviation (SD), median (interquartile range, IQR), or number (percentage,%).
Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ALAT, alanine aminotransferase; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor-neprilysin inhibitor; ASPAT, aspartate aminotransferase; BMI, body mass index; CABG, coronary artery bypass grafting; CD14, cluster of differentiation 14; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; ET-1, endothelin-1; HF, heart failure; Htx, orthotopic heart transplantation; IQR, interquartile range; MRA, mineralocorticoid receptor antagonist; *n*, number of patients; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PCI, percutaneous coronary intervention; PPI, proton pump inhibitors; SD, standard deviation; SGLT-2, sodium-glucose cotransporter 2 inhibitors; TIA, transient ischemic attack; WBC, white blood cells.

in this group [26 (81%) vs 10 (50%) and 11 (34%) vs 2 (10%), respectively]. Among baseline laboratory parameters, the Htx group had higher bilirubin levels [1.25 (0.9-2.3) vs 0.8 (0.6-0.9) mg/dL] compared with the non-Htx group. The non-Htx cardiac surgery control group was treated with left ventricular assist device implantation in 10 cases (50%), and the remaining underwent coronary artery bypass grafting with or without valve surgery. All procedures were performed with extracorporeal circulation. In addition, 15 healthy volunteers were recruited.

Baseline Levels of Gut Microbiota-Derived Metabolites in the Study Population

Baseline levels of GMDMs in the study group were as follows: TMAO 0.77 (0.52-1.45) ug/mL, indoxyl sulfate 1.29 (0.70-2.65) ug/mL, and p-cresol sulfate 9.90 (4.03-18.21) ug/mL. When compared with the healthy cohort, the study group was characterized by significantly higher levels of TMAO 0.28 (0.15-0.51) ug/mL ($P = 0.002$), p-cresol sulfate 4.45 (0.79-8.35) ug/mL ($P = 0.011$), but not of indoxyl sulfate 1.43 (0.89-1.54) ug/mL ($P = 0.586$). However, there were no significant differences with the control group undergoing non-Htx cardiac surgery (TMAO 0.54 [0.22-1.30] ug/mL, indoxyl sulfate 0.82 [0.41-1.49] ug/mL, p-cresol sulfate 6.02 [2.34-10.70] ug/mL; all $P > 0.05$; **Table 2**).

Changes of Gut Microbiota-Derived Metabolites Over the Follow-Up Period

There were no significant differences in GMDMs during the follow-up period (1, 3, and 6 months) in the group undergoing Htx and in the group undergoing other cardiac surgeries. During the follow-up period, data loss resulted from patients ultimately not undergoing Htx (n = 4), patient deaths (n = 4), loss to follow-up, or technical issues affecting the measurement of individual metabolites. Incomplete longitudinal follow-up in the control group precluded a corresponding complete-case repeated-measures analysis due to insufficient number of paired observations during the study period between the first and third month. Therefore, pairwise comparisons were performed using the Wilcoxon signed-rank test when sufficient paired

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Table 2. Changes in biomarker levels over the follow-up period in the study group.

Biomarker	Baseline (IQR) (n)	1 month (IQR) (n)	3 months (IQR) (n)	6 months (IQR) (n)	Friedman *P* value (n)
TMAO	0.77 [0.52-1.45] (30)	0.60 [0.34-1.07] (22)	0.78 [0.58-1.31] (18)	0.50 [0.36-0.91] (14)	0.85 (14)
IND	1.29 [0.70-2.65] (29)	1.27 [0.71-2.14] (22)	1.48 [0.88-2.06] (18)	1.47 [1.23-2.16] (14)	0.65 (13)
CRE	9.90 [4.03-18.21] (28)	5.49 [0.95-12.80] (22)	9.06 [0.59-13.19] (19)	7.79 [5.25-18.61] (14)	0.94 (12)

Continuous variables are presented as median (interquartile range [IQR]). Abbreviations: TMAO, trimethylamine N-oxide; IND, indoxyl sulfate; CRE, p-cresol sulfate; n, number of patients.

Table 3. Changes in biomarker levels over the follow-up period in the control group.

Biomarker	Baseline (IQR) (n)	1 month (IQR) (n)	3 months (IQR) (n)	6 months (IQR) (n)	Baseline vs 1 month *P* value (paired n)	3 months vs 6 months *P* value (paired n)
TMAO	0.54 [0.22-1.30] (19)	0.60 [0.36-2.26] (6)	0.62 [0.17-4.76](7)	0.48 [0.33-0.57] (5)	0.35 (6)	0.67 (5)
IND	0.82 [0.41-1.49] (19)	0.93 [0.67-2.25] (6)	0.52 [0.43-3.14] (7)	0.85 [0.45-3.68] (5)	0.75 (6)	0.22 (5)
CRE	6.02 [2.34-10.70] (19)	6.47 [1.24-10.42] (6)	8.81 [2.88-33.33] (7)	5.45 [5.38-17.46] (5)	0.60 (6)	0.50 (5)

Continuous variables are presented as median (interquartile range [IQR]). Abbreviations: TMAO, trimethylamine N-oxide; IND, indoxyl sulfate; CRE, p-cresol sulfate; n, number of patients.

observations were available. Changes in available biomarker levels measurements over follow-up for the study group are presented in **Table 2** and for the control group in the **Table 3**.

Relationship Between Gut Microbiota-Derived Metabolites, Systemic Inflammatory Biomarkers and Vascular-Immune Biomarkers in Patients After Htx Over the Follow-Up Period

We analyzed the correlations between GMDMs and markers of systemic inflammation (CRP, WBC, procalcitonin), as well as biomarkers of the immune-vascular axis (CD14, ET-1) as 2 separate hypothesis families. Bonferroni correction was applied within each hypothesis family, with a significance threshold of $P < 0.006$ for systemic inflammation analyses and $P < 0.008$ for immune-vascular axis analyses. The division into hypothesis families was based on distinct pathophysiological pathways that reflect the systemic inflammatory response and immune-endothelial activation. Hence, during the follow-up period (1, 3, and 6 months) after Htx, TMAO was the only GMDM that correlated with the aforementioned biomarkers. Specifically, TMAO correlated with ET-1 at 1 month after Htx ($r = 0.59$, $P = 0.004$, $n = 22$) and a strong significant correlation was also found for TMAO and procalcitonin at 6 months after Htx ($r = 0.94$, $P = 0.005$, $n = 6$). The correlations between the tested metabolites and inflammatory markers are summarized in **Table 4**.

Prevalence of ACR and Correlation With Gut Microbiota-Derived Metabolites

Out of 32 patients initially qualified for Htx, 4 did not undergo transplantation within the observational study period, 4 died during follow-up, and 16 experienced at least 1 episode of ACR within the 6-month follow-up. We evaluated whether pre-interval GMDM concentrations were associated with subsequent ACR episodes across predefined observational intervals. The interval-specific subgroup sizes were as follows: (1) baseline to 1 month: 8 patients with subsequent ACR vs 19 without ACR; 109 endomyocardial biopsies; (2) 1 to 3 months: 9 patients with subsequent ACR vs 16 without ACR; 69 endomyocardial biopsies; and (3) 3 to 6 months: 4 patients with subsequent ACR vs 18 without ACR; 57 endomyocardial biopsies.

Across all evaluated intervals, no statistically significant differences in serum concentrations of available TMAO, indoxyl sulfate, or p-cresol sulfate measurements were detected between patients with and without subsequent ACR (all $P > 0.05$; **Table 5**). However, given the limited sample sizes, these

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Table 4. Correlation between gut microbiota metabolites and inflammatory biomarkers.
Correlations between tested metabolites and biomarkers in patients after heart transplantation.

	1 month			3 months			6 months		
	TMAO	IND	CRE	TMAO	IND	CRE	TMAO	IND	CRE
CRP	*r* = -0.16 *P* = 0.53 n = 18	*r* = -0.12 *P* = 0.64 n = 18	*r* = -0.18 *P* = 0.46 n = 19	*r* = -0.10 *P* = 0.70 n = 18	*r* = 0.06 *P* = 0.80 n = 18	*r* = -0.03 *P* = 0.09 n = 19	*r* = 0.49 *P* = 0.19 n = 9	*r* = 0.48 *P* = 0.19 n = 9	*r* = 0.21 *P* = 0.59 n = 9
WBC	*r* = -0.07 *P* = 0.77 n = 21	*r* = 0.12 *P* = 0.60 n = 21	*r* = 0.15 *P* = 0.52 n = 21	*r* = 0.02 *P* = 0.94 n = 18	*r* = 0.05 *P* = 0.83 n = 18	*r* = 0.13 *P* = 0.59 n = 19	*r* = 0.30 *P* = 0.43 n = 9	*r* = 0.00 *P* = 1.00 n = 9	*r* = -0.28 *P* = 0.46 n = 9
Pct	*r* = 0.08 *P* = 0.76 n = 18	*r* = 0.23 *P* = 0.35 n = 18	*r* = -0.21 *P* = 0.40 n = 19	*r* = 0.28 *P* = 0.26 n = 18	*r* = 0.07 *P* = 0.77 n = 18	*r* = -0.22 *P* = 0.36 n = 19	***r* = 0.94*** ***P* = 0.005** **n = 6**	*r* = 0.76 *P* = 0.08 n = 6	*r* = 0.39 *P* = 0.44 n = 6
CD14	*r* = -0.11 *P* = 0.64 n = 22	*r* = 0.38 *P* = 0.08 n = 22	*r* = -0.18 *P* = 0.43 n = 22	*r* = 0.06 *P* = 0.81 n = 17	*r* = 0.10 *P* = 0.70 n = 17	*r* = -0.15 *P* = 0.55 n = 18	*r* = 0.09 *P* = 0.77 n = 14	*r* = 0.25 *P* = 0.38 n = 14	*r* = 0.33 *P* = 0.25 n = 14
ET-1	***r* = 0.59*** ***P* = 0.004** **n = 22**	*r* = 0.48 *P* = 0.02 n = 22	*r* = 0.22 *P* = 0.32 n = 22	*r* = 0.45 *P* = 0.08 n = 16	*r* = 0.16 *P* = 0.56 n = 16	*r* = 0.02 *P* = 0.93 n = 17	*r* = 0.10 *P* = 0.73 n = 13	*r* = 0.35 *P* = 0.25 n = 13	*r* = 0.34 *P* = 0.25 n = 13

Abbreviations: CD14, cluster of differentiation 14; CRE, p-cresol sulfate; CRP, C-reactive protein; ET-1, endothelin-1; Htx, orthotopic heart transplantation; IND, indoxyl sulfate; n, number of observations; Pct, procalcitonin; *r*, correlation coefficient; TMAO, trimethylamine N-oxide; WBC, white blood cells. * Bonferroni correction was applied, with significance thresholds of $P < 0.006$ for systemic inflammation markers (CRP, WBC, PCT) and $P < 0.008$ for immune-vascular axis biomarkers (CD14, ET-1).

Table 5. Gut microbiota- derived metabolites levels compared between acute cellular rejection (ACR) and non-ACR groups before and after orthotopic heart transplantation (Htx).

	Non-ACR group	ACR group	*P* value
Baseline to 1 month after Htx			
TMAO, baseline (µg/mL)	0.69 [0.51-1.14] (17)	0.86 [0.29-1.30] (8)	1.00
p-Cresol sulfate, baseline (µg/mL)	9.24 [3.77-16.37] (16)	18.64 [3.46-48.54] (7)	0.22
Indoxyl sulfate, baseline (µg/mL)	1.57 [0.38-2.85] (16)	0.99 [0.75-2.37] (8)	0.98
1 to 3 months after Htx			
TMAO, 1 month (µg/mL)	0.68 [0.44-1.01] (13)	0.35 [0.22-1.76] (9)	0.74
p-Cresol sulfate, 1 month (µg/mL)	3.46 [0.68-12.80] (13)	5.94 [4.94-12.38] (9)	0.26
Indoxyl sulfate, 1 month (µg/mL)	1.12 [0.71-2.12] (13)	1.31 [1.18-2.26] (9)	0.35
3 to 6 months after Htx			
TMAO, 3 months (µg/mL)	0.75 [0.41-0.97] (14)	0.74 [0.61-1.50] (3)	0.85
p-Cresol sulfate, 3 months (µg/mL)	10.74 [7.32-13.24] (14)	12.49 [0.59-13.19] (3)	0.95
Indoxyl sulfate, 3 months (µg/mL)	1.48 [1.03-2.05] (14)	1.52 [0.40-2.40] (3)	0.95

Continuous variables are presented as median (interquartile range) Abbreviation: TMAO, trimethylamine N-oxide.

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analyses possessed limited statistical power. Consequently, the lack of statistically significant differences should not be interpreted as definitive evidence of an absence of association, but rather as exploratory and hypothesis-generating observations that warrant verification in larger multicenter cohorts.

Discussion

The intestinal microbiota is a protective tool, separating the internal environment from the external environment in the digestive tract. Intestinal bacteria are the first barrier to pathogens and inhibitors of inflammatory reactions. On the other hand, disturbances in intestinal colonization promote abnormal inflammatory reactions. This phenomenon is frequently observed in HF and other cardiovascular diseases. The effect of intestinal bacterial endotoxemia on the intensity of inflammatory processes and the occurrence of cellular rejection in patients undergoing Htx has not yet been usefully studied. In response, this study provides insight into potential patomechanisms in patients undergoing organ transplantation and is also a source of new research hypotheses in the area of clinic transplantology. This has been achieved by the characterization of key gut microbiota metabolite profiles in serum and their changes in patients undergoing Htx. In our analysis, we showed that the group qualified for Htx had abnormal concentrations of metabolites in the blood, which indirectly may indicate abnormal gut microbiota composition and/or dysfunction [26]. Interestingly, the study in kidney recipients showed an increase in bacteria responsible for chronic inflammation in the T-cell-mediated rejection group, concluding that microbial-specific structure might be a noninvasive biomarker of the rejection group [27].

We observed significantly higher levels of the microbial metabolites p-cresol sulfate and TMAO among the advanced HF group compared with the healthy controls. However, indoxyl sulfate did not differ between the Htx and control groups at baseline. These findings partially align with current knowledge, as p-cresol sulfate and indoxyl sulfate, as uremic toxins, have already been indicated to worsen the prognosis of patients with HF [28]. Importantly, chronic kidney failure is commonly associated with an increase in the aforementioned metabolites due to their impaired clearance and higher synthesis [29]. Specifically, in end-stage renal disease, p-cresol sulfate and indoxyl sulfate are involved in the development of cardiomyopathy [30]. Nevertheless, uremic toxins' effects on heart dysfunction have been suggestedto be reversible by kidney transplant and the subsequent lowering of metabolite levels [30,31]. Likewise, p-cresol sulfate and indoxyl sulfate levels were also shown to predict 5-year all-cause mortality among patients with normal kidney function [32].

Regarding strong correlations ($|r| \geq 0.5$) between microbial metabolites and inflammatory biomarkers by 6 months, the strongest positive correlation was noted between TMAO and procalcitonin, which aligns with TMAO's contribution to systemic inflammation [33]. Nevertheless, this correlation should be interpreted as hypothesis-generating due to the limited sample size. These hypotheses would probably be relevant to the established discussions regarding TMAO's contribution to cardiovascular disease development as being well-established and driven by its proatherogenic and systemic proinflammatory role. It is also worth mentioning that higher levels of TMAO increased 5-year mortality and the need for transplantation among patients with HF [34].

The correlation between TMAO and ET-1 may reflect ET-1-related vasoconstrictive pathways that have been linked to accelerated cardiac allograft vasculopathy and late mortality in transplant recipients [35,36]. However, in our study, this correlation was observed only 1 month after Htx, and we do not have data on long-term mortality in this cohort. Therefore, this correlation at that time may reflect higher gut leakage along with increased bacterial toxin concentration, resulting in systemic inflammation.

The differences in GMDMs levels between ACR and non-ACR groups did not reach statistical significance. These results should be interpreted as hypothesis-generating only, due to the limited sample size, and require further evaluation in larger cohorts. To date, the only metabolite previously considered regarding ACR in patients with Htx is TMAO, whose predictive role in ACR in the 3-year follow-up was not confirmed [21]. However, as a complication, the increase in the level of TMAO's precursor, γ-butyrobetaine, was shown to be a positive predictor of rejection [21].

GMDMs are considered potential therapeutic targets in cardiovascular diseases. An optimal therapeutic approach for reducing these metabolites has not yet been established. Solely using beta-glucan supplementation has been shown to reduce p-cresol sulfate levels and improve endothelial vascular reactivity in healthy individuals [37]. Additionally, vancomycin was shown to lower the levels of uremic toxins among patients with end-stage renal disease [38]. However, modification of uremic toxin levels may be possible to some extent, as it is suggested that these uremic co-metabolite levels are also moderately dependent on host genetics [39]. Moreover, the role of ET-1 blockage was investigated in animal models, showing some promise [40]. Overall, further research is needed to evaluate the suspected role of GMDMs in the field of transplantology and the clinical utility of these therapies in patients with Htx.

Limitations

The main limitations of this study are its small sample size, small interval-specific ACR groups, and single-center design.

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Moreover, missing metabolite measurements additionally limit available data. Adjusted analyses were not performed, due to small sample size and event counts, thus residual confounding represents a limitation of the study. In addition, minor deviations from the predefined biopsy schedule were driven by patient-related, clinical or logistical reasons. These constraints lower data generalizibility and precision; therefore, presented results should be interpreted with caution and interpreted as hypothesis-generating.

Conclusions

TMAO and p-cresol sulfate were significantly higher among patients with advanced HF. Importantly, we identified significant correlations between TMAO and ET-1 at 1 month after Htx and between TMAO and procalcitonin at 6 months after Htx. No significant differences in GMDM levels were observed between the ACR and non-ACR groups; however, these results should be interpreted as hypothesis-generating.

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The figure was Created in BioRender. Florek, K (2026) https://BioRender.com/78nsezq. The authors hold publication licences. A part of this project has already been presented in the abstract form, but the present article represents a different methodological approach [41].

Ethics Statement

The study protocol was approved by the Bioethics Committee of Wrocław Medical University and was conducted in accordance with the Declaration of Helsinki (KB 678/2022, 3.10.2022).

Data Availability Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Patient Consent and Confidentiality

Written informed consent was obtained from all patients in the study.

Institution Where Work Was Done

Institute of Heart Diseases, University Hospital, Wrocław, Poland.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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