ORIGINAL ARTICLE

Relationship between Pro-Inflammatory Cytokines and Gut Microbiome in Chronic Coronary Syndrome Patients Undergoing Coronary Angiography: A Cross-Sectional Study

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Background: Chronic coronary syndrome (CCS) patients have a high mortality rate globally. Atherosclerosis, a cause of CCS, is influenced by inflammation. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) have a key role in the process of atherosclerosis. Moreover, gut microbiota dysbiosis can lead to leaky gut syndrome, subsequently triggering abnormal immune responses and contributing to diseases, including atherosclerosis and coronary artery disease (CAD).

Objective: To study the relationship between pro-inflammatory cytokines and gut microbiome in CCS patients undergoing coronary angiography.

Material and Methods: Participants were divided into two groups by using statistical matching techniques with age and gender, as CCS patients and healthy participants. Each patient's blood was collected on the day of the appointment. All patients' feces were collected one day before an appointment. The present research was a cross-sectional study.

Results: Fifty-three patients, including 28 CCS patients and 25 healthy participants were enrolled. CCS patients had a higher level of TNF-α compared to healthy participants with statistical significance at 79.31 pg/mL. *Phascolarctobacterium*, *Sutterella*, and *Prevotella* could distinguish CCS patients from healthy participants based on receiver operating characteristic (ROC) analysis. *Proteus* and *Phascolarctobacterium* were positively correlated with TNF-α.

Conclusion: There is a potential relationship between gut microbiome composition and inflammatory biomarkers in CCS patients. Pro-inflammatory cytokines and specific bacterial genera may be related to indicate significant CAD in CCS patients undergoing coronary angiography.

Keywords: Cardiovascular disease; Interleukin-1; Interleukin-6; Tumor necrosis factor-alpha; Pro-inflammatory cytokines; Gut microbiome; Chronic coronary syndrome

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Coronary artery disease (CAD) is associated with an atherosclerosis process, typically involving the formation of atherosclerotic plaque in the lumen of coronary arteries, resulting in vascular occlusion and thus, insufficient blood and oxygen supply to the myocardium[1]. CAD can be categorized into acute coronary syndrome (ACS) and chronic coronary syndrome (CCS)[2]. Globally, CAD remains to be the leading cause of premature death. In Asia, the mortality of patients with CAD has also dramatically increased from 23% to 35% between 1990 and 2019[3].

It is well-accepted that atherosclerosis and inflammation are closely linked[4]. As such, chronic infection creating an inflammatory milieu may also be related to atherogenesis. Studies have shown the association between atherosclerotic disease and chronic infections including cytomegalovirus, hepatitis C virus (HCV), and *Chlamydia pneumoniae*[5].

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Moreover, exposure to an aggregate number of pathogens, known as an infectious burden, further aggravates the inflammatory response and CAD risk[6].

Gut microorganisms play a crucial part in regulating the metabolic health of their human hosts. Hence, metabolic diseases, which include CAD, are also mediated by an imbalance of gut microbiome or gut dysbiosis[7-9]. For instance, certain gram-negative bacteria that produce lipo-polysaccharides (LPS), such as *Escherichia coli*, *Shigella*, *Veillonella*, *Haemophilus*, and *Klebsiella*, were more abundant in stool from patients with more severe CAD[10]. Dysbiosis of gut microbiota promotes an inflammatory response by modulating intestinal permeability and subsequently leading to intestinal inflammation evidenced by elevated levels of circulating pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP)[11-13]. There is no data about the relationship between gut dysbiosis and atherosclerosis in Thailand.

The present study, therefore, aimed to evaluate the relationship between TNF-α, interleukin-1 (IL-1), and IL-6 and gut microbiome in CCS patients undergoing coronary angiography. Moreover, the authors aimed to explore the utility of specific bacterial genera and pro-inflammatory cytokines to identify significant CAD in CCS patients undergoing coronary angiography.

Materials and Methods

Participants and study design

Patients between the ages of 35 and 70 hospitalized at Chulabhorn Hospital were recruited between February and July 2023. The present research was a cross-sectional study. Patients with CAD having at least one coronary artery with 70% stenosis by coronary angiography were included in the CCS group. The control group included healthy participants who were asymptomatic, did not have any cardiovascular risk factors, and had no prior history of CAD.

Patients who fulfilled one of the following criteria were excluded, 1) chronic kidney disease, liver disease, cancer, immunodeficiency, history of gastrointestinal disease, or other infections within four weeks; 2) use laxatives, probiotics, or antibiotics within four weeks; 3) alcoholism or smoking; 4) pregnant or lactating.

CCS patients were enrolled at the outpatient clinic, cardiovascular center, Chulabhorn Hospital in person within one to two weeks after the identification of the index case. Healthy participants were enrolled voluntarily in the project. The participants were divided into two groups, CCS patients and healthy participants. The sample size calculation was based on "Hypothesis testing and power calculations for taxonomic-based human microbiome data[14]". There were 25 patients in each group. This sample size may provide adequate power to differentiate gut microbiome or levels of cytokines between the two groups.

The research ethics committees at Chulabhorn Hospital and Srinakharinwirot University approved the present study (IEC No. 174/2564 and IEC No. SWUEC/E/M-100/2565E respectively), and the study was conducted with the Good Practices for Clinical Research in Thailand, Thai Clinical Trails Registry, TCTR20230428002, granted the study approval. Written informed consents were obtained from all study patients.

Sample collection and DNA sequencing of fecal samples

Each patient's blood was collected on the day of the appointment to evaluate fasting blood sugar (FBS), hemoglobin A1C (HbA1C), total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, high-sensitivity C-reactive protein (hs-CRP), IL-1, IL-6, and TNF-α.

In CCS patients and healthy participants' feces were collected one day before appointment in DNA/RNA shield fecal collection tubes (Zymo Research, CA, USA) and immediately frozen at –20°C for 48 hours before analysis. DNA was extracted by using the QIAamp Stool Mini kit (Qiagen, USA). Nanodrop and electrophoresis were used to evaluate the quantity and quality of DNA. The V4 hypervariable region of the 16S rRNA gene was amplified by PCR using 515 F and 806R primers and 2X KAPA hot-start ready mix. The PCR conditions included an initial denaturation at 94°C for three minutes, followed by 25 cycles of 98°C for 20 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for five minutes. The 16S amplicons were purified using AMPure XP beads and indexed using Nextera XT index kit, followed by eight cycles of the aforementioned PCR condition. The PCR products were then cleaned and pooled in preparation for cluster generation and Illumina® MiSeq™ 250-bp paired-end read sequencing.

Sequencing data analysis

To process the raw sequence data, the authors employed the q2-demux plugin for demultiplexing. To enhance data quality, the authors utilized DADA2 (via q2-dada2) to remove reads with expected errors (maxEE) exceeding 3.0. Subsequently, the authors employed the classify-sklearn naive Bayes taxonomy classifier to classify ASVs against the Greengenes 13_8 99% operational taxonomic units (OTUs) reference sequences. Correlation between gut microbiota and TNF-α, IL-1, and IL-6 were investigated using Spearman's correlation coefficients. Heat map visualization was generated using the ggplot2 R package. A p-value less than 0.05 was considered statistically significant and was labeled in the figure.

TNF-α, IL-1, and IL-6 measurement

Each patient's pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) were measured using a cytokine-specific quantitative enzyme-linked immunosorbent assay (ELISA, R&D Systems in Minneapolis, Minnesota, USA), according with the manufacturer's instructions. Briefly, mouse anti-human TNF-α antibodies were used as capture antibodies and overnight coated on 96-well microtiter plates. To reduce non-specific binding, wells were blocked with 300 microliters of 1% (w/v) bovine serum album (BSA: Sigma, USA) in PBS (reagent diluent) for two hours. Recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) was used as standard. Standard or samples were added to appropriate wells and plates were incubated overnight. Biotinylated goat anti-human TNF-α antibodies, (R&D Systems, Minneapolis, MN, USA) were added as detection antibodies and incubated for two hours. The plates were then incubated with streptavidin-horseradish peroxidase conjugate for 20 minutes (R&D Systems, Minneapolis, MN, USA). TMB substrate (tetramethyl benzidine: BioFX, USA) was added to the plates as a color indicator and incubated for 20 minutes. A stopping reagent consisting of H_2SO_4 was added to stop the reaction. Absorbance was measured at 450 nanometers using a BioTek Synergy H1, USA. In each step, the plate was washed three times with PBS containing 0.05% Tween 20. For IL-1 and IL-6 measurements were used specific antibodies and standard of IL-1 and IL-6, respectively. The entire process was carried out at room temperature. Cytokine concentrations were quantified from the standard curve and expressed as picogram per milliliter (pg/mL) of serum. Results were reported as means of triplicate experiments with standard deviations (SD). The statistical differences were evaluated by using the student's t-test with a one-tailed distribution. The number of experiments conducted was indicated by the letter "n", and a p-value of 0.05 was regarded as statistically significant.

Statistical analysis and visualization

To assess differences between the CCS patients' group and the healthy participants' group, the authors used Fisher's exact test or chi-square test for category data. A p-value of less than 0.05 was considered significant. Descriptive statistics were presented as numbers (percentages).

Descriptive statistics for continuous data were shown as mean±SD in the case of regularly distributed data or as median (interquartile range, IQR) in the case of non-normally distributed data. For inferential statistics, the independent t-test was used if the data were normally distributed or the Mann-Whitney U test was used if the data were not normally distributed, which then tested the normal distribution with Shapiro-Wilk test statistics. When the p-value was less than 0.05, statistics were considered significant. The statistical information was examined using Stata/SE 16.1 (StataCorp LLC, College Station, TX, USA). The receiver operating characteristic (ROC) curves were created by GraphPad Prism 9.1.2. The variables in the logistic regression equation were not adjusted. As for the combination of variables, to ascertain whether or if the combination of particular bacterial genera and pro-inflammatory cytokines would increase prediction efficiency, all forms had been combined.

Results

Fifty-three patients were included and divided into two groups as CCS patients and healthy participants with 28 and 25 patients in each group, respectively. The patients were 39.62% female, with a median age of 58 years, and 45.28% had hypertension. There was no statistically significant difference in gender and age between the two groups. CCS patients had a higher proportion of obesity, metabolic syndrome, diabetes mellitus, and dyslipidemia at 42.86%, 32.14%, 28.57%, and 60.71%, respectively. Characteristics of the patients are shown in Table 1.

Pro-inflammatory cytokine levels

According to pro-inflammatory cytokine results,

Table 1. Characteristics of the patients (n=53)

Parameters	CCS (n=28)	Healthy (n=25)	p-value
Age (years); median (IQR)	60 (55.5 to 66.5)	54 (50 to 59)	0.069[b]
Male; n (%)	18 (64.29)	14 (56.00)	0.538[c]
BMI (kg/m^2); median (IQR)	24.32 (22.39 to 26.47)	22.66 (21.00 to 23.44)	0.005[b]
Waist circumference (cm); mean±SD	87.50±8.82	77.74±6.34	<0.001[a]
History of CAD; n (%)	16 (57.14)	0 (0.00)	<0.001[c]
Medication; n (%)			
Antiplatelets	28 (100)	0 (0.00)	<0.001[c]
Antihypertensive drugs	23 (82.14)	0 (0.00)	<0.001[c]
Oral antidiabetic drugs	7 (25.00)	0 (0.00)	0.011
Statins	27 (96.43)	0 (0.00)	<0.001[c]
• Statin intensity			
Low intensity	1 (3.57)	0 (0.00)	
Moderate intensity	2 (7.14)	0 (0.00)	
High intensity	25 (89.29)	0 (0.00)	
• Duration ≥3 months	27 (96.43)	0 (0.00)	
Obesity+; n (%)	12 (42.86)	0 (0.00)	<0.001[c]
Abdominal obesity*; n (%)	11 (39.29)	3 (12.00)	0.025[c]
Hypertriglyceridemia**; n (%)	7 (25.00)	0 (0.00)	0.011[d]
Low HDL-C[#]; n (%)	12 (42.86)	0 (0.00)	<0.001[c]
Impaired fasting glucose[@]; n (%)	19 (67.86)	4 (16.00)	<0.001[c]
Metabolic syndrome; n (%)	9 (32.14)	0 (0.00)	0.002[d]
Hypertension; n (%)	24 (85.71)	0 (0.00)	<0.001[c]
Diabetes mellitus; n (%)	8 (28.57)	0 (0.00)	0.005[d]
Dyslipidemia; n (%)	17 (60.71)	0 (0.00)	<0.001[c]
Heart failure; n (%)	1 (3.57)	0 (0.00)	1.000[d]
Stroke; n (%)	0 (0.00)	0 (0.00)	1.000[d]
PAD; n (%)	1 (3.57)	0 (0.00)	1.000[d]
SBP (mmHg); median (IQR)	125 (113.5 to 136.5)	120 (115 to 126)	0.101[b]
DBP (mmHg); mean±SD	72.75±7.72	76.24±10.93	0.182[a]
Laboratory data			
FBS (mg/dL); mean±SD	111.07±19.92	89.72±9.15	<0.001[a]
HbA1C (mg/dL); median (IQR)	5.80 (5.45 to 6.30)	5.20 (5.00 to 5.60)	0.004[b]
Total Cholesterol (mg/dL); median (IQR)	141.5 (114.5 to 168.5)	182 (170 to 189)	0.006[b]
Triglyceride (mg/dL); median (IQR)	104 (77 to 157)	83 (63 to 101)	0.047[b]
LDL-C (mg/dL); median (IQR)	73.1 (54.5 to 101)	108.9 (98.6 to 128.1)	<0.001[b]
HDL-C (mg/dL); median (IQR)	41.5 (38.0 to 49.5)	61 (50 to 74)	<0.001[b]
Serum creatinine (mg/dL); median (IQR)	0.93 (0.84 to 1.09)	0.71 (0.65 to 0.78)	<0.001[b]
AST (IU/L); median (IQR)	20 (19 to 25.5)	16 (14 to 20)	0.001[b]
ALT (IU/L); median (IQR)	23.5 (15 to 31.5)	14 (11 to 17)	0.001[b]
hs-CRP (mg/dL); median (IQR)	1.35 (0.80 to 3.93)	0.91 (0.52 to 1.75)	0.047[b]
TNF-α (pg/mL); median (IQR)	79.31 (76.16 to 81.04)	75.96 (74.76 to 78.32)	0.028[b]
IL-1 (pg/mL); median (IQR)	23.15 (22.33 to 25.66)	23.81 (23.12 to 26.03)	0.149[b]
IL-6 (pg/mL); median (IQR)	39.23 (34.25 to 57.19)	33.67 (31.61 to 41.44)	0.064[b]

ALT=alanine aminotransferase; AST=aspartate aminotransferase; BMI=body mass index; CAD=coronary artery disease; CCS=chronic coronary syndrome patients; DBP=diastolic blood pressure; FBS=fasting blood sugar; HbA1C=hemoglobin A1C; healthy=healthy participants; hs-CRP=high-sensitivity C-reactive protein; HDL-C=high-density lipoprotein cholesterol; IL-1=interleukin-1; IL-6=interleukin-6; IQR=interquartile range; LDL-C=low-density lipoprotein cholesterol; PAD=peripheral artery disease; SBP=systolic blood pressure; SD=standard deviation; TNF-α=tumor necrosis factor-alpha

[a] Independent t-test, significant when p<0.05, [b] Mann-Whitney U test, significant when p<0.05, [c] Chi square test, significant when p<0.05, [d] Fisher's exact test, significant when p<0.05, + BMI ≥25 kg/m^2, * waist circumference >90 cm for male, waist circumference >80 cm for female, ** triglyceride ≥150 mg/dL, [#] HDL-C <40 mg/dL for male, HDL-C <50 mg/dL for female, [@] FBS ≥100 mg/dL

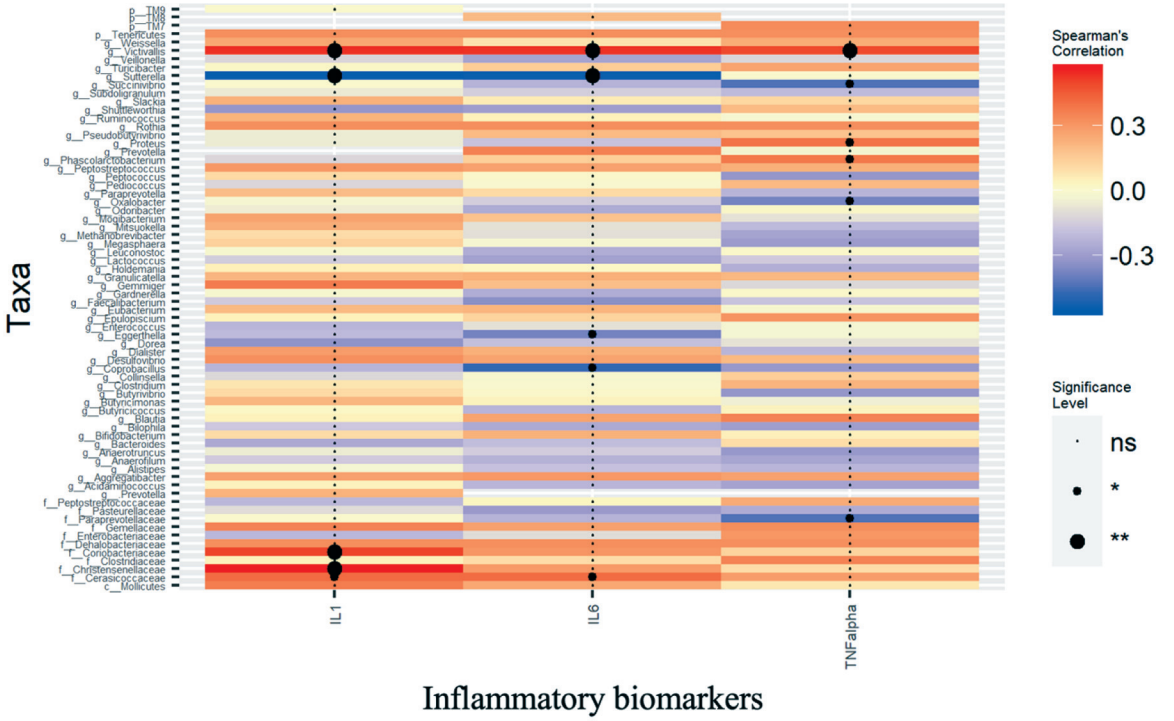

Figure 1. Spearman's correlation analysis between pro-inflammatory cytokines and the gut microbiome in CCS patients (n=28). The color represents positive (red) or negative (blue) correlations.

* $p<0.05$, ** $p<0.01$, IL-1=interleukin-1; IL-6=interleukin-6; TNF-α=tumor necrosis factor-alpha

TNF-α was higher in CCS patients compared to the control group with statistically significant at 79.31 pg/mL. The IL-6 level was higher in CCS patients than in healthy participants. Moreover, CCS patients had a higher level of hs-CRP at 1.35 mg/dL than healthy participants with statistical significance.

The relationship between pro-inflammatory cytokines and gut microbiome

Spearman correlation coefficient analysis showed that *Proteus* and *Phascolarctobacterium* were positively correlated with TNF-α. *Victivallis* had a positive association with IL-1, IL-6, and TNF-α. Family *Christensenellaceae* and *Coriobacteriaceae* were positively correlated with IL-1. On the other hand, *Sutterella* was negatively correlated with IL-1 and IL-6 (Figure 1).

The prediction model of the area under the curve based on receiver operating characteristic analysis

In the present study, ROC analysis revealed that TNF-α, IL-1, IL-6, and hs-CRP could distinguish CCS patients from healthy participants with area under the curve (AUC) values of 0.67 (95% CI 0.53 to 0.82), 0.62 (95% CI 0.46 to 0.77), 0.65 (95% CI 0.50 to 0.80), and 0.66 (95% CI 0.51 to 0.81, respectively (Figure 2a). The ROC analysis of the genera of gut microbiome demonstrated that the AUC values of *Phascolarctobacterium*, *Sutterella*, and *Prevotella* were 0.58 (95% CI 0.42 to 0.74), 0.67 (95% CI 0.52 to 0.81), and 0.59 (95% CI 0.43 to 0.74), respectively (Figure 2b). The AUC values for the combinations of TNF-α and IL-6 and TNF-α, IL-6, and hs-CRP were 0.70 (95% CI 0.56 to 0.85) and 0.70 (95% CI 0.55 to 0.84), respectively (Figure 2c, d).

Discussion

The present study is the first investigation on the association between the gut microbiome and TNF-α, IL-1, and IL-6 in CCS patients undergoing coronary angiography. Moreover, pro-inflammatory cytokines may be related to CCS patients in the present study. According to the present study, TNF-α was statistically significantly greater in CCS patients than in the other group, which is consistent with other studies. One of the most potent pro-inflammatory cytokines is TNF-α. In the elderly group, a high prevalence of atherosclerosis was linked to high TNF-α levels[15]. The study of mice showed that TNF-α had a significant role in the development

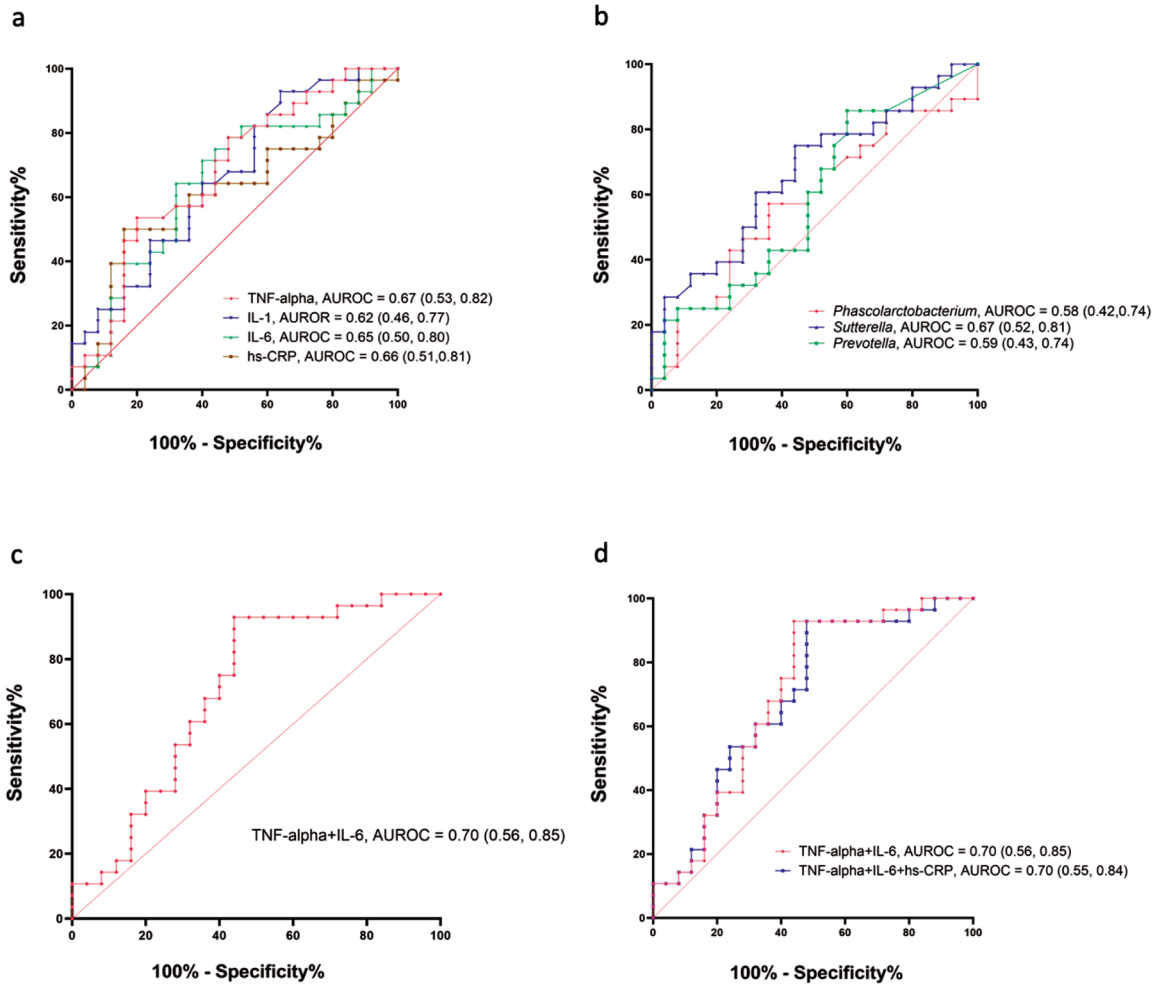

Figure 2. Gut microbiome and clinical features could effectively distinguish CCS patients from healthy participants. TNF-α, IL-1, IL-6, and hs-CRP (a), gut microbiome features (b), the combination of TNF-α and IL-6 (c), the combination of TNF-α and IL-6 and TNF-α, IL-6, and hs-CRP (d) to build the prediction model yielded an AUC based on ROC analysis.

AUC=area under the curve; CCS=chronic coronary syndrome; hs-CRP=high-sensitivity C-reactive protein; IL-1=interleukin-1; IL-6=interleukin-6; ROC=receiver operating characteristic; TNF-α=tumor necrosis factor-alpha

of atherosclerosis[16]. On the other hand, TNF-α inhibition resulted in reduced atherosclerosis[17,18].

There is a causal connection between CAD and the gut microbiome. A decrease in the abundance of the gut microbiome and the production of butyrate, along with an increase in systemic inflammation, are indicative of the progression of CAD[19]. Inflammation is linked to a leaky gut. An inflammatory cascade triggered by microbial translocation has the potential to worsen pre-existing conditions or cause CAD[20]. Zhu et al. showed that in the Chinese CAD patients displayed fewer OTUs overall, as well as reduced richness and diversity of gut microbiome, according to the gut dysbiosis hallmarks of the disease. This investigation also showed that the CAD group had high concentrations of infections, such as *Enterococcus* and *E. coli*[21].

It is well recognized that variables, including genetics, food, lifestyle, and environment, can affect the gut microbiome[8,22]. Thai people's gut microbiota showed Firmicutes and Bacteroidetes predominated. The first three prevalent genera were determined to be *Bacteroides*, *Prevotella*, and *Faecalibacterium*[23]. In Thailand, there is no data about the gut microbiome of CCS patients. Patients with metabolic syndrome who smoked and drank heavily seemed to have significantly higher levels of *Prevotella*[24]. In Western patients, a correlation has been observed between gut microbiome, CAD, and the Western diet[25]. Foods high in choline, betaine, and phosphatidylcholine,

found in most Western recipes such as eggs, fish, red meat, soybeans, and peanuts are key sources of trimethylamine-N-oxide (TMAO), a potent risk factor for the development of CAD[26,27]. Proteobacteria, particularly by Enterobacteriaceae and Firmicutes is the abundance of bacteria producing TMAO precursor[28]. Along with increased intestinal permeability, TMAO is associated with raised blood levels of the endotoxin LPS, endothelial dysfunction, and CRP. Platelet hyperreactivity, which affects the advancement of CAD, can also result from it[29].

There was a trend that showed lower diversity CCS patients than in healthy volunteers (unpublished data). The most prevalent bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria[30]. Previous studies have reported that *Prevotella* and *Streptococcus* genera had a close relationship with metabolic syndrome, atherosclerosis, and CAD[31-34]. It suggested that the changes in the abundance of *Prevotella* and *Streptococcus* were the characteristics of the bacterial microbiota of the CCS patients. The greatest associations were found for *Streptococcus anginosus* and *Streptococcus oralis*, according to Sayols-Baixeras et al.'s study of the correlation between *Streptococcus* spp. and subclinical coronary atherosclerosis[35].

Specific bacterial genera, such as *Sutterella*, *Prevotella*, and *Phascolarctobacterium* may be related to CCS patients from healthy participants. This finding has not been reported before. *Prevotella*'s impact on human health is debatable. Recent studies have linked the increased *Prevotella* abundance and specific strains to metabolic syndrome, obesity, insulin resistance, and low-grade systemic inflammation due to the stimulation of epithelial cells to produce IL-1, IL-6, interleukin-18 (IL-8), and interleukin-23 (IL-23). Additionally, it enhanced mucosal helper T-cell (Th17) immune responses[36]. Because of its functions in inflammation and immunology, it is a potential major pathogen linked to CAD[37].

In the CCS patients' group, *Proteus* and *Phascolarctobacterium* were positively correlated with TNF-α. Genus *Proteus* are gram-negative bacilli, in the Enterobacteriaceae family. They produce LPS and should be correlated with a rise in TNF-α.

Gram-negative bacteria that generate LPS, including *E. coli*, *Shigella*, *Proteus*, *Veillonella*, and *Klebsiella*, increased the severity of CAD[10]. Lipoprotein (LP) isolated from *E. coli* was found to increase the production of TNF-α and IL-6. LP is a major part of bacteria in Enterobacteriaceae. In a mice study, the production of cytokines was synergistically stimulated by both LP and LPS from macrophages via different receptors and signal pathways in septic shock[38]. Not only CAD patients but also major depression (MDD), HIV infection, inflammatory bowel disease, and rheumatoid arthritis are accompanied by leaky gut with an increased translocation of LPS from gram-negative enterobacteria through increased IL-6 and interferon-gamma[39-41]. Even though *Veillonella parvula* LPS is less effective than Enterobacteriaceae LPS, it may still cause the production of cytokines such as TNF-α, IL-1, IL-6, and IL-10, via Toll-like receptor (TLR) pathways in humans and mice[42].

Prior studies suggested that *Bifidobacterium*, a short-chain fatty acids (SCFAs)-producing bacteria, is a protective microorganism against CAD due to its ability to produce SCFAs, modulating the effect of the inflammatory reaction brought by TNF-α and IL-6[43-45]. A randomized clinical trial showed *Bifidobacterium adolescentis*, *B. bifidum*, *B. animalis*, and *Butyricicoccus porcorum*, detected in the probiotic group, added benefits to CAD patients with significantly lower IL-6 and LDL-C level[46]. Moreover, *B. lactis* has the ability to lower cholesterol levels, TNF-α, IL-6, and BMI, which may lower the risk of cardiovascular disease in metabolic syndrome patients[44]. *B. breve* and *B. longum* may be effective in treating TMAO-related diseases[47]. CAD mice and patients have a lower relative abundance of *Bifidobacterium*[48]. However, this bacterium was not found to have statistically significant association in the present study.

The difference of TNF-α and hs-CRP between the two groups is not massive. Normally, pro-inflammatory cytokines are small amount. It is difficult to identify the differences between the two groups. The authors did not expect this finding. However, the difference between the two groups is statistically significant.

The research had limitations, including that 1) it did not experimentally investigate the specific function and metabolites of the gut microbiota, 2) the authors were unable to control other potential confounding factors such as obesity, impaired fasting glucose, and polypharmacy, which may have affected the results of the present study, 3) a shotgun sequencing approach yield more information (vs 16S rRNA V4 region sequencing) given the larger set of genes and ability to profile metabolic pathways, however, there were budget limitations, and 4) the present study was cross-sectional design thus, the study's design limited the ability to infer

causality between gut microbiome changes and CCS patients.

The present study found that gut microbiome in specific genera and pro-inflammatory cytokines may be related to CCS patients from healthy participants. Moreover, the correlation between pro-inflammatory cytokines and the gut microbiome in CCS patients was demonstrated. The present research should be continued to increase the data of Thai CCS patients in multi-centers. This will increase the understanding of the impact of pro-inflammatory cytokines, specific bacterial genera, and significant CAD in CCS patients undergoing coronary angiography and extend these findings before translational applications.

Conclusion

There is a potential relationship between gut microbiome composition and inflammatory biomarkers in CCS patients. Pro-inflammatory cytokines and specific bacterial genera may be related to indicate significant CAD in CCS patients undergoing coronary angiography.

What is already known on this topic?

Atherosclerosis, a cause of CCS, is influenced by inflammation. TNF-α, IL-1, and IL-6 have a key role in the process of atherosclerosis. Moreover, gut microbiota dysbiosis can lead to leaky gut syndrome, subsequently triggering abnormal immune responses and contributing to diseases, including atherosclerosis and CAD.

What does this study add?

Pro-inflammatory cytokines and the composition of the gut microbiota may be related in CCS patients. TNF-α, IL-1, IL-6, and specific bacterial genera may be related to indicate significant CAD in CCS patients undergoing coronary angiography.

Authors' contributions

Conceptualization, WL, PP, and MT; data curation, WL, PP, and MT; format analysis, WL and MT; funding acquisition, WL, and MT; investigation, WL, and MT; methodology, WL and MT; project administration, WL; resources, WL, PP, and MT; software, WL, PP, and MT; supervision, PP, MT, and EE; validation, WL, PP, and MT; visualization, WL, PP, MT, and WP; writing - original draft preparation, WL; writing - review and editing, WL, PP, MT, and SF; All authors have read and agreed to the published version of the manuscript.

Data availability

The raw sequence data are available under BioProject PRJNA1000984 from the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1000984?reviewer=kpesdmrsqch7ijls8j4i6ai1bo, and Biosample SAMN36786011.

Funding disclosure

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Conflicts of interest

The authors declare no conflict of interests.

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