Co-toxicity of Endotoxin and Indoxyl Sulfate, Gut-Derived Bacterial Metabolites, to Vascular Endothelial Cells in Coronary Arterial Disease Accompanied by Gut Dysbiosis

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Abstract

Gut dysbiosis, alongside with high-fat diet and cigarette smoking, is considered one of the factors promoting coronary arterial disease (CAD) development. The present study aimed to research whether gut dysbiosis can increase bacterial metabolites concentration in the blood of CAD patients and what impact these metabolites can exert on endothelial cells. The gut microbiome of 15 CAD patients and age-matched 15 healthy controls was analyzed by metagenome sequencing. The in vitro impact of LPS and indoxyl sulfate at concentrations present in patients sera on endothelial cells was investigated. A metagenome sequencing analysis revealed gut dysbiosis in CAD patients, further confirmed by elevated levels of LPS and indoxyl sulfate in patients sera. CAD was associated with depletion of Bacteroidetes and Alistipes. LPS and indoxyl sulfate in meager concentrations demonstrated co-toxicity to endothelial cells inducing reactive oxygen species, E-selectin, and monocyte chemoattractant protein-1 (MCP-1) production and promoting thrombogenicity of endothelial cells confirmed by monocyte adherence. The co-toxicity of LPS and indoxyl sulfate was associated with harmful effects on endothelial cells, strongly suggesting that gut dysbiosis-associated increased intestinal permeability can initiate or promote endothelial inflammation and atherosclerosis progression.

Key words: coronary artery disease, gut microbiome, dysbiosis, obesity, Bacteroidetes, LPS, indoxyl sulfate
Graphical abstract

1. Dysbiotic flora produce toxins that enter the circulation through the leaky blood-gut barrier.

2. Toxins transported with the blood reach the coronary vascular endothelium and induce inflammation.

3. Chronic inflammation leads to the formation of coronary plaques and ischemic heart disease.
Research manuscript section

1. Introduction

Coronary artery disease (CAD) includes stable and unstable angina, myocardial infarction, and sudden cardiac death, the primary cause of morbidity and mortality worldwide [1]. The mechanisms and risk factors involved in the pathogenesis of CAD have been well-documented over the last decades. However, early inducers triggering the cascade of inflammation and atherosclerotic plaques formation are only putative.

Gut dysbiosis related to the change in diversity and abundance of resident intestinal microbiota plays a vital role in cardiovascular diseases [2,3]. Gut dysbiosis induces chronic inflammation resulting in increased intestinal epithelial permeability and leakage of bacterial metabolites into the bloodstream [4]. Some of these metabolites can activate endothelial cells (ECs) and promote atherosclerosis. Moreover, bacterial metabolites may interact with each other increasing their mutual cytotoxicity to the ECs directly or indirectly via the effect on monocyte/macrophage.

To date, few bacterial metabolites that exert deleterious effects on ECs have been recognized. One of those is indole, a tryptophan derivative produced by gut microbiota, oxidized to indoxyl sulfate (IS) in the liver and then removed from the body throughout kidneys filtration. Elevated indoxyl levels induce oxidative stress, pro-inflammatory response, and enhanced expression of adhesion molecules in ECs. The toxic effect of indoxyl on ECs contributes to chronic kidney disease (CKD) and is involved in cardiovascular diseases pathogenesis in CKD patients [5]. However, the impact of indoxyl on CAD pathogenesis in patients without renal impairment is still lacking.

Another well-known bacterial metabolite with deleterious effects on the ECs is endotoxin (LPS), originating from the outer membrane of Gram-negative bacteria. LPS influences the endothelium indirectly via pro-inflammatory cytokines or affects ECs directly. In ECs, LPS upregulates nitric oxide synthase (iNOS), increases endothelium permeability, and enhances the expression of leukocytes' adhesion molecules. Furthermore, LPS induces the conversion of the endothelial surface from anti-coagulant to pro-coagulant and affects multiple signaling pathways via the TLR4 receptor [6]. Significant risk factors for the development of CAD are lipoproteins and the high-fat diet as their source. Low-density lipoproteins (LDL) entering arterial intima from the blood are considered the primary driver of atherogenesis [7]. Diet is also an essential factor regulating the composition and metabolic activity of the intestinal microbiota. Hence, an unhealthy diet results in gut dysbiosis and leakage of bacterial metabolites into the circulation [8]. Plasma lipoproteins such as HDL, LDL, and VLDL can sequester LPS from circulation to limit its harmful impact on the host cells [9]. LPS sequestration by plasma lipids, however, may become a pathway of its delivery into monocytes/macrophages infiltrating vascular endothelium activated by cytokines released by monocytes in response to bacterial metabolites. Moreover, at the site of clot formation, LPS alone or combined with indoxyl via ROS formation in ECs can promote lipids oxidation and the formation of foamy macrophages.

The present study focused on the association of gut dysbiosis and an in vitro analysis of the direct and indirect effects of indoxyl and LPS at the concentrations present in the sera of dysbiotic CAD patients on vascular endothelial cells. The results of the study demonstrated gut dysbiosis in CAD patients and elevated levels of bacterial metabolites, i.e., LPS and indoxyl sulfate in their sera. In vitro co-toxicity of LPS and indoxyl sulfate in meager concentrations was associated with deleterious effects on endothelial cells strongly suggesting that overweight-associated increased intestinal permeability can initiate or promote endothelial inflammation.
2. Materials and Methods

Study participants
Fifteen CAD patients and 15 healthy individuals were enrolled in the study. CAD was confirmed by coronary angiography, and patients with ≥ 50% stenosis in single or multiple vessels were qualified for the study. Study participants were recruited at Regional Specialist Hospital between March and September 2020. Biochemical parameters were assessed employing standard techniques at the Specialist Hospital's Laboratory (Table 1). The exclusion criteria included renal disease defined as an abnormal creatinine serum level (>2 mg/dl), malignancy, ongoing infectious disease, hepatic disease, and use of antibiotics within four weeks before sample collection. All participants of the study gave their written consents; the study followed guidelines of the Helsinki Declaration and was approved by Wroclaw Medical University's Ethics Committee (authorization number KN-209/2020).

Table 1. Baseline clinical characteristics of the study cohort.

|                          | CAD (average) | Range   | HC (average) | Range   | p-value |
|--------------------------|---------------|---------|--------------|---------|---------|
| Age (years)              | 67.2±9        | 54 – 90 | 57±11,1      | 47– 74  | p>0.5   |
| Sex                      | 11♂/4♀       |         | 5♂/10♀      |         |         |
| BMI                      | 29.4±5.05     | 23.6-39.26 | 25.95±4.21  | 18.71-36.25 | p<0.05 |
| CAD type                 | 10 SA; 5- ACS | NO      | NO           |         |         |
| Indoxyl sulfate (µM)     | 13.1±4.1      | 3.6 – 21.2 | 6.9±3.5     | 2.1 – 15.7 | p=0.41 |
| LPS (ng/mL)              | 2.8±1.5       | 0.9 – 5.5  | 0.009±0.01   | 0.0 – 0.007 | p<0.0001 |

Microbiome analysis
Stool samples from all participants, collected and transported to the laboratory in an ice bag, were stored at -80°C until processed. DNA was extracted from stool specimens using the QIAamp DNA Stool Mini Kit. The hypervariable V3-V4 regions of the 16S ribosomal RNA (rRNA) gene was amplified. Quantitative real-time polymerase chain reaction amplification was performed in triplicate. The products were purified, and then pyrosequencing was performed using the MiSeq system (Illumina, San Diego, CA). The entire workflow for microbiome analysis was run in Snakemake [10], cutadapt [11], USEARCH3 algorithm [12], phyloseq object [13], decipher idtaxa [14], with SILVA as reference database [15] (detailed description of the workflow is provided in the methods section of the supplementary file).

Endotoxin, indoxyl sulfate, and IL-6 measurements in patients sera
LPS level was determined using PierceTM LAL chromogenic assay (ThermoFisher Science), whereas IS was measured in plasma samples with Human Indoxyl Sulphate ELISA Kit (MyBioSource). Both assays were performed according to the manufacturer’s instructions.

Cell cultures and conditioned medium preparation
Endothelial cells (primary human umbilical vein endothelial cell line HUVEC C-015-10C was obtained from ThermoFisher Scientific) were routinely cultured in an EBM-2 bullet kit medium (Lonza). For experiments, HUVECs were trypsinized and seeded into cell culture plates at the density of 4 x 10⁵ cells/ml in M199 medium (ThermoFisher Scientific) supplemented with 10% foetal bovine serum (FBS) and cultured overnight. Human monocytic THP-1 (EP-
CL-0233 was obtained from Elabscience) cell line was cultured in RPMI-1640 medium (ThermoFisher Scientific) with 10% FBS and 1% antibiotics solution (penicillin, streptomycin). THP-1 cells were differentiated into monocyte-derived macrophages (MDM) with 50 ng/ml PMA (phorbol 12-myristate 13-acetate; Sigma) for 48 h. The obtained monocyte-derived macrophages (MDM) were stimulated for 18 h with LPS (from Salmonella Typhimurium, Sigma-Aldrich) and indoxyl sulfate (Sigma-Aldrich) at concentrations corresponding to a mean concentration detected in patients sera and ten times higher concentrations. Thus, LPS was used at concentrations 3 ng/ml or 30 ng/ml and indoxyl at 13 µM and 130 µM. The mixtures of both metabolites at concentrations LPS 3 ng/ml + indoxyl 13 µM, LPS 30 ng/ml + indoxyl 130 µM were used to assess the co-toxicity of these both metabolites. The negative control included MDM in RPMI-1640 medium without LPS and indoxyl. Next, the culture medium was drawn and centrifuged for 10 min at 1500 rpm and filter-sterilized (0.22 µm). The resultant supernatants called conditioned medium (CM) were frozen at -70°C for further study. CM from macrophages apart from LPS and indoxyl contains cellular metabolites and cytokines in concentrations toxic for cultured cells. Hence, CM diluted with cell culture medium to a concentration of 25% was used (hereafter referred to as CM25).

**Cells viability and ROS measurement**

HUVECs and MDM viability was assessed with 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich). The cells were treated with LPS and IS at appropriate concentrations for 18 h followed incubation with MTT for 2 h at 37°C in an atmosphere with 5% CO₂. The MTT solution was removed, and 100 µl DMSO was added to wells to dissolve violet formazan crystals. The reaction was read at 570 nm in a spectrophotometer UV/VIS 340. The 2’, 7’-dichlorodihydrofluorescein diacetate (H2DCF-DA; Sigma-Aldrich) was used to assess ROS production in HUVECs treated with LPS IS at appropriate concentrations for 5 h. After treatment, cells were washed three times with Hank's Balanced solution (HBSS), and 10 µM H2DCF-DA in HBSS was added to cells for 30 min. The fluorescent product was quantified with a spectrophotometer Tecan Infinite M200 plate reader at 488/525 nm.

**Endothelial cells thrombogenicity assessment**

The effect of LPS and indoxyl on HUVECs thrombogenicity was investigated by monocyte adherence, E-selectin, and monocyte chemoattractant protein-1 (MCP-1) investigation. HUVECs were pre-treated for 5 h with LPS and indoxyl at appropriate concentrations in M199 medium supplemented with 10% FBS and CM25 diluted in M199 medium. THP-1 cells at the density 1 x 10⁶ cell/ml were stained with 10 µg/ml calcein-AM for 1 h and added to stimulated HUVEC cells for 4 h. Following three washes in HBSS, the number of THP-1 cells adhering to HUVECs was quantified under a fluorescence microscope (from six different fields of view) and expressed as the percentage of adhering monocytes relative to negative control, i.e., unstimulated HUVEC cells, considered 100%.

E-selectin level in HUVEC cells stimulated with LPS and indoxyl, and CM25 was assessed according to the protocol described by Grabner et al. [16]. Briefly, a confluent HUVECs layers in a 6-well plate stimulated with CM25 or LPS and IS for 18 h was washed twice with PBS and fixed with cold 0.5% formalin in PBS for 2.5 min. Then, cells were washed with HBSS-BSA (0.5% w/v) before adding FITC-conjugated mouse monoclonal anti-human E-selectin antibody (R&D Systems) at concentration 10 µg per 1 x 10⁶ cells in HBSS with 0.02% saponin (w/v). The reaction was run for 45 min at 4°C in darkness, followed by two washes in HBSS-saponin and detachment with 0.25% trypsin. Detached cells were collected and centrifuged at 1000 rpm at 4°C for 10 min, washed twice with cold PBS, and finally diluted in PBS. A 100 µl sample was added to a 96-well black-walled plate in triplicate and read using a Tecan Infinite
M200 plate reader at 488/535 nm. HUVECs incubated at the same conditions with an isotype mouse FITC-conjugated antibody (Sigma-Aldrich) served as a negative control.

MCP-1 protein was assessed in the culture media from HUVECs stimulated with CM25 and LPS and IS antigens for 5 h using the MCP-1 Human ELISA kit (Invitrogen) performed according to the manufacturer's instruction. The level of MCP-1 was calculated from a standard curve.

**Statistical analysis**

For alpha diversity analyses, reads were normalized to 5000 reads by subsampling without replacements. Alpha diversity was assessed using Chao1 and Fisher’s alpha (richness), Bulla and Simpson indices (evenness), and dominance index. In addition, beta-diversity was assessed using principal coordinate analysis (PCoA) weighted UniFrac, unweighted UniFrac, and Bray-Curtis distances. Statistical significance was tested with PERMANOVA using 9999 permutations. Student’s *t*-test was used to analyze differences in a vitro qualitative study results.

### 3. Results

Bacterial metabolites leaking from the gut into the bloodstream can affect the vascular endothelium directly or indirectly by factors released from white blood cells upon contact with bacterial antigens. To better understand the role of gut dysbiosis in the development of atherosclerosis, the study aimed to evaluate, first, the gut microbiome in CAD patients and healthy controls, second, the levels of LPS and IS in the sera of CAD patients and their direct and indirect effects on the vascular endothelium.

There are five core bacterial phyla within the gut microbiome. The most numerous *Firmicutes* make up 65%, followed by *Bacteroidota* accounting 30%, *Proteobacteria* and *Verrucomicrobiota* constituting 2%, *Actinobacteria* comprising 1%, and other phyla contributing <1% [17]. Despite the small sample size, the metagenome analysis revealed the altered composition of the gut microbiome in CAD patients compared to HC, confirming gut dysbiosis in CAD patients (Figure 1). At the phylum and class levels, CAD patients had decreased *Bacteroidota* compared to healthy controls (HC) (35.2%±11.5% vs. 48.2%±21.1, respectively; *p*=0.01). On the contrary, *Firmicutes* were more prevalent in CAD than in HC, but statistically insignificantly. Similarly, at the order level, *Bacteroidales* depletion was associated with CAD patients compared to HC (36.01%±11.6 vs. 49.03%±21.06%, respectively; *p*=0.03). Moreover, *Coriobacteriales* increase was observed in CAD compared to HC (2.32%±2.1% vs. 1.0%±1.06%, respectively; *p*=0.04). Besides, *Enterobacteriales* increase was registered in the CAD group but statistically insignificant. The analysis at the family level indicated decreased *Rikenellaceae* of *Bactroidales* order in the CAD cohort compared to HC (3.3%±4.6% vs. 4.7%±2.8%, respectively; *p*=0.054), although the difference did not reach statistical significance. The *Rikenellaceae*, *Prevotellaceae*, and *Tannerelaceae* increase was observed in HC, whereas *Ruminococcaceae* were more abundant in CAD. However, none of these differences reached statistical significance. At the genus level, a significant depletion of *Alistipes* of the *Rikenellaceae* family was detected in CAD compared to HC (3.2%±4.6 vs. 5.1%±3.4%, respectively; *p*=0.01).
Thus, our next question was whether, in patients with CAD, bacterial metabolites could leak from the intestine into the bloodstream. The targeted metabolites for further study were LPS and indoxyl. The mean indoxyl level in CAD patients sera (13.3 µM), although twice as high as in healthy subjects (6.8 µM), was not significantly different from that in controls (p=0.41). In contrast, the mean LPS level in CAD patients' sera (2.8 ng/ml) was up to 330-fold higher than in sera from healthy subjects (0.009 ng/ml) (p<0.0001) (Table 1).

Further, we investigated whether LPS and indoxyl at concentrations detected in patients sera could, directly and indirectly, affect ECs thrombogenicity. The direct effect of both metabolites on ECs was investigated by applying LPS and indoxyl to cultured cells. The indirect effect was analyzed with conditioned medium (CM25) from MDMs stimulated with appropriate concentrations of LPS and indoxyl. Neither CM25 medium nor LPS and indoxyl or their combinations directly applied to endothelial cells significantly affected HUVECs viability (Figure 2). Both metabolites at concentrations studied did not affect MDM viability except the highest ones used (Figure 2). The LPS (30 ng/ml) combined with indoxyl (130 µM) decreased MDM viability by >10% (p=0.0024).

![Diagram showing gut microbiome abundance in CAD patients and healthy controls.](image)

**Fig.1.** Community abundance (%) in gut microbiome of CAD patients and healthy controls (HC).
Increased production of reactive oxygen species (ROS) by endothelial cells is a hallmark of cardiovascular diseases and endothelial damage [18]. Hence, we investigated whether such low concentrations of LPS and indoxyl present in patients sera could induce ROS. The results showed that LPS either at low and high concentrations (3 ng/ml and 30 ng/ml) increased ROS production in ECs of 160-fold ($p<0.0001$) and >200-fold ($p<0.00001$), respectively. Indoxyl at both concentrations (13 µM and 130 µM) increased ROS production more than LPS, i.e., by >240-fold ($p<0.00001$) and >260-fold ($p=0.00001$), respectively. Moreover, LPS combined with indoxyl induced higher ROS levels than each metabolite individually (>330-fold for low concentrations and >400-fold for 10-times higher concentrations), indicating their co-toxicity for HUVECs (Figure 2).

Endothelial dysfunction induced by bacterial toxins and pro-inflammatory cytokines results in leukocyte-endothelial interactions and procoagulant activities, ultimately leading to thrombosis and atherosclerosis [19]. To determine whether LPS and indoxyl at concentrations detected in patients sera could affect ECs thrombogenicity, E-selectin and MCP-1 levels, and monocytes’ adherence to HUVECs were assessed. The results demonstrated that even...
meager LPS (3 ng/ml) and indoxyl (13 µM) concentrations found in patients sera significantly increased E-selectin in HUVECs compared to the negative control (p<0.05) (Figure 3). Both metabolites used at 10-fold higher concentrations (LPS 30 ng/ml and IS 130 µM) increased E-selectin levels accordingly. Moreover, both metabolites at low (LPS 3 ng/ml and IS 13 µM) and 10-fold higher concentrations exerted a cumulative effect on increased E-selectin levels. Similar results were observed in HUVECs treated with CM25, although lesser (p>0.05). The CM25 obtained from MDM treated with indoxyl at 13 µM and 130 µM combined with LPS induced E-selectin levels to be equal or higher than the same metabolites applied to HUVECs directly.

MCP-1 levels in CM25 used for HUVECs stimulation were significantly lower than in the culture media from HUVECs directly treated with LPS or indirectly stimulated with CM25 (p<0.05) (Figure 3). MDM treated for 18 h with indoxyl at 13 µM, and 130 µM released significantly lower amounts of MCP-1 than MDM treated with LPS (p<0.05). Both metabolites combined at low concentrations (LPS 3 ng/ml and indoxyl 13 µM) induced higher MCP-1 levels than a combination of LPS and indoxyl at higher concentrations (LPS 30 ng/ml and indoxyl 130 µM) (p<0.05). These results pointed out that indoxyl in a concentration-dependent manner inhibited MCP-1 secretion by MDM. MCP-1 levels in culture media from HUVECs treated with CM25 or directly stimulated with LPS and indoxyl were significantly higher than MCP-1 levels detected in CM25 (p<0.05), signifying de novo production of the chemokine by HUVECs in response to bacterial metabolites. Furthermore, the levels of MCP-1 secreted by HUVECs were not affected by the concentration of LPS and indoxyl, inducing the secretion of comparable MCP-1 levels, ca. 16 ng/ml (p>0.05).
Fig. 3. The thrombogenicity study. (a) MCP-1 levels in HUVECs stimulated with LPS and indoxyl sulphate (IS); (b) E-selectin levels in HUVECs treated directly with LPS and indoxyl sulfate (IS) and with 25% conditioned medium from MDM treated with LPS and IS at appropriate concentrations; RFU, relative fluorescence units; (c) Monocyte adherence to endothelial cells stimulated with LPS and IS. The student's t-test determined the significance of direct and indirect HUVEC stimulation with LPS and IS. All plots: a black asterisk indicates differences among directly stimulated HUVECs and negative control (NC); a red asterisk indicates the differences among indirectly stimulated HUVECs and NC; a green asterisk indicates the differences among MDM and NC; blue values of p display the differences between HUVECs treated directly with LPS and IS, and indirectly via CM; (d) fluorescence images presenting calcein-stained monocytes adhering to HUVECs stimulated directly and indirectly (e) via CM.
Direct and indirect HUVECs treatment with LPS and indoxyl significantly augmented monocyte adhesion. However, indoxyl generated reduced adherence of monocytes to HUVECs compared to LPS alone or combined with indoxyl (1.7-fold and 2.8-fold decrease for low concentrations respectively; \( p=0.01 \); 2.8-fold and 2.9-fold decrease for 10-fold higher concentrations respectively; \( p=0.00001 \)). CM25 had a more pronounced effect on monocyte adhesion (about a 3-fold increase for low and high concentrations; \( p=0.001 \)) than combined metabolites applied directly to HUVECs. These results confirmed the deleterious synergistic effects of these two metabolites on endothelial cells.

4. Discussion

Intact vascular endothelium displays an antithrombotic surface, preventing thrombosis and governing homeostasis. Endothelial dysfunction triggers a cascade of reactions shifting endothelial surface from antithrombotic to prothrombotic. As a result, endothelial activation promotes thrombus formation with two opposite implications for the host. First, the clot is digested and homeostasis restored; second, the thrombosis cascade progresses to CAD [20].

The gut microbiota plays a vital role in atherosclerosis, heart failure, diabetes, and obesity, acting as an independent risk factor [21]. A western diet providing an excess of amino acids and proteins promotes the growth of proteolytic bacteria producing indole processed in the liver to indoxyl sulfate. In turn, proteolytic gut microbiota metabolism favors gut dysbiosis [22]. The altered gut microbiota composition was confirmed in a metagenome study of patients with atherosclerosis and healthy individuals, strongly suggesting correlation between CAD and dysbiosis [23,24,25]. In the study, a comparison of the gut microbiome of CAD patients and healthy controls revealed significant depletion of Bacteroidetes in the CAD cohort supporting previous observations [2,21,26]. Bacteroidetes are known to play a pivotal role in maintaining a healthy gut ecosystem, stimulating immunity, providing short-chain fatty acids (SCFA), and facilitating digestion of macronutrients [27]. Bacteroidetes depletion can result from a high-fat diet which changes the gut microbial profile and reduce its diversity. The depletion of Bacteroidetes combined with increased Firmicutes elevates circulating LPS levels leading to endotoxemia. Thus, Bacteroidetes may have the potential to regulate atherosclerosis progression [23]. Moreover, lipid A of LPS from Bacteroides is an antagonist of immune stimulation and inflammatory cytokine response [24,28].

Although not reported in other studies, the prevalence of Coriobacteriaceae observed in the study in the CAD cohort could play a role in the pathogenesis of atherosclerosis. In the animal model, the abundance of Coriobacterium correlates with higher cholesterol absorption from the gut [29]. At the genus level, in the CAD group, a marked decrease in Alistipes belonging to the family Rikenellaceae of Bacteroidetes was observed, supporting previous findings [24,30,31]. The Alistipes genus comprises species found primarily in the gut of healthy humans and is a potent SCFA producer essential for maintaining balanced gut microbiota [29]. According to various studies, Alistipes play a protective role in cardiovascular diseases [25,30,31].

Considering that gut dysbiosis underlies the leakage of some bacterial metabolites into the circulation, the study analyzed levels of the endotoxin and indoxyl sulfate, a co-metabolite of indole, in CAD patients sera and an in vitro effects of these metabolites on vascular endothelium. The results showed that LPS and indoxyl at concentrations detected in CAD patients sera induced oxidative stress in cultured endothelial cells. Similarly, Loffredo et al. [32] showed that LPS at a similar low concentration (3 ng/ml) induced oxidative stress in patients with peripheral arterial disease. Moreover, they found the correlation between LPS and zonulin of tight junctions, suggesting gut permeability
as a trigger for LPS translocation. In turn, Adesso et al. [33] demonstrated that indoxyl increased ROS and inflammatory cytokines, i.e., TNFα and IL-6 in mouse peritoneal macrophages stimulated with LPS.

Oxidative stress is a potent risk factor for atherogenesis. Pathological endothelial activation results in the secretion of factors attracting monocytes and induces the expression of monocyte-binding adhesion molecules [34]. E-selectin plays an essential role in priming and amplifying the innate immune response of ECs, leukocyte rolling, and adhesion [35]. LPS directly increases the expression of E-selectin and integrin counterpart receptors in ECs [6]. In the study, LPS and indoxyl, directly and indirectly, increased E-selectin levels in endothelial cells in a concentration-independent manner reaffirming earlier findings [36]. A mixture of LPS and indoxyl enhanced E-selectin levels more than every metabolite individually, indicating mutual amplification of their effect on endothelial cells.

MCP-1 is a significant chemokine recruiting monocyte/macrophage to the injured vascular endothelial cells. LPS activates ECs and monocyte/macrophage to secrete proinflammatory cytokines, i.e., IL-6 and IL-8, and MCP-1 among them [37,38]. Our results showed that LPS and indoxyl at low concentrations induced in ECs synthesis de novo and secretion of MCP-1. On the contrary, indoxyl alone or in combination with LPS, in a concentration-dependent manner, inhibited MCP-1 secretion by macrophages but not by ECs, which corroborates results from other studies. Ferreira et al. [39] demonstrated that indoxyl at a 53 mg/ml concentration decreased MCP-1 production by monocytes. In turn, according to Tumur et al. [40], indoxyl, in a time and concentration-dependent manner, increased mRNA MCP-1 expression in HUVECs. Considering the deleterious impact of LPS and indoxyl on ROS, MCP-1 and E-selectin demonstrated in our study, the effect of both metabolites on monocyte adherence was further examined. Just five hours of exposure of ECs to LPS or CM significantly increased monocyte adhesion. However, this effect diminished when ECs were stimulated with indoxyl or the combination of LPS and indoxyl. Contradictory results have been described by Shunsuke et al. [41], who demonstrated that indoxyl significantly enhanced monocyte adherence to LPS-activated HUVECs under physiological share conditions. However, these discrepancies with our results are associated with higher IS concentrations (i.e., 200-fold higher) these Authors used in the study, and more prolonged endothelium stimulation, lasting 20 h. This suggests a concentration- and exposure-time-dependent effect of indoxyl on monocyte adhesion to vascular endothelium.

It has long been known that LPS and indoxyl induce inflammation [42,43]. However, findings to date refer to high or even very high LPS and indoxyl concentrations accompanying sepsis or CKD, which rarely relate to the actual blood levels of both metabolites in CAD patients without kidney failure. Similarly, studies of indoxyl toxicity to endothelium are based on high concentrations of the metabolite detected in CKD patients sera. Although the study results hardly reflect the scenario in vivo, they contribute to a better understanding of the influence of bacterial metabolites on atherogenesis and the role of gut dysbiosis in this process. A significant limitation of the study is the small group of patients enrolled which was associated with strict recruitment criteria excluding patients with various chronic diseases often associated with CAD. Nonetheless, the study indicated that bacterial metabolites leaking from dysbiotic gut to blood, even in meager concentrations, could adversely affect vascular endothelium, promoting the development or progression of CAD. Moreover, the study demonstrated co-toxicity of bacterial metabolites entering blood during gut dysbiosis for the first time.

Conclusions. A metagenome sequencing analysis revealed gut dysbiosis in CAD patients that was further confirmed by elevated levels of bacterial metabolites, i.e., LPS and indoxyl sulfate in patients sera. Both metabolites
demonstrated co-toxicity in meager concentrations to endothelial cells inducing ROS, E-selectin, and MCP-1 production and promoting thrombogenicity of endothelial cells confirmed by monocyte adherence.
Back matter

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Author contributions
MC, BS, TR, RJ Conceptualization, Investigation, BS, WU, MC Methodology, KL, ŁL, MC Software, Validation, Data Curation, MC, BS Original Draft Preparation, Writing – Review & Editing, MC, BS MW Writing – Review & Editing, Supervision, BS Project Administration, Visualization, MC Funding Acquisition. All authors reviewed the manuscript.

Institutional Review Board Statement
All participants of the study gave their written consents; the study followed guidelines of the Helsinki Declaration and was approved by Wroclaw Medical University’s Ethics Committee.

Conflict of interest
The authors declare no known competing financial interests or personal relationships that could influence the work reported in this paper.

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