ASSOCIATION OF GUT MICROBIOTA WITH DYSLIPIDEMIA AND CHRONIC CORONARY SYNDROME PATIENTS UNDERGOING CORONARY ANGIOGRAPHY

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ASSOCIATION OF GUT MICROBIOTA WITH DYSLIPIDEMIA AND CHRONIC CORONARY SYNDROME PATIENTS UNDERGOING CORONARY ANGIOGRAPHY

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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THE DISSERTATION TITLED

ASSOCIATION OF GUT MICROBIOTA WITH DYSLIPIDEMIA
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BY

WONGSAKORN LUANGPHIPHAT

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Dyslipidemia is a significant risk factor for chronic coronary syndrome (CCS), which has a high death rate. Dysbiosis of the gut microbiota, its metabolites, and inflammation have an impact on atherosclerosis, which is the etiology of CCS. The objective was to compare the gut microbiota composition and diversity among CCS patients undergoing coronary angiography and dyslipidemia patients to healthy volunteers in Thailand, as well as to examine the clinical characteristics associated with these conditions. A total of 91 patients met the inclusion criteria. According to our findings, CCS patients exhibited higher risk factors, inflammatory markers, and high-sensitivity C-reactive protein (hs-CRP) than others. We demonstrated that compared to the healthy group, patients with CCS and dyslipidemia had reduced alpha diversity. The gut microbiota compositions of three groups differed significantly. In CCS patients, there was a significant rise in the relative abundance of Proteobacteria, Fusobacteria, Enterobacteriaceae, *Prevotella*, and *Streptococcus*, but a decrease in *Roseburia, Ruminococcus,* and *Faecalibacterium.* Lachnospiraceae, Peptostreptococcaceae, and *Pediococcus* showed favorable correlations with hs-CRP in CCS patients. The genera *Sutterella* and *Roseburia* showed a negative correlation with low-density lipoprotein cholesterol (LDL-C) in CCS patients. Megasphaera showed a substantial positive correlation with triglyceride (TG) levels in patients with dyslipidemia, but it showed a negative correlation with high-density lipoprotein cholesterol (HDL-C). The changes in clinical factors linked to the onset of coronary artery disease (CAD) in CCS patients were linked to altered gut microbiota. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) have an important role in the process of atherosclerosis. Moreover, gut microbiota dysbiosis can lead to leaky gut syndrome, subsequently triggering abnormal immune responses and contributing to several diseases, including atherosclerosis and CAD.

Keyword : cardiovascular disease, chronic coronary syndrome, dyslipidemia, gut microbiome, pro-inflammatory cytokines, 16S rRNA sequencing

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ABBREVIATIONS

ACS: acute coronary syndrome

ASCVD: atherosclerotic cardiovascular disease

ALT: alanine transaminase

AST: aspartate transaminase

Apo B-48: Apolipoprotein B-48

AUC: the area under the curve

AUROC: the area under the curve of receiver operating characteristic

BA: bile acid

CAD: coronary artery disease

CCS: chronic coronary syndrome

CKD: chronic kidney disease

CVD: cardiovascular disease

DNA: deoxyribonucleic acid

EDTA: ethylene diamine tetra acetic

ELISA: enzyme-linked immunosorbent assay

FMO3: flavin monooxygenase 3

FPG: Fasting plasma glucose

FXR: farnesoid X receptor

HbA1C: hemoglobin A1C

HDL-C: high-density lipoprotein cholesterol

HeFH: heterozygous familial hypercholesterolemia

hHSP60: human heat-shock protein 60

HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryt-coenzyme A reductase

hs-cTn T: high-sensitivity cardiac troponin T

hs-CRP: high sensitivity C-reactive protein

IDL: Intermediate density lipoprotein

LDL-C: low-density lipoprotein cholesterol

IFN: interferon

IL-1: interleukin-1

IL-6: interleukin-6

LDA: linear discriminant analysis

LEfSe: linear discriminant analysis effect size

LPS: lipopolysaccharide

MVD: multivessel disease

MyD88: myeloid differentiation primary response gene

Na: sodium

NGS: next-generation sequencing

NF-**κ**B: nuclear factor kappa B

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

RA: rheumatoid arthritis

RNA: ribonucleic acid

ROC: receiver operating characteristic

SCFA: short-chain fatty acids

SVD: single-vessel disease

TC: total cholesterol

TG: triglyceride

TINA: turbidimetric inhibition immunoassay

TMA: trimethylamine

TMAO: trimethylamine-*N*-oxide

TLRs: toll-like receptors

TNF-**α**: tumor necrosis factor-alpha

VLDL: very low-density lipoprotein

WHO: World Health Organization

CHAPTER I
INTRODUCTION

Cardiovascular disease (CVD) is one of the major problems in Thailand[1] and the leading cause of death worldwide.[2] There are many risk factors involved in this disease e.g. diabetes mellitus,[3] dyslipidemia,[4] hypertension,[5] obesity,[6] insulin resistance,[7] and metabolic syndrome.[8] At present, the risk factors of CVD can be reduced through lifestyle modifications, such as exercising, eating healthy food, and reducing salt intake to lower blood pressure levels in combination with medications such as antihypertensive medications, statins, and blood glucose-lowering agents. However, morbidity and mortality rates which are still extremely high in Thailand and throughout the world, are also impacted by CVD.

While there are additional risk factors for coronary artery disease (CAD), dyslipidemia is a significant one.[9] The timing of the onset of signs and symptoms distinguishes the two categories of CAD, acute and chronic coronary syndromes. Along with chronic coronary syndrome (CCS), CAD is a chronic, progressive disease that can be stabilized.[10]

Previous studies found that more than 1,100 types of bacteria are associated with many symptoms and diseases such as cancer, diabetes mellitus, obesity, and CVD.[11] Therefore, consuming the right amount of healthy food can prevent and cure CVD, including metabolic syndrome. Most microorganisms in the gastrointestinal tract cannot be cultured.[12] Therefore, the type and quantity of these microorganisms have to be studied by the genes of the microorganism, known as the microbiome.

A type of microbe called the gut microbiota resides in the digestive system of humans. Several external factors, including geographic origin, age, genetics, food, and the use of prebiotics and antibiotics, determine and influence the quantity and kind of microbes in the gastrointestinal tract.[13,14] Numerous microorganisms, including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria, are found throughout the gastrointestinal tract. These kinds of organisms differ in their modes of

action and habitats.[15] *Lactobacillus* and *Bifidobacterium* are important gastrointestinal bacteria which existed since birth.[16] The complex gut microbiota that influences the immune system, nutrition metabolism, and the stabilization of the gastrointestinal tract lining (gut epithelium) is gaining attention in modern times. In addition, the gut microbiota aids in the body's synthesis of vitamins and hormones and breaks down some substances that the stomach is unable to break down. There is also a complex relationship between the gut microbiome and the body via metabolites, including bile acid (BA), short-chain fatty acids (SCFA), and trimethylamine-*N*-oxide (TMAO). These metabolites can influence the function of several organs. Gut microbiota and metabolites modulate the process of atherosclerosis, a cause of CVD.

The involvement of gut dysbiosis in various diseases, such as atherosclerosis and numerous cardiovascular conditions, has been brought to light by recent research. These bacteria, along with their surroundings, diet, and medications, are inherited from the mother at birth.[17] If the gastrointestinal system has an imbalance of bacteria (dysbiosis), altering the intake of macronutrients causes metabolic syndrome.[18] A diet heavy in fat, low in fiber, and high in sugar has an adverse effect on the digestive system, including the gut ecology, and changes in gut microbiota will raise the risk of developing a number of diseases. In contrast to bacteria that produce butyric acid, like Ruminococcaceae and Lachnospiraceae, which decrease the onset of CAD, an increase in the abundance of gram-negative bacteria that produce lipopolysaccharides (LPS), such *as Escherichia, Klebsiella, Shigella, Veillonella,* and *Haemophilus*, increased the severity of CAD. Furthermore, the gut microbiota from a number of species, such as *Eubacterium rectale, Roseburia intestinalis, Faecalibacterium prausnitzii,* and Bacteroidetes phylum, are also protective against CAD.[19–21]

One of the methods that is used to study microbes is next-generation sequencing (NGS). NGS is used to discover microbes in many aspects because NGS does not rely on culture techniques. The advantages of NGS include its ability to identify more unique species than culture methods and the capacity to perform parallel sequencing of microbiome. Most commonly, the target is the bacterial 16S ribosomal

RNA (rRNA) gene. The 16S rRNA gene is the target because it is ubiquitous in for bacteria.

To the best of our knowledge, no study in Thailand has examined if the gut microbiome is associated with the parameters of patients with dyslipidemia and CVD. The purpose of this exploratory study is to identify potential relationships among relative gut microbiota composition and related parameters in patients with dyslipidemia and CVD in Thailand.

Hypothesis

1. Bacterial diversity is lesser in patients with dyslipidemia and chronic coronary syndrome than in healthy participants.
2. There are differences in gut microbiota composition among healthy participants and patients with dyslipidemia and cardiovascular disease.
3. Gut microbiota composition is related to blood parameters associated with chronic coronary syndrome.

Objectives

Primary objective

To evaluate the association between gut microbiota composition and related parameters in dyslipidemia and chronic coronary syndrome patients.

Secondary objective

1. To analyze gut microbiota diversity among patients with dyslipidemia, chronic coronary syndrome, and healthy participants.
2. To determine gut microbiota composition in patients with dyslipidemia and with chronic coronary syndrome patients.

Operative definition

Acute coronary syndrome

A term that is diagnosed by a physician and requires an electrocardiogram and a history of the signs and symptoms of cardiac ischemia. The common electrocardiographic abnormalities included T-wave tenting or inversion, ST-segment elevation or depression, and pathological Q waves. Troponin T or I generally is the most sensitive marker of acute coronary syndrome.

Atherosclerosis process

A term is used to describe the thickening and stiffness of the arteries caused by a buildup of plaque in the inner lining of arteries. This is the main process of coronary artery disease.

Chronic coronary syndrome

The new term has been introduced to replace the previous term 'stable coronary artery disease'. Coronary anatomy by coronary angiography is greater than 70% stenosis in coronary arteries that are larger than 2.5 mm in one view of a coronary angiogram, greater than 50% stenosis in coronary arteries in two views of coronary angiograms and greater than 50% stenosis in the left main coronary artery.

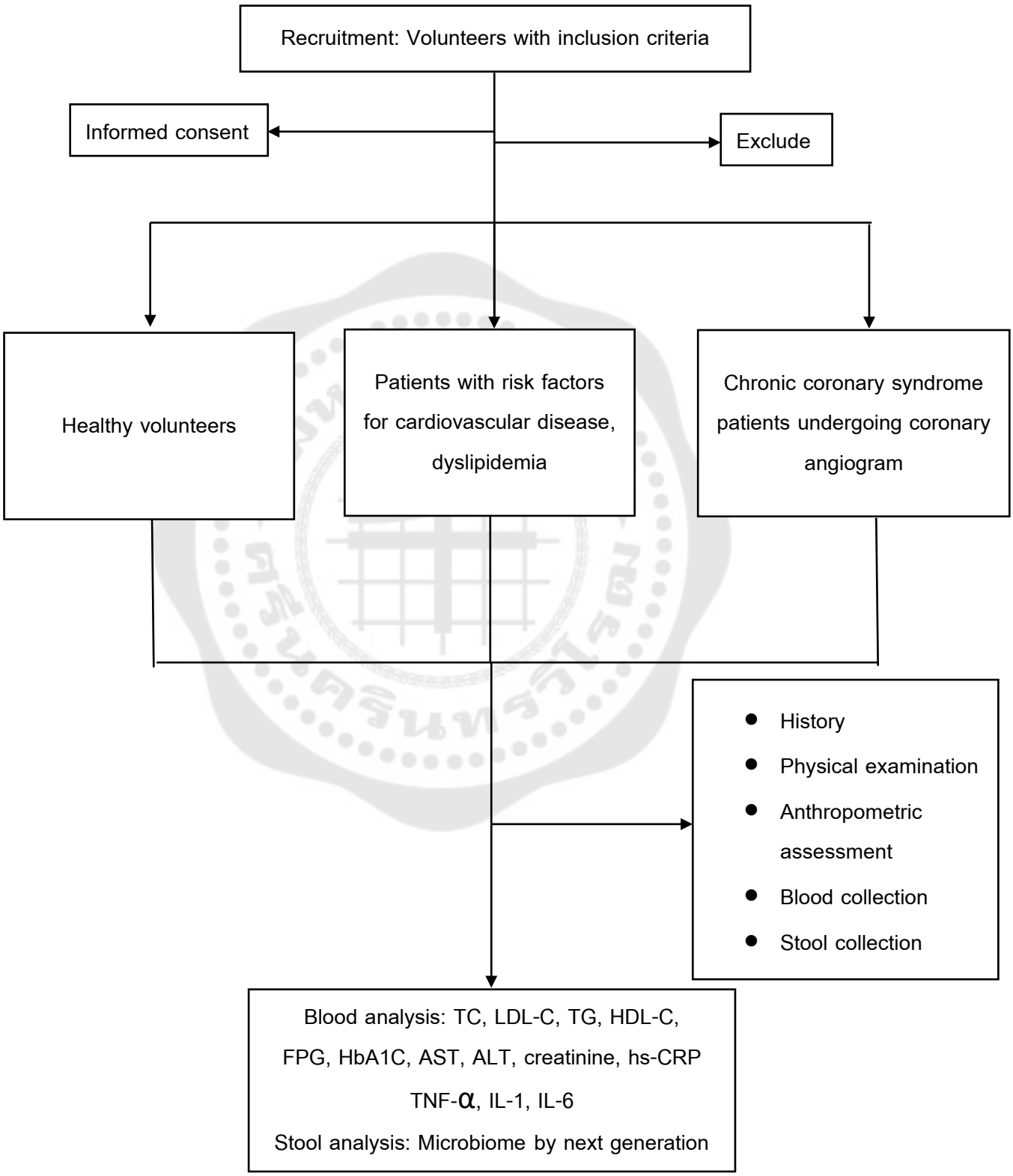
Coronary angiography

A term is used to describe a procedure that uses x-ray imaging to evaluate coronary arteries. This procedure is performed by interventional cardiologists. If there's any arterial luminal narrowing or any restriction in blood flow, it can be seen in coronary angiography.

Gut microbiota

A term is used to describe the microorganisms that live in the digestive tracts of humans. Gut microbiota is often composed of bacteria and has a direct relationship with the human body. Stool samples will be collected 1 day before the intervention and immediately stored at -20 °C. DNA will be extracted and the quantity and quality of DNA will be examined by nanodrop and electrophoresis. The V4 hypervariable region of the 16S rRNA gene is PCR amplified. The PCR products are cleaned and pooled for cluster generation and 250-bp paired-end read sequencing on the Illumina® MiSeq™. Microbiome bioinformatics are performed with QIIME 2 2019.10.

Experimental design



CHAPTER II
LITERATURE REVIEW

1. Coronary anatomy

Coronary arteries are the arterial blood vessels for coronary circulation. There are two main branches: the left main coronary artery and the right coronary artery. The left side of the heart muscle receives blood supply from the left main coronary artery. The left anterior descending artery splits off from the left coronary artery and feeds blood to the left side of the heart's anterior region. The circumflex artery surrounds the heart muscle and splits out from the left coronary artery. The rear and outside of the heart are supplied with blood by this artery. The right atrium, right ventricle, and the AV (atrioventricular) and SA (sinoatrial) nodes—which control heart rhythm—are all supplied with blood by right coronary artery. The right coronary artery assists in supplying blood to the heart's septum or middle chamber, along with the left anterior descending artery.

Coronary arteries are the first branch of the aorta, which consists of three layers: tunica intima (the inner layer), tunica media (the middle and elastic layer), and adventitia (the outer layer). Tunica intima normally consists of a single endothelial layer. The migration of smooth muscle cells from the tunica media to the tunica intima causes the intima's structure to alter with age and become multilayered. One of the key stages in the development of atherosclerosis is thought to be the multilayering of inflammatory cells and tunica intima. The heart receives its blood supply from the coronary arteries and their branches. They deliver blood that is oxygenated to the heart muscle. For the heart to function properly, oxygen must be continuously supplied. The heart is completely encircled by the coronary arteries. The arteries supply different areas of the heart. The reduced blood flow of the coronary arteries can decrease the flow of oxygen and nutrients to the heart. This can affect the ability of the heart to pump blood throughout the body.[22]

2. Cardiovascular disease

CVD is one of the major health problems in Thailand[1] and the leading cause of death worldwide.[2] There are many risk factors involved in this disease e.g. diabetes mellitus,[3] dyslipidemia,[4] hypertension,[5] obesity,[6] insulin resistance,[7] and metabolic syndrome.[8] At present, the risk factors of CVD can be reduced through lifestyle modifications, such as exercising, eating healthy food, and reducing salt intake to lower blood pressure levels in combination with medications. Nonetheless, CVD has an impact on both mortality and morbidity rates, which are still high in Thailand and other countries.

The term "CAD" describes a condition where the arteries supplying the heart muscle narrow or block. This is primarily due to the buildup of fat in the blood vessel walls, which thickens the endothelium layer of the blood channel. The heart's ability to receive oxygen and nutrients might be compromised by a reduction in coronary artery blood flow. The heart's capacity to circulate blood throughout the body may be impacted by this. Coronary arteries contain three layers: tunica intima, tunica media, and adventitia. The heart receives its blood supply from the coronary arteries and their branches. For the heart and the rest of the body to pump blood, cardiac muscles require oxygen and nourishment.[22] Patients present with signs and symptoms when arteries are narrowed by 50 percent or more, the most common symptoms include angina, palpitations, sweating, tiredness at work, fainting, unconsciousness, or sudden death. According to the onset of the signs and symptoms presentation, CAD is divided into acute and CCS.[23] However, the focus of this research proposal is on chronic CAD.

3. Chronic coronary syndrome

Definition and criteria of CCS[24]

The clinical settings of patients with CCS are:

1. Suspected CAD and stable anginal patients
2. New onset of left ventricular dysfunction patients
3. Stabilized symptoms <1 year after acute coronary syndrome patients
4. >1 year after initial diagnosis or revascularization patients
5. Suspected vasospastic or microvascular disease patients
6. Asymptomatic individuals in whom CAD is found during screening

Epidemiology of chronic coronary syndrome

The prevalence of CCS in the population is complex. The incidence of CCS falls continuously in developed countries over 20-30 years. In contrast, in developing countries, the incidence of CCS progressively increases due to unhealthy diets, sedentary lifestyles, and ineffective treatment.[25,26] CVD is the leading cause of death for people of most racial and ethnic groups in the United States.[27] About 6.7% of 20-year-old and older patients have CAD (18.2 million).[28] In Thailand, the prevalence of coronary heart disease was 9.9/1,000 and CVD is the second leading cause of death for 11.53% (51,305 patients) of total deaths in 2020.[27,29]

Risk factors

Atherosclerosis is one of the common forms of arterial disease in which lipid deposition forms a plaque in the blood vessel walls (Figure 1). A patient's lifestyle can be changed to lower a number of risk factors.

There are two groups of risk factors: modifiable and fixed risk factors. The modifiable risk factors e.g. hypertension, dyslipidemia, type 2 diabetes mellitus, smoking, obesity (especially abdominal obesity), lack of exercise, and alcohol consumption (more than two drinks per day) can be adjusted and controlled by the patients. The fixed risk factors include old age, male gender, and family history of premature CVD among first-degree relatives (< 55-year-old man and < 65-year-old woman).[30–32]

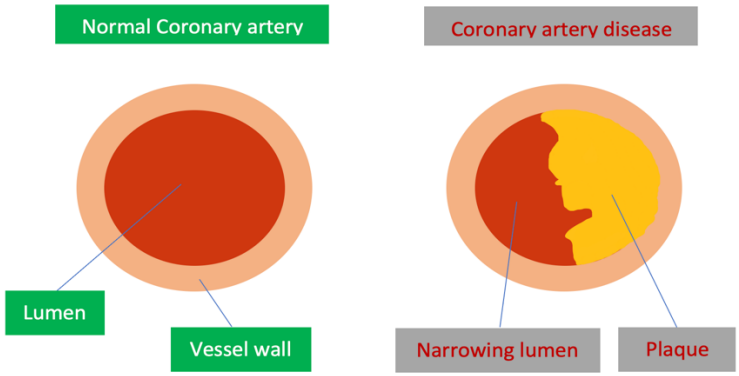


Figure 1 Image demonstrating a cross-section view of coronary heart disease (Reference: Adapted from Coronary heart disease-atherosclerosis by National Heart, Lung and Blood Institute. License: Public Domain, edited by Lecturio.)

Dyslipidemia is a significant risk factor in patients with CAD. From the INTERHEART study, dyslipidemia emerged as the most critical risk factor compared to hypertension, diabetes, smoking, and obesity (Figure 2).[33,34]

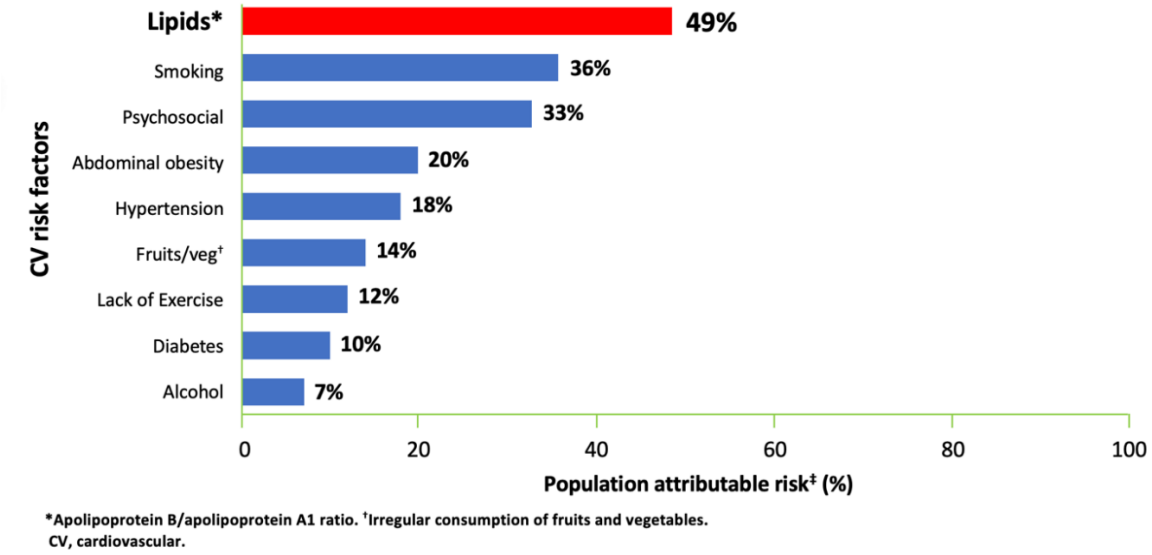


Figure 2 Lipids emerged as the most critical risk factor in the INTERHEART study (Reference: Adapted from Yusuf S, et al. Lancet 2004;364:937-52.)

Pathophysiology of chronic coronary syndrome

The atherosclerosis process is the evolution of the atherosclerotic plaque. Blood plasma's LDL-C particles penetrate the endothelium and oxidize. An inflammatory reaction happens when the endothelium sustains initial injury. Platelets stick to the damaged location as monocytes travel through the circulation and enter the arterial wall. The monocytes undergo macrophage differentiation and become foam cells after consuming oxidized LDL-C. In response to pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) released by injured endothelial cells and macrophages, there is migration and proliferation of smooth muscle from the tunica media into the intima. Atheromatous plaques develop in the artery's tunica intima as a result of the inflammation.[35]

The key mechanism is to maintain the blood supply to the heart muscles. At rest, in patients with CAD (luminal narrowing >50%), their post-stenotic pressure will drop and arteriole and precapillary will dilate to compensate for adequate blood volume. At exertion, the blood reserve of the heart muscles will be used up; therefore, the patients will have myocardial ischemia and angina pectoris.[36,37]

Angina pectoris is caused by myocardial ischemia, which has two main mechanisms:

1. **Increase myocardial oxygen demand**

   This is seen during increases in heart rate and blood pressure such as exertion and emotional/mental stress.

2. **Decrease myocardial oxygen supply**

   This is usually the result of epicardial artery stenosis or blockage (but maybe constriction of blood vessels at the microvascular level). Epicardial artery stenosis is due to the accumulation of atherosclerotic plaque build-up on the walls of blood vessels or is caused by atherosclerotic plaque rupture and causing thrombosis or possibly coronary vasospasm.[38]

At a resting state, the heart needs a small amount of blood and oxygen. During exercise, the body and heart work harder and faster to increase the amount of blood

delivered throughout the body and the heart muscles. The adaptation of the body and heart to maintain regular blood flow is called “auto-regulation”.[38]

In the presence of coronary artery stenosis with >50% luminal stenosis, the blood supply to the heart muscle is still sufficient during rest but is insufficient (demand ischemia) during exercise. Angina pectoris and dyspnea during physical activity and symptoms will subside when resting.

Treatment

There are two main goals for the treatment of CCS.

1. To relieve angina and improve quality of life

2.To prevent acute coronary syndrome, which is often caused by the patients’ death.[39,40]

There are two options for CCS treatment

1. Symptom-based method or Conservative medical method is treatment with medication, behavior modification (lifestyle modification), and cardiac rehabilitation.[41,42]

2. A coronary angiogram-based method is revascularization, catheter-based therapy (Interventional), or coronary bypass graft surgery.

Both options require behavioral modification (lifestyle modification) and medication to reduce risk factors together.[43,44] A group of drugs used to treat CCS are:

1. Drugs to reduce myocardial ischemia and suppress angina.

2. Drugs to prevent acute coronary syndrome and slow progression of atherosclerosis.[24,45]

Prevention of chronic coronary syndrome

There are several ways to reduce patients’ risks of developing CAD, such as lowering your blood pressure and cholesterol levels, consuming a healthy and balanced diet (avoiding food containing saturated fats and too much sugar, increasing food with high unsaturated fat), being more physically active, keeping to normal weight, giving up smoking, reducing alcohol consumption and taking any prescribed medication regularly.[46–49]

4. Dyslipidemia

Normal blood lipid levels are as follows: (1) total cholesterol (TC) < 200 mg/dL; (2) triglyceride (TG) < 150 mg/dl; (3) high-density lipoprotein-cholesterol (HDL-C) > 40 mg/dl; and (4) low-density lipoprotein-cholesterol (LDL-C) < 100 mg/dl. Patients with high TC and LDL-C levels have a higher risk of heart and blood vessel diseases.[50]

Definition and criteria of dyslipidemia

The definition of dyslipidemia is different from the medical practice guidelines used. Dyslipidemia refers to patients with abnormalities in lipoprotein metabolism, resulting in changes in blood lipid levels, which is a risk factor for atherosclerosis. Generally, dyslipidemia is determined by total cholesterol (TC) levels in blood more than 200 mg/dL, triglyceride (TG) greater than 150 mg/dL, and low-density lipoprotein cholesterol (LDL-C) greater than 160 mg/dL, etc. (Table 1).[51]

Table 1 Definition and criteria of dyslipidemia

Lipid Parameter	High
Total cholesterol	≥ 200 mg/dL
LDL-C	≥ 160mg/dL
Triglyceride	≥ 150 mg/dL

(Reference: Handelsman Y, et al. Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of CVD algorithm - 2020 Executive summary. Endocr Pract. 2020 Oct;26(10):1196-224.)

Epidemiology of dyslipidemia

The World Health Organization (WHO) estimates the prevalence of dyslipidemia (defined as blood levels of TC > 190 mg/dL) in Southeast Asia, the Western Pacific, Europe, and the Americas were 30.3%, 36.7%, 53.7%, and 47.7%, respectively.[52] The prevalence in Thais with some forms of dyslipidemia was 66.5%.[53] The elevation of

plasma LDL-cholesterol levels was the 15th leading risk factor of death in 1990, rising to 11th and 8th in 2007 and 2019, respectively. The global burden of dyslipidemias has increased over the past 30 years.(54)

Causes of dyslipidemia

There are two main causes of dyslipidemia which are the primary and secondary causes.

The primary cause is unknown. It is believed that it may be caused by genetic abnormalities in regulating the synthesis and metabolism of LDL cholesterol levels. This leads to high levels of LDL cholesterol in the blood. The other cause is abnormalities in genes in the regulation of creating LDL receptors in the liver which results in reducing the number of LDL receptors.

The secondary causes are varied, such as weight gain in the elderly, pregnancy, high carbohydrate diet, high saturated fat diet, diabetes, low thyroid hormone, chronic renal failure, liver disease, and certain medications, such as steroids, and oral contraceptives. The cause of low HDL-cholesterol levels and high triglyceride levels are obesity, smoking, and lack of exercise.(55)

Lipid metabolism

Since water-insoluble fat in the body binds to protein to form lipoproteins allowing it to move in the circulatory system and organs. One lipoprotein, also known as one particle, is composed of phospholipid, cholesterol, and protein named apolipoprotein presented on the outer wall. In the inner part which consists of triglyceride and cholesterol ester apolipoprotein plays an important role in ligand binding to the receptor for stimulating or inhibiting enzymes affecting lipoprotein metabolism. There are six types of lipoproteins.

1. Chylomicrons, the largest lipoproteins, are composed of more than 80% triglyceride. Apolipoprotein B-48 (Apo B-48), Apo-C, Apo-E, and Apo-A are involved in the transportation of triglycerides from the gut to the tissues and liver.

2. Chylomicron remnants are formed from chylomicrons that are broken down triglycerides by lipoprotein lipase enzymes and have an increased proportion of cholesterol, containing Apo B-48 and apolipoprotein E (Apo E).

3. Very low-density lipoprotein (VLDL) contains approximately 50% triglyceride and 20% cholesterol, with apolipoprotein B-100 (Apo B-100), Apo-E, and Apo-C. It is responsible for transporting triglycerides from the liver to other tissues.

4. Intermediate density lipoprotein (IDL) has properties between VLDL and LDL.

5. Low-density lipoprotein (LDL) is smaller than VLDL. Cholesterol is the most common constituent compared to other types of lipoproteins, which is about 50% cholesterol and about 10% triglyceride. It is synthesized by the liver. It is responsible for transporting cholesterol to the cells of the body, with Apo B-100.

6. High-density lipoprotein (HDL), the smallest lipoprotein, is synthesized in the liver and intestines. The primary responsibility is to transport excess cholesterol from cells back to the liver, a known process as the “reverse cholesterol pathway”, with apolipoprotein Al (Apo A-1) as the main constituent. [56,57]

There are two pathways for creating and removing lipoproteins in the body: the exogenous pathway and the endogenous pathway.[57]

- The exogenous lipoprotein pathway is the process of transferring fatty acid from food to muscles and tissues for energy and/or to store and carry cholesterol to the liver for use in the production of VLDL, bile acid, and secreted back into the intestines. In normal situations, this can manage dietary fat intake up to 100 grams a day without affecting serum triglyceride.[57]

- The endogenous lipoprotein pathway is initiated by the process of VLDL formation in the liver from triglyceride, cholesterol ester, and Apo B-100. Triglyceride level is a factor in Apo B-100 and VLDL formation. When VLDL is released into the bloodstream and tissues, triglyceride hydrolysis occurs and fatty acid is used as energy. After that, VLDL remnants or IDL, which have higher cholesterol ester ratios, receive Apo E from HDL. About 50% of IDL is removed from the bloodstream by binding to the LDL receptor in the liver, the remaining IDL is broken down by hepatic lipase and converted

to LDL. About 70% of LDL binds to the LDL receptor in the liver by Apo B-100 endocytosis enters the liver cells and 30% of LDL is taken out of the bloodstream in the tissues (extrahepatic tissue). Therefore, the activity of the LDL receptors in the liver determines both the formation and removal of LDL from the bloodstream.[56]

The amount of LDL receptors in the liver depends on the level of cholesterol present in the liver cells. Low cholesterol level in hepatocytes activates sterol regulatory element binding proteins (SREBPs), which are proteins that regulate the activity of genes involved in the formation of LDL receptors and other factors involved in the metabolism of lipids such as HMG-CoA reductase. This receptor can be destroyed by protein convertase subtilisin kexin type 9 (PCSK9) that is attached to the LDL particle-LDL receptor complex, and the LDL receptor is destroyed by lysosome degradation.[58,59]

Pathophysiology of dyslipidemia

The accumulation of cholesterol in the artery wall is an important mechanism of atherosclerosis. Cholesterol accumulated in the intima of the arterial wall is the cholesterol-containing Apo B-100 and has a diameter smaller than 70 nanometers. About 90% of the lipoprotein that contains Apo B-100 as a component is LDL. In addition, IDL and VLDL with Apo B-100 can bring cholesterol to accumulate in the arterial wall as well. These three groups of lipoproteins are known as “atherogenic lipoproteins”.[57]

From epidemiological studies, the Apo B-100 level directly relates to the risk of CVD. Apo B-100 measurement is rare and generally not measurable. Therefore, the level of LDL cholesterol (LDL-C), which is the total cholesterol in the LDL particle, correlates with the measurement of Apo B-100.[60]

The association of coronary heart disease in people with high LDL-C is due to several types of familial hypercholesterolemia (FH)[61–63] including:

- Abnormalities of the genes that regulate LDL-receptor (LDLR) make the LDL-C receptor abnormal, unable to carry LDL-C into the liver cells, resulting in high LDL-C levels.

- Controlled gene abnormalities of apolipoprotein B-100 (APOB) which are located in the receptor binding region of LDL-C, cause decreased clearance of LDL-C from the bloodstream.
- Abnormality of the genes that relate to the function of the PCSK9 protein that binds to the LDL receptor in the liver and causes the destruction of the LDL-C receptor.

In contrast, familial hypobetalipoproteinemia causes malfunction of the genes that regulate the production of apolipoprotein B by impairing its production, lowering LDL-C levels, and reducing the likelihood of CVD.[62]

Signs, symptoms, and associated diseases of dyslipidemia

Regarding the symptoms of dyslipidemia, most of the patients usually do not show any abnormal symptoms. In some patients, they may have symptoms from the complications of the disease, such as hardening of the walls of the arteries. This forces the heart to work harder to pump enough blood throughout the body. If this happens for a longer time, it leads to hypertension, CAD, cerebrovascular disease, and peripheral vascular disease.

In some cases, especially hereditary hypercholesterolemia, we may find yellow patches on the skin such as eyelids, elbows, knees, and palms, thicker Achilles tendon than normal, white circles between the rim of the eye and the sclera, etc.[64]

There are five risk categories based on the number and severity of major risk factors (Table 2-3). Each category has goals for LDL-C and other cholesterol, proportional to the degree of risk. For example, patients at extreme risk for ASCVD events including those diagnosed with progressive ASCVD or those with a history of premature ASCVD will have the goal for LDL-C < 55 mg/dL. LDL-C remains the main goal of efforts to improve lipid profiles in individuals at risk for ASCVD.[65]

Table 2 ASCVD risk categories and treatment goals.

Risk category	Risk factors and 10-year risk	Treatment goals (mg/dL)			
		LDL-C	Non-HDL-C	Apo B	TG
Extreme risk	• *Progressive ASCVD including unstable angina* • *Established clinical ASCVD plus diabetes or CKD ≥ 3 or HeFH* • *History of premature ASCVD (< 55 y, male; < 65 y, female)*	< 55	< 80	< 70	< 150
Very high risk	• *Established clinical ASCVD or recent hospitalization for ACS, carotid, or peripheral vascular disease, or 10-year risk > 20%* • *Diabetes with ≥ 1 risk factor(s)* • *CKD ≥ 3 with albuminuria* • *HeFH*	< 70	< 100	< 80	< 150
High risk	• *≥ 2 risk factors and 10-year risk 10-20%* • *Diabetes or CKD ≥ 3 with no other risk factors*	< 100	< 130	< 90	< 150
Moderate risk	• *< 2 risk factors and 10-year risk < 10%*	< 100	< 130	< 90	< 150
Low risk	• *No risk factors*	< 130	< 160	NR	< 150

Abbreviations: ACS = acute coronary syndrome; Apo B = apolipoprotein B; ASCVD = atherosclerotic cardiovascular disease; CKD ≥3 = stage 3-5 chronic kidney disease (estimated glomerular filtration rate ≤59 mL/min/1.73 m2); HeFH = heterozygous familial hypercholesterolemia; NR = not recommended; TG = triglycerides; y = years.

(Reference: Handelsman Y, et al. Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm - 2020 Executive summary. Endocr Pract. 2020 Oct;26(10):1196-224.)

Table 3 Major atherosclerotic cardiovascular disease risk factors

Major Atherosclerotic Cardiovascular Disease Risk Factors		
Major risk factors	**Additional risk factors**	**Nontraditional risk factors**
Advancing age[a,b,c,d]	Obesity, abdominal obesity[c,d]	Lipoprotein(a)
↑Total serum cholesterol level[a,b,d]	Family history of	↑Clotting factors
↑Non–HDL-C[d]	hyperlipidemia[d]	↑Inflammation markers
↑LDL-C[a,d]	↑Small, dense LDL-C[d]	(hsCRP; Lp-PLA2)
Low HDL-C[a,d,e]	↑Apo B[d]	↑Homocysteine levels
Diabetes mellitus[a,b,c,d]	↑LDL particle concentration	Apo E4 isoform
Hypertension[a,b,c,d]	Fasting/postprandial	↑Uric acid
Chronic kidney disease 3,4[h]	hypertriglyceridemia[d]	↑TG-rich remnants
Cigarette smoking[a,b,c,d]	PCOS[d]	
Family history of ASCVD[a,d,g]	Dyslipidemic triad[f]	

Abbreviations: apo = apolipoprotein; ASCVD = atherosclerotic cardiovascular disease; HDL-C = high-density lipoprotein cholesterol; hsCRP = high sensitivity C-reactive protein; LDL = low-density lipoprotein; LDL-C = low-density lipoprotein cholesterol; Lp-PLA2 = lipoprotein-associated phospholipase; PCOS = polycystic ovary syndrome.

[a]Risk factors identified in the Framingham Heart study.

[b]Risk factors identified in the MRFIT study (Multiple Risk Factor Intervention Trial).

[c]Risk factors identified in the INTERHEART study.

[d]Risk factors identified in guidelines and position statements (National Cholesterol Education Program Adult Treatment Panel III, American Association of Clinical Endocrinologists Polycystic Ovary Syndrome Position Statement, American Association of Clinical Endocrinologists Insulin Resistance Syndrome Position Statement, American Diabetes Association Standards of Care, American Diabetes Association/American College of Cardiology Consensus Statement on Lipoprotein Management in Patients with Cardiometabolic Risk, National Lipid Association, Clinical Utility of Inflammatory Markers and Advanced Lipoprotein Testing).

[e]Elevated high-density lipoprotein cholesterol is a negative risk factor.

[f]Hypertriglyceridemia; low high-density lipoprotein cholesterol; and an excess of small, dense low-density lipoproteins.

[g]Definite myocardial infarction or sudden death before 55 years of age in father or other male first-degree relative or before 65 years of age in mother or other female first-degree relative.

[h]Based on a pooled analysis of community-based studies (N = 22,634).

(Reference: Handelsman Y, et al. Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm - 2020 Executive summary. Endocr Pract. 2020 Oct;26(10):1196-224.)

Treatment of dyslipidemia

The treatment of dyslipidemia has the primary purpose to prevent atherosclerosis, including CAD, cerebrovascular disease, peripheral vascular disease, or abdominal aortic aneurysm. The prevention of atherosclerosis can be divided into two types. The primary prevention is for patients who have not previously developed atherosclerosis. The secondary prevention is to prevent recurrent cardiovascular events in patients who already had an atherosclerosis process.[66] There are several groups of lower LDL-C levels medications including drugs that inhibit cholesterol synthetic enzymes, reduce cholesterol absorption, and increase cholesterol uptake into cells.

Statins

Statins inhibit 3-hydroxy-3-methyl-glutaryt-coenzyme A reductase (HMG-CoA reductase), an important enzyme in the synthesis of cholesterol, resulting in decreasing cholesterol content in the liver, increasing the number of LDL receptors and uptaking cholesterol in the bloodstream and in the walls of blood vessels back into the liver.[67,68]

Several studies of statins were conducted in patients with no history of CVD or primary prevention, and in patients with a history of CVD or secondary prevention, either in patients with acute coronary syndrome or in patients with chronic CAD. They showed the benefit of statins for reducing cardiovascular complications and cardiovascular death.[69–72] The range of LDL-C reductions in dyslipidemia for each agent is depicted in Table 4.[64,73]

In addition, therapy with statins slows down the formation of the atheroma. It was demonstrated that if the LDL-C level was reduced by about 50%, or the LDL-C level was lower to 70 milligrams per decilitre or less, this could reduce the amount of the atheroma.[74]

Table 4 Effects of LDL-C lowering agents

Effects of LDL-C Lowering Agents	
Agent	LDL-C reductions
Moderate-intensity HMG-CoA reductase inhibitors (statins)	
Lovastatin 40 mg	–31% to –42%
Pravastatin 40-80 mg	–34% to –37%
Fluvastatin 40 mg BID	–36%
Fluvastatin XL 80 mg	–35%
Pitavastatin 2-4 mg	–38% to –45%
Simvastatin 20-40 mg	–29% to –41%
Atorvastatin 10-20 mg	–29% to –33%
Rosuvastatin 5-10 mg	–45% to –52%
High-intensity HMG-CoA reductase inhibitors (statins)	
Atorvastatin 40-80 mg	–50% to –60%
Rosuvastatin 20-40 mg	–55% to –63%
Cholesterol absorption inhibitor: ezetimibe	–12% to –17%
PCSK9 inhibitors	
Evolocumab 140 mg Q2W or 420 mg Q4W	–63% to –71%
Alirocumab 75-150 mg Q2W	–48% to –58%
Bile acid sequestrant: colesevelam	–8% to –16%
ACL inhibitor: bempedoic acid	–17% to –18%

Abbreviations: ACL = adenosine triphosphate-citrate lyase; BID = twice daily; HMG-CoA = hydroxymethylglutaryl-coenzyme A; LDL-C = low-density lipoprotein cholesterol; PCSK9 = proprotein convertase subtilisin/kexin type 9; Q2W = once every 2 weeks; Q4W = once every 4 weeks; XL = extended release

(Reference: Handelsman Y, et al. Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm - 2020 Executive summary. Endocr Pract. 2020 Oct;26(10):1196-224.)

Ezetimibe

Ezetimibe inhibits intestinal cholesterol absorption at the site of Niemann-Pick C1-like 1 (NPC1L1) protein. When used as monotherapy, it is effective in reducing LDL-C levels by approximately 19%. However, when combined with statins, it can decrease LDL-C on top of statin by 14%.[75,76]

PCSK9 inhibitors

Proprotein convertase subtilisin kexin type 9 (PCSK9) binds to the LDL particle-LDL receptor complex and causes the LDL receptor to be destroyed and unable to cycle through. The result is decreasing in the total amount of LDL receptors and increasing in LDL-C levels in the bloodstream. A monoclonal antibody has been discovered against PCSK9 and it can reduce LDL-C effectively by about 50-60%.[77–80]

5. Gut microbiota

The human gut microbiota is often composed of bacteria and has a direct relationship with the human body.[81] Imbalance of gut microbiota, known as gut dysbiosis, can lead to a range of diseases, including CVD, metabolic syndrome, gastrointestinal disorders, and cancers. Two major phyla *Bacteroidetes* and *Firmicutes*, account for approximately 90% of the gut microbiota.[82,83]

From birth to adulthood, several factors affect the gut microbiota including diet, underlying disease, antibiotic exposure, host genome, and geography (Figure 3).[13,14] Many microorganisms, including Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, with distinct habitats and modes of activity, are found in the gastrointestinal system.[15] *Lactobacillus* and *Bifidobacterium* are classified as good important gastrointestinal bacteria inherited from birth.[16] Nowadays, people pay attention to the diverse gut microbiome, which affects the immune system, nutrient metabolism, and the stabilization of the gastrointestinal tract lining (gut epithelium).

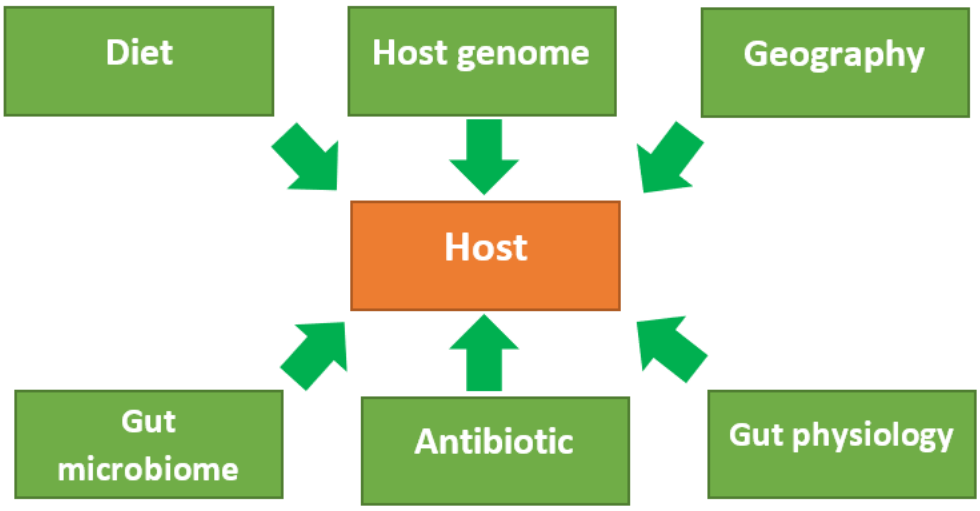


Figure 3 Factors affecting gut microbiome development
(Reference: Adapted from Novakovic M, Rout A, Kingsley T, et al. Role of gut microbiota in cardiovascular diseases. World J Cardiol 2020;12(4):110–22.)

The gut microbiota plays a crucial role in the absorption of nutrients, the synthesis of enzymes, vitamins, and amino acids, and the production of short-chain fatty acids (SCFAs). As a result of lowering the pH of the intestinal environment caused by the accumulation of SCFAs, the colon becomes less conducive to the growth of pathogenic bacteria and becomes more natural habit to beneficial intestinal microbes.[84]

It is noteworthy that vaginally born babies have gut microbiota containing *Lactobacillus, Prevotella*, and *Atopobium,* while caesarean section born infants have more *Staphylococcus*. As children grow up, the gut microbiota contains *Bifidobacteria* and Clostridia. In adults, the gut microbiota tends to be stable and changes again as we get older with an increase in anaerobes and a decrease in *Bifidobacteria* (Figure 4).[85,86]

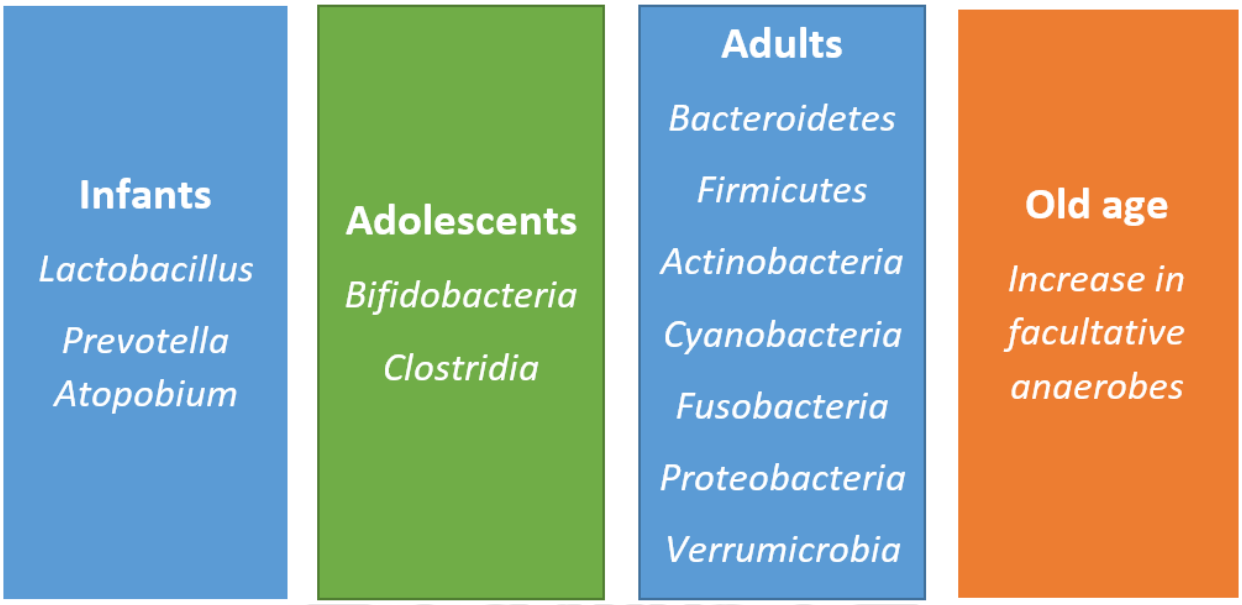


Figure 4 Evolution of gut microbiome with age
(Reference: Adapted from Novakovic M, Rout A, Kingsley T, et al. Role of gut microbiota in cardiovascular diseases. World J Cardiol 2020;12(4):110–22.)

In addition to assisting the body synthesis vitamins, including hormones, the gut microbiota is also in charge of breaking down some nutrients that the stomach is unable to break down. These microbes are inherited from food, medicine, the environment, and the mother at birth.[17] It has been demonstrated that altering the consumption of macronutrients can exacerbate metabolic syndrome in cases when the gastrointestinal system exhibits dysbiosis, or an imbalance of bacteria.[18]

In healthy people, the composition of gut microbiota remains stable. Bacteria are dominant in this environment belonging to 5 main phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia (Figure 5). However, due to variations in the host genome and lifestyle risk factors including drug usage, nutrition, and environmental exposure, there is heterogeneity in the bacterial diversity across the patients. The host and bacteria have a symbiotic relationship that continues from infancy until old age. The microbiome and host have a symbiotic interaction that encourages the

growth of commensal bacteria that are useful to the host while preventing the expansion of harmful bacteria.[87]

The creation of short-chain fatty acids (SCFAs) for metabolism, the synthesis of vitamins, the fermentation of indigestible food components, and the control of the intestinal epithelial mucosal barrier are among the normal physiological roles of the gut microbiome. A breach in the barrier will cause gut leakage, which will increase exposure to endotoxins and permit bacterial translocation. The gut microbiota plays a wide range of roles and affects the body in a variety of ways. The body and the gut microbiome interact intricately through metabolites such as trimethylamine-N-oxide (TMAO), bile acid (BA), and SCFAs. The intestinal epithelium allows these compounds to enter the circulation, which can affect how several organs operate.[88]

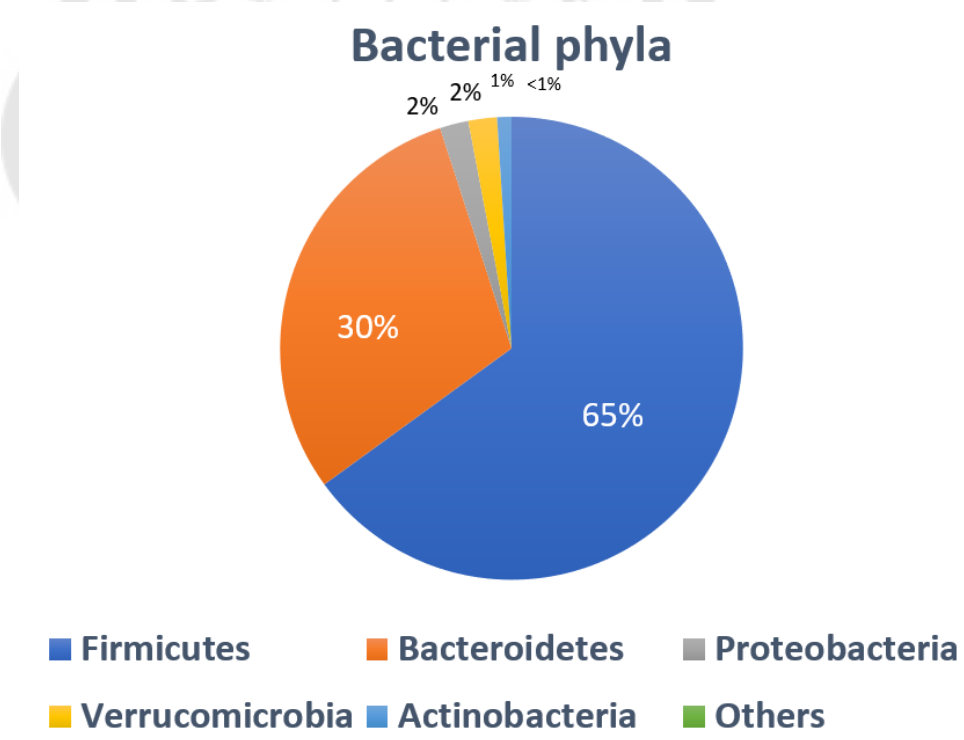


Figure 5 Relative distribution of the 5 main bacterial phyla

(Reference: Adapted from Yang X, Xie L, Li Y, Wei C. More than 9,000,000 unique genes in human gut bacterial community: estimating gene numbers inside a human body. PLoS One 4: e6074, 2009.)

Metabolites and gut microbiome influence the progression of atherosclerosis brought on by stroke, ACS, and CCS. The importance of gut dysbiosis in a number of disorders, including CVD, has been underlined by recent research. These conditions can alter the diversity, composition, and metabolic activities of gut microbiota, which can disrupt important physiological processes like glucose homeostasis, bacterial translocation, lipid metabolism, and inflammation. These changes may also have an impact on the onset and course of disease. Variations in the ratio of the major phyla of Firmicutes to Bacteroidetes have been suggested as a potential risk factor for CVD. These changes in the microbiome composition may contribute to the development of CVD. The pathophysiology of CAD is correlated with levels of gut microbiome metabolites, such as TMAO, SCFA, and BA. The relationship between the gut microbiota and CVD is being studied in great detail (Table 5).

Table 5 Summary of studies investigating associations between gut microbiome and CVD

Method	No. of Subjects	Disease Status	Main Findings
16S rRNA and metagenome sequencing	n = 1,046	Plasma lipids, glycemic traits	Microbiota is shaped predominantly by nongenetic factors and explains ≥% variance in HDL, fasting glucose, and obesity
Metagenome shotgun sequencing	n = 218 CVD, n = 187 controls	Atherosclerosis	In CVD, increased *Enterobacteriaceae* and *Streptococcus*
qPCR 16S rRNA	n = 15 CVD, n = 15 controls	Atherosclerosis	Overgrowth of *Veillonella* and *Streptococcus* in atherosclerotic plaque samples

CVD, cardiovascular disease; HDL, high-density lipoprotein; qPCR, quantitative polymerase chain reaction; 16S rRNA, 16S ribosomal ribonucleic acid.

(Reference: Adapted from Am J Physiol Heart Circ Physiol 317: H923–H938, 2019.)

Previous studies found that more than 1,100 types of bacteria are associated with many symptoms and diseases, such as cancer, diabetes mellitus, obesity, and CVD.[11] Therefore, consuming right amount of healthy food can prevent and cure CVD, including metabolic syndrome. Most microorganisms in the gastrointestinal tract cannot be cultured.[12] Therefore, the type and quantity of these microorganisms has been studied by the genes of the microorganism, known as the microbiome.

6. Gut microbiota and dyslipidemia

In patients with dyslipidemia, which directly affects the heart and blood vessels. Dyslipidemia is one of the most important factors in the occurrence of atherosclerosis, where cholesterol accumulates in the walls of blood vessels, causing the macrophage to transform into foam cells, which leads to an inflammatory process. No studies have shown the association between plaque microbiota and outcomes, including plaque vulnerability, plaque rupture, or cardiovascular events. However, pathogenic bacteria originating from the gut microbiota contribute to the formation of plaque in the blood vessels.[89,90]

People who consume a high-fat diet are usually associated with a higher level of lipopolysaccharides (LPS, an endotoxin) in the blood. LPS is a known inflammatory response inducer and is a part of the cell wall of gram-negative bacteria. Elevations in these bloodstream endotoxins, particularly those over 50 pg/mL, have been linked to a tripling of the risk of atherosclerosis.[91] Consuming a high-fat diet directly causes chylomicrons to accumulate, which in turn increases the local intercellular pressure, relaxes tight junctions, and affects the entry of bigger molecules like LPS. An indirect consequence of a high-fat diet is increased intestinal permeability through the stimulation of mast cell activation in the intestinal mucosa, which results in the release of histamine and inflammatory mediators.[92,93]

Cross-sectional studies in humans have demonstrated that patients with atherosclerosis have high amounts of *Collinsella,* Enterobacteriaceae, Streptococcaceae, and *Klebsiella* spp. and low amounts of SCFA-produced bacteria

Eubacterium, Roseburia, and *Ruminococcaceae* in the gut microbiota compared with the normal group.[94,95] The good microorganisms could reduce cholesterol levels due to increased gastrointestinal transit time and decreased absorption of cholesterol into the bloodstream.[96]

Some beneficial microorganisms, known as probiotics, have a cholesterol-lowering mechanism. They can lower the levels of fats and sugars in the body and can inhibit the absorption of fats, especially cholesterol. By the mechanism of creating enzyme bile salt hydrolase that can break down bile salts from the soluble form (conjugated bile salt) to the insoluble form (deconjugated bile salt) is excreted in the feces. The results of these processes are to impair the breaking down of fat (emulsification) which leads to decrease absorption of fat in the intestines. In addition, probiotics can directly extract the cholesterol present in the gastrointestinal tract by being used to build cell membranes. The amount of cholesterol in the body is reduced as well as the sugars presented in the intestines to be used directly as energy. Moreover, probiotics can secrete substances that have the effect of balancing cytokines, which help regulate fat and sugar levels in the bloodstream to reduce the progression of CVD. For example, in patients with antibiotic-associated diarrhea (AAD), irritable bowel syndrome (IBS), and *Clostridium difficile* colitis, probiotics help balance the gut microbiota and immune system. Many studies showed that *Lactobacillus acidophilus* strain was effective in reducing LDL-C but had no significant effect on reducing HDL-C or triglycerides.[97–100]

7. Gut microbiota and chronic coronary syndrome

CAD is associated with hyperlipidemia, diabetes mellitus, and various metabolic syndromes. Changes in the gut microbiota can increase the risk of CAD including the occurrence of atherosclerosis (Figure 6).

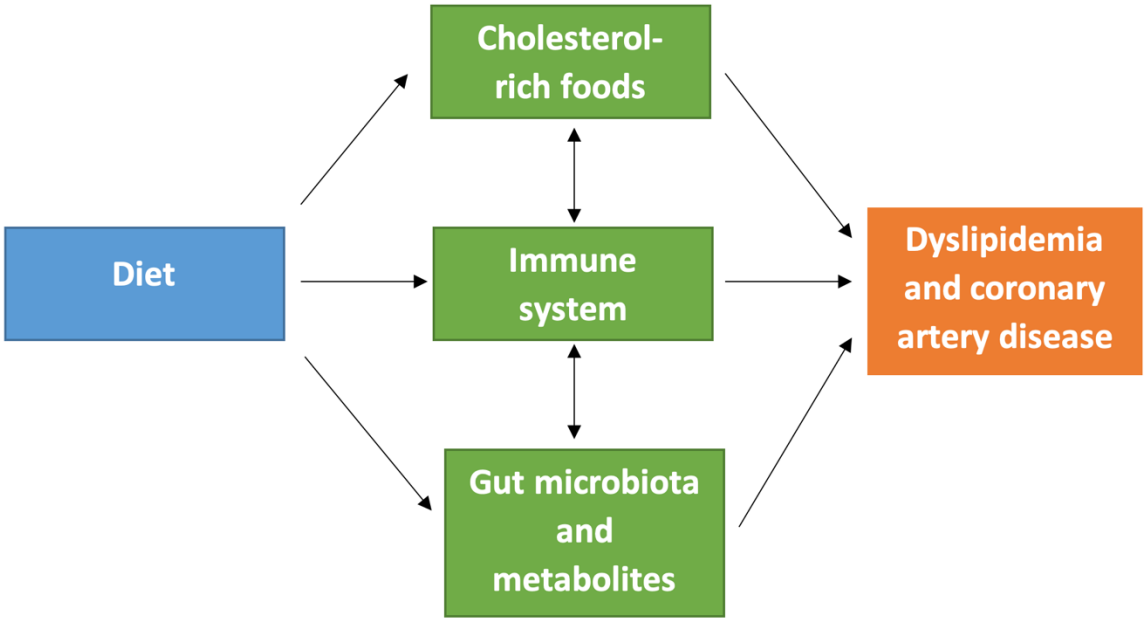


Figure 6 Gut microbiota and risk factors for CCS
(Reference: Adapted from Kazemian N, Mahmoudi M, Halperin F, et al. Gut microbiota and cardiovascular disease: opportunities and challenges. Microbiome 2020;8(1):36.)

The atherosclerosis process is caused by an inflammatory process. Moreover, infection is a process that causes inflammation in the body and affects the development of atherosclerosis via two mechanisms: direct infection of the blood vessel wall and indirect infection through proinflammatory mediators from the systemic immune response. Several species increase the risk of CAD such as *Porphyromonas gingivalis*, Hepatitis C virus, *Helicobacter pylori*, Influenza A virus, and *cytomegalovirus.*[101,102]

For direct infection, several types of bacteria are associated with plaques in the blood vessels. Firmicutes phylum, including *Lactobacillales, Clostridium, Eubacterium,*

and *Roseburia* genera, and Proteobacteria phylum are found in atherosclerotic plaques. Studies have shown that gut microbiota such as *Clostridium*, *Bacteroides,* and *Lactobacillales* can be found and are considered markers in CAD patients.[95,103]

In the case of an indirect infection, atherothrombosis—which is caused by surface erosion—can cause clots to form or rupture plaques damaged by cytokines, which impairs blood flow and results in CAD.[104] Many studies have shown that certain bacteria are associated with atherogenesis that the bacteria produce inflammatory cytokine and induce acute-phase reactants.[105–108]

The study discovered that inflammatory processes were brought on by cross-reactivity or molecular mimicry between self-antigens and bacterial antigens. For instance, the arterial endothelium expresses Human Heat-Shock Protein 60 (hHSP60) in response to stressors such high blood pressure and hypercholesterolemia. Patients with CAD also have hHSP60, which is the primary antigenic component of bacteria and a target of antibodies produced against them.[109–111]

Bacterial infection is triggered by innate immunity via Toll-like receptors (TLRs). When ligands such as lipopolysaccharides (LPS, an endotoxin) are captured, TLR is activated and sends a signal as a downstream signaling cascade, which will result in proinflammatory cytokines and chemokines. TLRs can be found in cardiovascular cells such as cardiomyocytes, endothelial cells, and macrophages.[112–115] TLR4 is activated by saturated fatty acids, which are similar to a ligand through pathways as for LPS, resulting in the formation of proinflammatory cytokines.[116–118] In addition, saturated fatty acids induce inflammation by affecting gut microbiota, particularly Gram-negative bacteria, which results in higher LPS levels. Studies in mice found that genetic deficiency of TLR4 gene could reduce lipid content in plaque, proinflammatory cytokines, and atherosclerosis.[119,120] Increased TLR1, TLR2, and TLR4 expression in atherosclerotic plaques has been linked to pathogenesis in human studies. Immune system alterations brought on by gut dysbiosis result in elevated inflammation and the development of atherogenesis.[121–123]

Plasma Trimethylamine-*N*-oxide (TMAO) is a gut microbiota metabolite from dietary phosphatidylcholine and choline which can be found in red meat, fish, eggs, dairy, and saltwater fish. Trimethylamine (TMA) is generated by gut bacteria and then transferred via the bloodstream to the liver where it becomes oxidized by the enzyme flavin monooxidases3 (FMO3) into TMAO.[124–126] FMO3 is regulated by farnesoid X receptor (FXR) that can be upregulated by bile acids. There are many mechanisms for atherogenesis via TMAO. For instance, increasing platelet hyperresponsiveness by the stimulus-dependent release of Ca2+, promoting proinflammatory proteins such as IL-6, intercellular adhesion molecule-1 cyclooxygenase-2, and E-cadherin via nuclear factor kappa B (NF-**K**B) signaling pathway, inhibiting reversion of cholesterol transport causing reduced cholesterol removal from peripheral macrophages and decreasing of the protective effects of high-density lipoprotein (Figure 7).[127,128]

Studies found a correlation between plasma TMAO levels and high-sensitivity cardiac troponin-T (hs-cTnT) and the correlation was stronger in subclinical myocardial damage patients (hs-cTnT $\geq$ 14 ng/L). Therefore, plasma TMAO is a predictor of subclinical myocardial damage in high atherosclerotic risk patients. In CCS, the higher hs-cTnT level, the more risk of cardiovascular events.[129,130] Elevation of trimethylamine N-oxide (TMAO) due to changes in gut microbiota was associated with mortality from CAD.[131] The studies in mice found that the species *Lactobacillus* could reduce atherosclerosis.[132] Seldin MM et al. conducted a study in mice by giving TMAO supplemented diet, TMAO was found to induce cellular and vascular inflammatory processes and induce dyslipidemia leading to atherosclerosis.[128] Tascanov MB et al. conducted a prospective study with 44 patients of less than 50 years and presented with acute myocardial infarction, the result of this study found that there was a higher level of trimethylamine N-oxide (TMAO) compared to the older group and normal group.[133] A 2016 study by Stubbs JR et al., included 220 patients with chronic kidney disease who had coronary artery catheterization, high TMAO levels are associated with increased arteriosclerosis.[134]

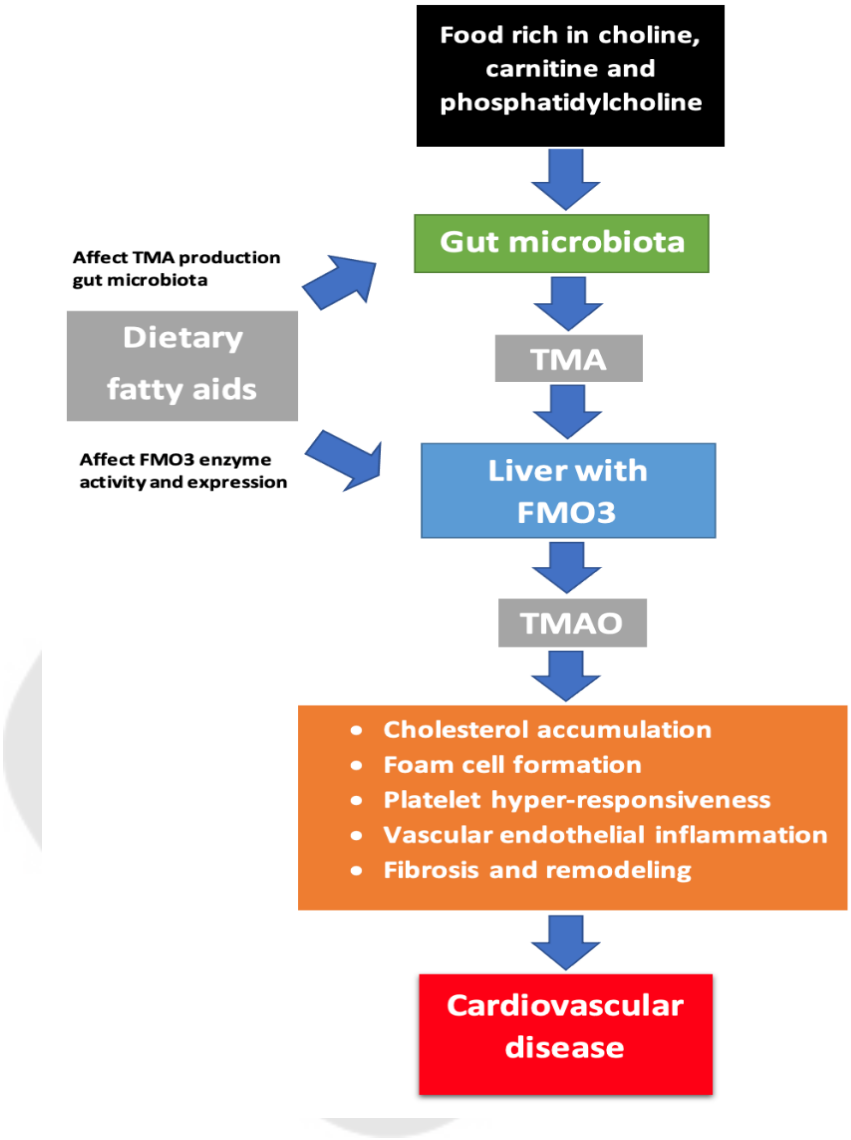


Figure 7 Pathway of plasma trimethylamine-N-oxide (TMAO) production

FMO3, flavin monooxidases3; TMA, trimethylamine.

(Reference: Adapted from He M, Tan CP, Xu YJ, et al. Gut microbiota-derived trimethylamine-N-oxide: A bridge between dietary fatty acid and cardiovascular disease? Food Research Inter 2020;138.)

Short-chain fatty acid (SCFA) is fermented from complex carbohydrates which affect host processes such as energy utilization and control of colonic pH with consequent effects on the gut microbiota. SCFA is correlated with *Roseburia,*

Bacteroides spp., and *Eubacterium rectale.* SCFA is a protective element in CAD development, it can decrease serum lipid levels by blocking cholesterol synthesis and/or redirecting them to the liver.[135–137] The association of gut microbiota with short-chain fatty acid and lipopolysaccharides (LPS) was reported. SCFA plays a role in preventing atherosclerosis, while LPS induces inflammation in the body and accelerates the formation of plaques in the blood vessels.[137–139]

Primary bile acid (BA) is secreted into the duodenum. BA's function is to emulsify vitamins and lipid-soluble food ingredients to aid in absorption and digestion. By removing the OH groups from primary BA, the gut microbiota changes it into secondary BA, which is less hazardous to bacteria. This process is accomplished by bacterial salt hydrolase activity. By eliminating cholesterol through excretion in feces, BA plays a critical role in lowering blood cholesterol levels and lowering the risk of plaque development. The synthesis of BA can be slowed down by intestinal bacteria, increasing LDL-C levels and the risk of atherosclerosis.[88]

A study by Zhu et al. reported that increasing the dose of *Faecalibacterium, Subdoligranum* and *Eubacterium* and decreasing the dose of *Escherichia, Enterococcus,* and *Shigella* was found in CAD patients. The latter group was believed to reduce inflammation.[140] While Yoshida et al. reported a reduction in *Bacteroides vulgatus* and *Bacteroides dorei* in patients with CAD, it showed that in healthy people, there were more of these two strains of bacteria to reduce inflammation in the body.[141] Studies have shown that *Lactobacillus rhamnosus, Lactobacillus sakei, Lactobacillus plantarum,* and *Lactobacillus reuteri* can reduce ischemic injury and improve heart function in patients with myocardial infarction. However, most of the studies were observational studies in mice.[142] The information on gut microbiome and parameters that are associated with CCS in Thai patients has not yet been reported.

8. Cytokines, atherosclerosis process, and coronary artery disease

Numerous risk factors activate the endothelium, which triggers the recruitment of immune cells by chemokines. This is the first step towards atherosclerosis. The fatty

streak's "foam cells" are caused by macrophages consuming lipoproteins and poor cholesterol efflux. Vascular smooth muscle cell migration and proliferation with an inflammatory response occur as atherosclerosis advances. Smooth muscle cells' extracellular matrix stabilizes plaques, while macrophages' matrix metalloproteinase destabilizes them. Acute coronary syndrome is caused by unstable plaque rupture. Atherosclerosis involves cytokines at every stage.[143]

For the atherosclerosis process, macrophages polarize into M1 macrophages. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) stimulate M1 macrophages, which then release pro-inflammatory cytokines such IL-1, IL-6, and TNF-α. These secretions have the power to eradicate infectious agents like bacteria and viruses; the macrophages phagocytose the dead cells. An ongoing inflammatory process may result from overreactions of the immune system.[144] Atherosclerosis is significantly influenced by inflammation.[145] Many studies examined the associations between atherosclerotic diseases like CAD and chronic infections by microorganisms including cytomegalovirus, hepatitis C virus (HCV), and *Chlamydia pneumoniae* because chronic infection can cause chronic inflammation. According to several studies, the development of atherosclerosis may include more infectious pathogens than a single one, known as the infectious burden.[146] Ishizaka et al. demonstrated the potential link between chronic HCV infection and atherosclerosis.[147] The study with fluorodeoxyglucose positron-emission tomography (FDG PET) imaging showed that one arterial territory's inflammation was linked to other arterial territories' inflammation, and the quantity of various circulating biomarkers in the blood reflects the severity of local arterial inflammation.[148]

The number of diseases, including CAD, will increase if there is an imbalance of microorganisms in the gastrointestinal system (gut dysbiosis). Gram-negative bacteria that produce lipopolysaccharides (LPS), such as *Escherichia coli, Shigella, Veillonella, Haemophilus*, and *Klebsiella*, were more abundant with the severity of CAD.[20] Intestinal permeability is potently induced by elevated levels of circulating pro-inflammatory cytokines such as TNF-α, IL-6, and C-reactive protein (CRP). There was a mice study

that showed the modulation of IL-6 in intestinal tight junction permeability was controlled by c-jun N-terminal kinase (JNK) pathway activation of the claudin-2 gene.[149]

9. Next-generation sequencing

Since next-generation sequencing (NGS) approaches do not depend on culture procedures, NGS is utilized to find bacteria in various aspects. Compared to culture methods, NGS can detect a greater number of distinct species, and it can sequence numerous samples in concurrently. Microbial DNA or RNA from stool, blood, and tissue samples is directly sequenced using NGS techniques. The bacterial 16S ribosomal RNA (rRNA) gene is most frequently the target of PCR amplification. Amplicon sequencing is hence also known as 16S rRNA sequencing or analysis. Since the 16S rRNA gene is so widely distributed among bacteria, it is the target.[150]

While amplification of hypervariable regions might be useful for differentiating between certain species within a genus, it can also skew the results, under- or overrepresent particular taxa. The whole 16S rRNA gene has just been sequenced by NGS, and with the use of more sophisticated analytical techniques, this technology may be able to resolve strain and species in the microbiome. Like QIIME, the platforms are user-friendly. Because of the possibility of horizontal transmission of the 16S rRNA locus and the occurrence of numerous bacterial species and strains that are more than 97% identical, 16S rRNA sequencing is restricted to taxonomic categorization at the genus level or higher. There is not much projected functional information available from 16S rRNA analysis. Due to its lower cost and bioinformatics burden, 16S rRNA sequencing is probably a better way to identify the prevalent bacteria in a sample.[151]

CHAPTER III
MATERIALS & METHODS

1. Study population

1.1 Sample size calculation

"Hypothesis Testing and Power Calculations for Taxonomic-Based Human Microbiome Data" served as the foundation for the sample size calculation in this study (Table 6).[152] A function of the number of sequence reads equal to 50,000 with three groups, the value of Alpha (α) = 5% and power = 80%. With a 20% dropout rate, a total of 25 volunteers were recruited, making a total of 90 volunteers—30 volunteers for each group.

Table 6 Power calculation as a function of number of sequence reads and sample size for the comparison of p from the subgingiva and supragingiva populations Using as a reference the taxa frequencies obtained from the 24 samples and 5% significant levels.

Alpha = 5%	Reads							
Subjects	500	1,000	2,500	5,000	10,000	20,000	50,000	1,000,000
10	51.96%	52.79%	53.14%	52.91%	53.20%	53.57%	53.16%	53.34%
15	76.01%	77.10%	77.90%	77.88%	77.98%	78.00%	77.92%	78.09%
25	96.50%	96.80%	97.02%	97.02%	97.13%	97.17%	97.09%	97.10%
50	99.99%	99.99%	99.99%	99.99%	99.99%	99.99%	99.99%	99.99%

(Reference: Adapted from La Rosa PS, Brooks JP, Deych E, Boone EL, Edwards DJ, Wang Q, et al. Hypothesis testing and power calculations for taxonomic-based human microbiome data. White EP, editor. PLoS ONE. 2012;7(12):e52078.)

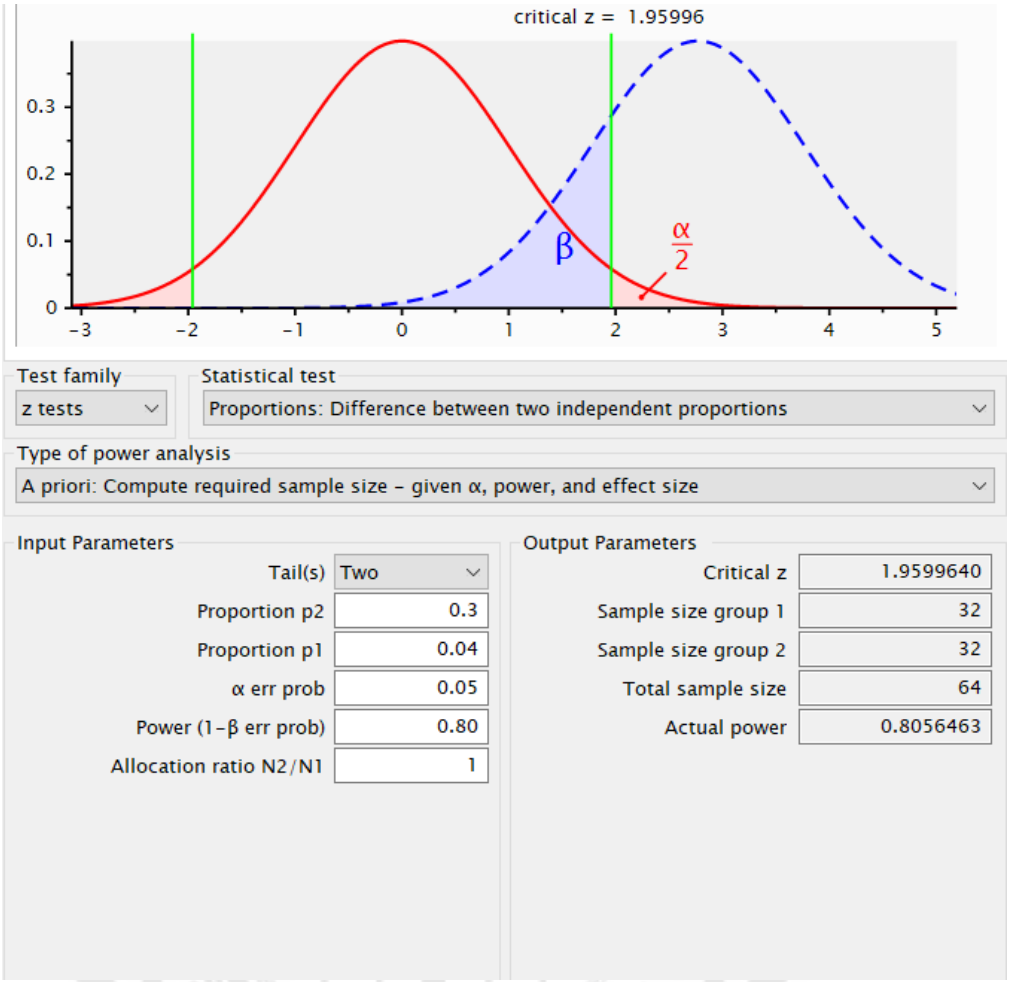


Figure 8 The sample size calculated by using G*Power version 3.1.9.7.

(Reference: URL: http://www.gpower.hhu.de/)

The sample size calculation was also analyzed by using proportions: the difference between two independent proportions with values **α** = 0.05, power = 80%, proportion prevalence 1= 0.04,[153] proportion prevalence 2= 0.30,[53] 1:1 ratio, the sample size of 32 volunteers per group was obtained and determine the dropout rate of 20%, a total of 39 volunteers per group, a total of 117 volunteers (Figure 8).

1.2 Clinical criteria

Inclusion criteria

The first group: Healthy volunteers

1. Volunteers were 35-70 years old

2. Volunteers had no any of the following risk factors for CVD; type 2 diabetes mellitus, hypertension, dyslipidemia or metabolic syndrome.

3. Volunteers had no ACS or CCS

4. Volunteers were willing to participate in the research by signing the consent forms

The second group: Patients with cardiovascular risk factors, dyslipidemia

1. Volunteers were 35-70 years old

2. Volunteers had a risk factor for CVD, dyslipidemia (TC ≥ 200 mg/dL or TG ≥ 150 mg/dL or LDL-C ≥ 160 mg/dL)[154]

3. Volunteers had no any of the following risk factors for CVD

- Hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg at least two different days)[155]
- Type 2 diabetes mellitus (FPG ≥ 126 mg/dL twice separately)[156]
- Metabolic syndrome, Volunteers had at least three of the following risk factors: waist circumference ≥ 90 cm and ≥ 80 cm for men and women, respectively, blood pressure ≥ 130/85 mmHg, TG ≥ 150 mg/dL, HDL-C ≤ 40 mg/dL for men and ≤ 50 mg/dL for women, and FPG ≥100 mg/dL

4. Volunteers were willing to participate in the research through the permission forms' signatures.

The third group: Patients with CCS undergoing coronary angiography

1. Volunteers were 35-70 years old

2. Volunteers with CCS undergoing coronary angiography

CCS patients are referred to the patients with angina pectoris due to demand-supply mismatch which causes myocardial ischemia.[24,157,158] Coronary anatomy is greater than 70% stenosis in coronary arteries that are larger than 2.5 mm in

one view of coronary angiogram, greater than 50% stenosis in coronary arteries in ≥ two views of coronary angiogram.[159]

3. Volunteers were willing to participate in the research by signing the consent forms

Exclusion criteria

1. Patients with underlying medical conditions such as cancer, inflammatory bowel disease, thyroid dysfunction, chronic kidney disease (eGFR < 60 ml/min/1.73 m2 ≥ 3 months), liver disease (AST/ALT > 5 times), and immunodeficiency

2. Patients who had used immunosuppressants, probiotics, synbiotics, herbal supplements, antacids, laxatives, or antibiotics within the four weeks prior to participation.

3. Patients who had a history of gastrointestinal disorders and other infections within the previous four weeks

4. Smoking volunteers

5. Alcoholic volunteers

6. Patients who were lactating or pregnant

7. Patients with COVID-19 infection within four weeks

1.3 Sampling allocation

Volunteers participated in the study at the cardiovascular center, Chulabhorn Hospital. After the volunteers met inclusion criteria and without exclusion criteria, they were categorized into three groups that included 30 volunteers per group; a group of healthy volunteers, a group of patients with cardiovascular risk factors, dyslipidemia, and a group of patients with CCS undergoing coronary angiography. Volunteers were collected by statistical matching methods with age and sex. The study received approval from Chulabhorn Hospital and Srinakharinwirot University research ethics committees (IEC No: 174/2564, dated: 22/07/2022 and IEC No: SWUEC/E/M-100/2565E, dated: 22/02/2023, respectively), and the study was conducted in accession with the Good Practices for Clinical Research in Thailand. The study was approved by Thai

Clinical Trails Registry (TCTR) (TCTR20230428002). Written informed consent was obtained from all study participants.

Materials

1. DNA/RNA shield fecal collection tubes (Zymo Research, CA, USA)
2. QIAamp Stool Mini kit (Qiagen, USA)
3. NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA)
4. Autoclave (J.P. Selecta, Spain)
5. Water bath (J.P. Selecta, Spain)
6. Biosafety cabinet (Nuaire, USA)
7. Incubator (J.P. Selecta, Spain)
8. Centrifuge (Sartorious Stedim, Germany)
9. Blood pressure monitors
10. Waist tape
11. Weighing apparatus
12. Vacuum blood collection tube; EDTA tube, heparin tube, sodium fluoride tube
13. Syringe (NIPRO, Thailand)
14. ELISA plate: 96- well plate: High binding (Corning, USA)
15. ELISA kit (R&D Systems, USA)
16. BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA)
17. Bovine serum album (BSA: Sigma, USA)
18. Recombinant human TNF-α (R&D Systems, USA)

2. Biochemical analyses

The volunteers were instructed to fast for 10-12 hrs. The blood samples were collected as followed: 2.5 mL (NaF), 2.5 mL (Na-heparin), and 2.5 mL (Na-EDTA). Blood samples were centrifuged for 10 minutes at 4000 rpm at 4°C and serum was stored at -80 °C until analysis. LDL-C, triglyceride, total cholesterol, HDL-C, fasting plasma glucose, aspartate transaminase (AST), alanine transaminase (ALT) (International Federation of Clinical Chemistry (IFCC) without pyridoxal-5'-phosphate), and creatinine

were analyzed by Cobas 6000 analyzer series (Roche, USA). All blood parameters were collected 1 day before coronary angiography.

3. Measurement of tumor necrosis factor-alpha, interleukin-1, interleukin-6, and high-sense-CRP

Tumor necrosis factor-alpha (TNF-α) production in serum was measured with cytokine-specific sandwich quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, USA). Briefly, 96-well microtiter plates (Corning, USA) were coated overnight with 100 µl mouse anti-human TNF-α antibodies as capture antibodies diluted in phosphate-buffered saline (PBS) pH 7.4. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) to remove excess capture antibody. To reduce non-specific binding, wells were blocked with 300 µl of 1% (w/v) bovine serum album (BSA: Sigma, USA) in PBS (reagent diluent) for two hours and were washed three times with PBS-T. Recombinant human TNF-α was used as standard diluted in reagent diluent at the concentrations of 15.625, 31.5, 62.5, 125, 250, 500, and 1,000 pg/ml. Reagent diluent was used for blank. Standard or samples were added (100 µl volume) to appropriate wells, and plates were incubated overnight. After washing three times with PBS-T, 100 µl per well of biotinylated goat anti-human TNF-α antibodies diluted in reagent diluent were added as detection antibodies and incubated for two hours. The plates were washed three times with PBS-T and incubated with 100 µl per well of streptavidin horseradish peroxidase conjugate for 20 minutes. After washing three times, 100 µl per well of TMB (tetramethyl benzidine) substrate was added to the plates as a color indicator and incubated for 20 minutes. Stopping reagent consisting of H_2SO_4 were added (50 µl to each well) to stop the reaction. Absorbance was measured at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). All procedures were performed at room temperature. The results of cytokine concentration were quantified from the standard curve and expressed as pg/ml of culture medium.

For the measurement of interleukin-1β (IL-1β), after preparing all reagents and working standards, excess microplate strips were removed from the plate frame,

returned to the foil pouch containing the desiccant pack, and resealed. Briefly, 50 μL of Assay Diluent RD1-63 were added to each well, 100 μL of standard, control, or sample were added per well and covered with the adhesive strip provided. After that, a horizontal orbital microplate shaker (0.12" orbit) was incubated for two hours at room temperature on set at 500 ± 50 rpm. A plate layout was provided to record standards and samples assayed. Each plate was aspirated well and washed, repeating the process three times for a total of four washes. Wash Buffer (400 μL) was washed using a squirt bottle, manifold dispenser, or autowasher. Complete removal of the liquid at each step was essential to good performance. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate and blot were inverted against clean paper towels. Each well was added with 200 μL of Human IL-1β Conjugate, covered with a new adhesive strip and plates were incubated for one hour at room temperature on the shaker. Repeat washing again with the previous steps. Each well was added with 200 μL of Streptavidin Polymer-HRP (1X), covered with a new adhesive strip and plates were incubated for 30 minutes at room temperature on the shaker. Each well was added with 200 μL of Substrate Solution and plates were incubated for 30 minutes at room temperature on the benchtop. Each well was added with 50 μL of Stop Solution. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. All procedures were performed at room temperature.

For the measurement of interleukin-6 (IL-6), after preparing all reagents and working standards. Excess microplate strips were removed from the plate frame, returned to the foil pouch containing the desiccant pack, and resealed. Briefly, 100 μL of Assay Diluent RD1W were added to each well. 100 μL of standard, control, or samples were added per well, covered with the adhesive strip provided and plates were incubated for two hours at room temperature. A plate layout was provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process three times for a total of four washes. Each well was washed by filling with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. The plate and blot were inverted against clean paper towels. Each well was added with 200 μL of

Human IL-6 Conjugate and covered with a new adhesive strip and plates were incubated for two hours at room temperature. Repeat the aspiration/wash as in the previous steps. Each well was added with 200 µL of Substrate Solution and plates were incubated for 20 minutes at room temperature. Protected from light. Each well was added with 50 µL of Stop Solution. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. All procedures were performed at room temperature.

For the measurement of C-reactive protein (CRP), after preparing all reagents and working standards. Excess microplate strips were removed from the plate frame, returned to the foil pouch containing the desiccant pack, and resealed. Each well was added with 100 µL of Assay Diluent RD1F. 50 µL of standard, control, or sample were added to per well, covered with the adhesive strip provided and plates were incubated for two hours at room temperature. A plate layout was provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process three times for a total of four washes. Each well was washed by filling with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. After the last wash, removed any remaining Wash Buffer by aspirating or decanting. The plate and blot were inverted against clean paper towels. Each well was added with 200 µL of Human CRP Conjugate, covered with a new adhesive strip and plates were incubated for two hours at room temperature. Repeat the aspiration/wash as in the previous steps. Each well was added with 200 µL of Substrate Solution to incubate for 30 minutes at room temperature on the benchtop. Then each well was added with 50 µL of Stop Solution. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. All procedures were performed at room temperature.

4. Stool sampling and sequencing

Stool samples were collected in DNA/RNA shield fecal collection tubes (Zymo Research, CA, USA) 1 day before the intervention and immediately stored at -20 °C. DNA was extracted from stool samples using the QIAamp Stool Mini kit (Qiagen, USA).

The quantity and quality of DNA were examined by nanodrop and electrophoresis. The V4 hypervariable region of the 16S rRNA gene was PCR amplified using 515 F and 806R primers and 2X KAPA hot-start ready mix. The PCR conditions were as follows: a three-minute initial denaturation at 94 °C; 25 cycles of 98 °C for 20 s; 55 °C for 30 s; 72 °C for 30 s; and a final extension step lasting five minutes at 72 °C. The 16S amplicons were subjected to eight cycles of the previously described PCR condition after being purified using AMPure XP beads and indexed using a Nextera XT index kit. The PCR products were then cleaned and combined in preparation for the Illumina® MiSeqTM 250-bp paired-end read sequencing and cluster formation. Table 7 contains a summary of the examination of the blood and stool.

5. Sequencing data analysis

QIIME 2 2019.10 was utilized for the bioinformatics of microbiomes. The q2-demux plugin was used to demultiplex the raw sequence data, and DADA2 (via q2-dada2) used expected errors (maxEE) greater than 3.0 to discard the data. Using the SEPP q2-plugin, a phylogeny was built by inserting brief sequences into the reference phylogenetic tree, sepp-refs-gg-13-8.qza.[160]

Q2-diversity was used to estimate the alpha-diversity metric, beta-diversity metric, and Principal Coordinate Analysis (PCoA) following the samples' rarefaction (subsampling without replacement) to a minimal read. The classify-sklearn naive Bayes taxonomy classifier was used to assign taxonomy to ASVs based on comparison with the Greengenes 13_8 99% operational taxonomic units (OTUs) reference sequences. Using Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, respectively, statistical tests of alpha and beta diversity were carried out.

Furthermore, utilizing the algorithm module on the Galaxy platform at http://huttenhower.sph.harvard.edu/galaxy, the significantly differential abundance analysis of microbiota was carried out using LEfSe.[161] Initially, characteristics that were significantly distributed among classes ($p < 0.05$) were selected using nonparametric

factorial Kruskal-Wallissum-rank testing. Their effect sizes were estimated using the linear discriminant analysis (LDA) model, which was backed by 30-fold bootstrapping (cutoff = logarithmic LDA score of > 2.0). Additionally, using the Benjamini and Hochberg false discovery rate correction, significant p-values linked to the microbiological traits that LEfSe found to be significantly different were adjusted for multiple hypothesis testing.

The Random Forests Classification model was trained at the genus level to find a group of the most crucial predictive microbiota able to differentiate among SVD and MVD patient groups. All study figures were processed using MicrobiomeAnalyst website tools.[162]

Table 7 Blood and stool analysis

Blood and stool parameters	Technique/ Instruments
LDL-C, TG, TC, HDL-C, fasting plasma glucose	Cobas 6000 analyzer series
AST, ALT	Cobas 6000 analyzer series
Creatinine	Cobas 6000 analyzer series
TNF-α, IL-1β, IL-6, hs-CRP	Sandwich ELISA
Stool collection	DNA shield (Zymo, USA)
DNA extraction	Zymo Stool Mini kit (Zymo, USA)
DNA concentration and purity	Nanodrop and gel electrophoresis
Amplification	V4 regions of the 16S rRNA genes
Nucleotide sequences	Illumina® MiSeq™, analyzed with UCLUST from QIIME 2 2019.10 software in conjunction with the Zymo database (Zymo Research, CA, USA)

(Reference: Adapted from Wensel CR, Pluznick JL, Salzberg SL, et al. Next-generation sequencing: insights to advance clinical investigations of the microbiome. J Clin Invest 2022;132(7))

6. Statistical analysis

Utilizing Stata/SE 16.1 software (StataCorp LP, College Station, TX, USA), the statistical data was examined. Data were deemed noteworthy when the p-value was less than 0.05. Descriptive statistics analysis was performed on all study variables, and the results were given as mean ± standard deviation (SD) or median for nonnormal quantitative data and frequency (%) for categorical data. If the distribution of the quantitative data, including age and laboratory test results, was normal, one-way analysis of variance (ANOVA) and post hoc analysis using the Scheffe test with p-value < 0.05 were used. If the distribution of the data was not normal, the Kruskal-Wallis test and the post hoc Mann-Whitney U test with p-value < 0.017 were used.

Following was an analysis of the Spearman's correlation coefficient: anthropometric measures, physical examination, blood pressure, and blood tests. Using the ggplot2 R program, correlation heat map visualization was done. Figure labeling applied to p-values less than 0.05, which were deemed statistically significant. Furthermore, using the metacoder R program, the phylogenetic heat tree was shown. It was GraphPad Prism 9.1.2 that produced the receiver operating characteristic (ROC) curves.

CHAPTER IV

RESULTS

1. Clinical characteristics

Ninety-six patients were enrolled; however, because stool samples could not be obtained, the microbiota data of four CCS patients and one patient with dyslipidemia were not included in the analysis. Ninety-one patients were involved, with 30, 32, and 29 patients in each group—CCS patients undergoing coronary angiography, dyslipidemia patients, and healthy volunteers, respectively. With a mean age of 57.57 ± 8.35 years and 26.37% had hypertension, the patients were 43.96% female. Among the groups, there was no gender or age difference that was statistically significant.[163] Baseline characteristics are shown in Table 8.

Table 8 Clinical characteristics of the patients (N=91) in this study.

Parameters	Total (n = 91)	CCS (n = 30)	DLP (n = 32)	Healthy (n = 29)	p-value
Age (years)	57.57 ± 8.35	59.60 ± 9.04	57.44 ± 8.88	55.62 ± 6.60	0.066[b]
Male (%)	51 (56.04)	20 (66.67)	16 (50.00)	15 (51.72)	0.356[c]
BMI (kg/m^2)	24.10 ± 4.07	24.63 ± 3.47	25.19 ± 5.45	22.37 ± 1.78	0.003[b]
Waist circumference (cm)	82.32 ± 9.76	88.20 ± 8.92	81.25 ± 10.59	77.43 ± 6.04	<0.001[b]
History of CAD (%)	17 (18.68)	17 (56.67)	0 (0.00)	0 (0.00)	<0.001[c]
Medication					
Antiplatelets	30 (32.97)	30 (100.00)	0 (0.00)	0 (0.00)	<0.001[c]
Antihypertensive drugs	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	<0.001[c]
Oral antidiabetic drugs	7 (7.69)	7 (23.33)	0 (0.00)	0 (0.00)	<0.001[d]
Statins	58 (63.74)	29 (96.67)	29 (90.63)	0 (0.00)	<0.001[d]
Low intensity	2 (3.45)	0 (0.00)	2 (6.90)	0 (0.00)	<0.001[d]
Moderate intensity	18 (31.03)	2 (6.90)	16 (55.17)	0 (0.00)	
High intensity	38 (65.52)	27 (93.10)	11 (37.93)	0 (0.00)	
Duration ≥ 3 months	54 (93.10)	29 (100.00)	25 (86.21)	0 (0.00)	0.112[d]
Obesity (%)	19 (21.11)	13 (43.33)	6 (19.35)	0 (0.00)	<0.001[c]
Abdominal obesity (%)[*]	21 (23.08)	13 (43.33)	5 (15.63)	3 (10.34)	0.005[c]
Hypertriglyceridemia (%)[**]	18 (19.78)	8 (26.67)	10 (31.25)	0 (0.00)	0.005[c]
Low HDL-C (%)[#]	18 (19.78)	13 (43.33)	5 (15.63)	0 (0.00)	<0.001[c]
Impaired fasting glucose (%)[@]	28 (30.77)	21 (70.00)	3 (9.38)	4 (13.79)	<0.001[c]
Metabolic syndrome (%)	10 (10.99)	10 (33.33)	0 (0.00)	0 (0.00)	<0.001[d]
Hypertension (%)	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	<0.001[c]
Diabetes mellitus (%)	9 (9.89)	9 (30.00)	0 (0.00)	0 (0.00)	<0.001[d]

Parameters	Total (n = 91)	CCS (n = 30)	DLP (n = 32)	Healthy (n = 29)	p-value
Dyslipidemia (%)	49 (53.85)	17 (56.67)	32 (100.00)	0 (0.00)	<0.001[c]
Heart failure (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
Stroke (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.000[d]
PAD (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
SBP (mmHg)	121.65 ± 11.88	124.23 ± 14.34	122.41 ± 10.12	118.14 ± 10.35	0.174[b]
DBP (mmHg)	73.99 ± 9.64	72.27 ± 7.76	73.59 ± 10.57	76.21 ± 10.20	0.283[a]
Laboratory data					
FBS (mg/dl)	99.74 ± 18.81	114.31 ± 23.98	95.59 ± 11.00	89.76 ± 8.53	<0.001[b]
HbA1C (mg/dL)	5.69 ± 0.75	6.04 ± 0.97	5.74 ± 0.50	5.28 ± 0.47	<0.001[b]
Total Cholesterol (mg/dL)	175.76 ± 44.19	144.33 ± 41.24	202.93 ± 43.67	178.28 ± 22.28	<0.001[b]
Triglyceride (mg/dL)	119.26 ± 74.86	135.83 ± 92.69	136.44 ± 76.38	83.17 ± 25.98	0.003[b]
LDL-C (mg/dL)	106.72 ± 39.97	77.11 ± 34.44	129.96 ± 40.78	111.70 ± 21.97	<0.001[b]
HDL-C (mg/dL)	56.31 ± 17.89	45.90 ± 15.08	59.84 ± 18.09	63.17 ± 15.88	<0.001[b]
Serum creatinine (mg/dL)	0.86 ± 0.22	0.98 ± 0.20	0.83 ± 0.21	0.77 ± 0.19	<0.001[b]
AST (IU/L)	22.90 ± 11.23	24.50 ± 11.96	25.19 ± 10.60	18.72 ± 10.29	<0.001[b]
ALT (IU/L)	23.22 ± 15.45	26.10 ± 12.79	25.59 ± 10.94	17.62 ± 20.43	<0.001[b]
hs-CRP (mg/dL)	2.36 ± 2.99	3.46 ± 4.26	2.03 ± 2.05	1.58 ± 1.80	0.043[b]
TNF-α (pg/mL)	76.64 (74.84 – 79.96)	79.31 (76.16 – 81.04)	76.08 (73.54 – 77.98)	75.96 (74.76 – 78.32)	0.01[b]
IL-1 (pg/mL)	23.74 (22.65 – 26.45)	23.15 (22.33 – 25.66)	23.90 (23.29 – 25.93)	23.81 (23.12 – 26.03)	0.28[b]
IL-6 (pg/mL)	34.61 (31.17 – 50.24)	39.23 (34.25 – 57.19)	32.37 (30.22 – 53.72)	33.67 (31.61 – 41.44)	0.09[b]

Categorical variables are expressed as frequency and percentage. Continuous variables are expressed as mean ± standard deviation. a One-way ANOVA, post hoc test analysis using Scheffe, significant when p-value <0.05, b Kruskal-Wallis test, post hoc test analysis using Mann-Whitney U test, significant when p-value <0.017 (0.05/3), c Chi square test, significant when p-value <0.05, d Fisher's exact test, significant when p-value <0.05, +Obesity, BMI $\geq$25 kg/m2, *Abdominal obesity = waist circumference >90 cm for male, waist circumference >80 cm for female, **Hypertriglyceridemia = triglyceride $\geq$150 mg/dl, #Low HDL-C = HDL-C <40 mg/dL for male, HDL-C <50 mg/dL for female, @Impaired fasting glucose = FBS $\geq$100 mg/dL, ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; CCS, chronic coronary syndrome patients; DBP, diastolic blood pressure; DLP, dyslipidemia patients; FBS, fasting blood sugar; HbA1C, hemoglobin A1C; healthy, healthy volunteers; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; IL-1, interleukin-1; IL-6, interleukin-6; IU/L, international units per liter; LDL-C, low-density lipoprotein cholesterol; mmHg, millimeters of mercury; mg/dL, milligrams per deciliter, ng/L, nanogram per liter; PAD, peripheral artery disease; SBP, systolic blood pressure; TNF-α, tumor necrosis factor-alpha.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

The CCS patients were 59.60 ± 9.04 years old on average, and 56.67% of them had a history of CAD. Compared to other groups, a higher proportion of patients had metabolic syndrome, hypertension, and diabetes mellitus (33.33%, 80%, and 30% with statistical significance, respectively). Furthermore, this group had higher levels of HbA1C, hs-CRP, and FPG than other groups with statistically significant differences.

The mean age of patients with dyslipidemia was 57.44 ± 8.88 years. In this group, 9.38% of the patients had impaired fasting glucose, but the average FPG and HbA1C were within normal limits. With statistical significance, the levels of TC and LDL-C were greater than the others.

Interestingly, TC and LDL-C were found to be the lowest in CCS patients at 144.33 ± 41.24 and 77.11 ± 34.44 vs 202.93 ± 43.67 and 129.96 ± 40.78 vs 178.28 ± 22.28 and 111.70 ± 21.97, respectively, compared to patients with dyslipidemia and healthy volunteers. According to this study, 96.67% of CCS patients utilized statins, with a high-intensity statin rate of 93.10%. All patients took statins for a minimum of three months. Patients with dyslipidemia used statins at a rate of 90.63% with only high-intensity statins at 37.93%.[163]

2. Pro-inflammatory cytokine analysis

According to pro-inflammatory cytokine results (Table 8), out of the three groups, TNF-α was highest in CCS patients and was statistically significant at 79.31 pg/mL. In comparison to patients with dyslipidemia and healthy participants, CCS patients also had the highest amount of IL-6. Moreover, CCS patients had the highest level of hs-CRP at 3.46 mg/dL among the three groups with statistical significance.[164]

3. Gut microbiota profile

3.1 Gut microbiota diversity

To calculate the Shannon diversity index and Chao1 index, the alpha

diversity analysis was applied (Figures 9a and 9b). There was a tendency that demonstrated less diversity in patients with dyslipidemia and CCS compared to healthy volunteers.

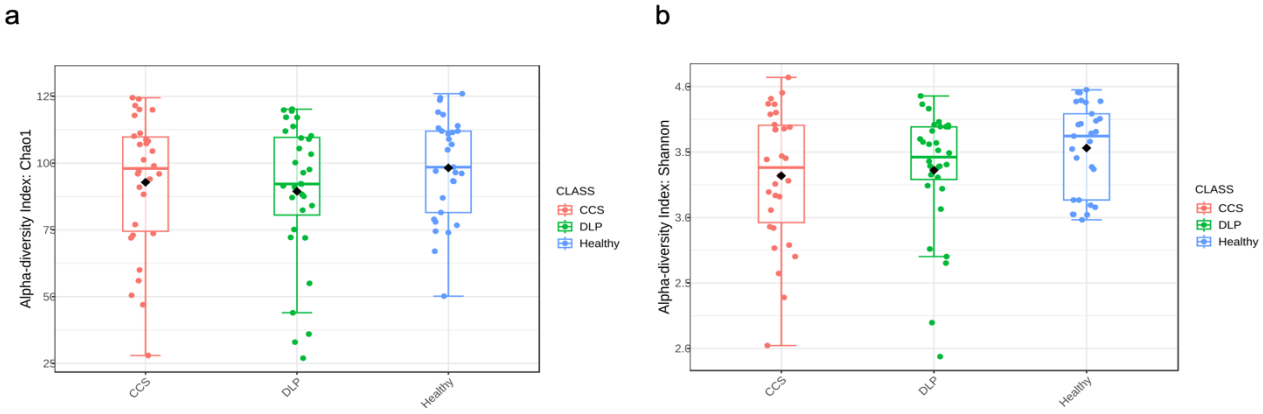


Figure 9 Analysis of alpha diversity of microbial composition in the three patient groups.

Diversity within bacterial communities was measured by The Chao1 index (a) and the Shannon diversity index (b). Kruskal-Wallis H test was used in the statistical test of the alpha diversity (No statistically significant difference found). CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

Using weighted and unweighted UniFac distance, the beta diversity was utilized to identify the similarities and differences in the composition structure of microbial communities. A statistically significant difference (PERMANOVA, $p<0.01$) was seen in the clustering of fecal samples between the gut microbiome of patients with dyslipidemia, patients with CCS, and healthy volunteers, as demonstrated by principal coordinates analysis (Figures 10a and 10b).[163]

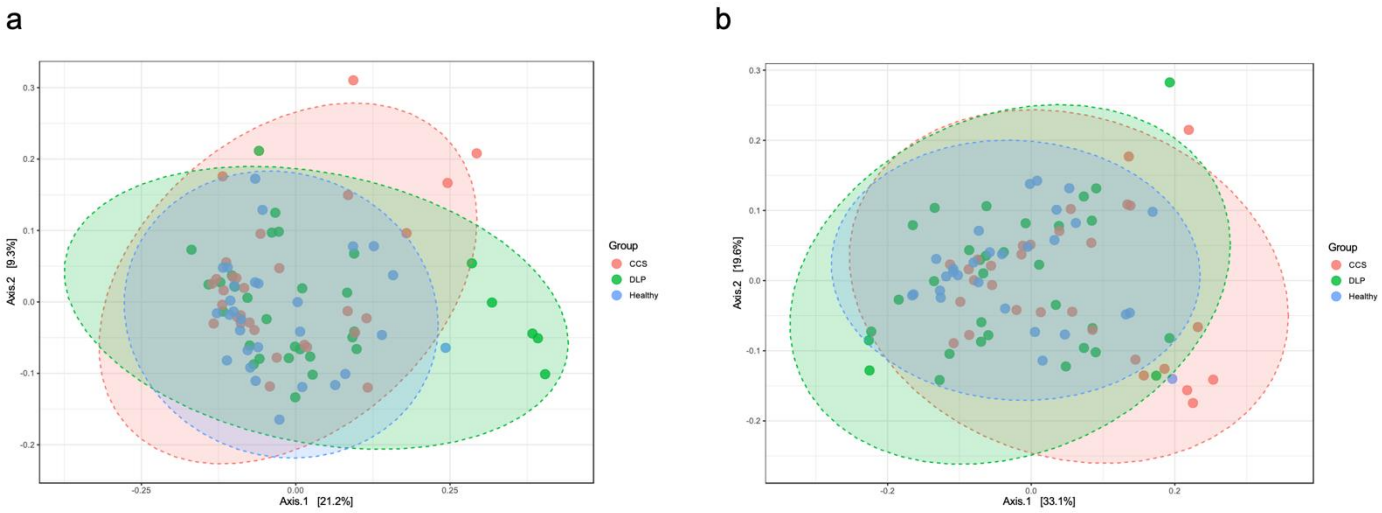


Figure 10 Analysis of beta diversity of microbial composition in the three patient groups.

Diversity within bacterial communities was measured by principal coordinate analysis (PCoA) of beta diversity based on the unweighted UniFrac distance (a), and the weighted UniFrac distance (b). Permanova (Permutational multivariate analysis of variance) test was used in the statistical test of the beta diversity. Statistically significant differences were found at $p<0.001$. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

3.2 Gut microbiota taxonomic composition

At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the most prevalent bacterial phyla in each of the three groups. Proteobacteria was found in higher relative abundance in CCS patients (Proteobacteria percentages in CCS patients, dyslipidemia patients, and healthy volunteers were 8.22,

4.79, and 4.16, respectively). Additionally, there was an increase in the relative abundance of Fusobacteria in CCS patients (the percentages of Fusobacteria in CCS, dyslipidemia patients, and healthy volunteers were 1.48, 1.07, and 0.89, respectively). Conversely, of the three categories, Verrucomicrobia and Actinobacteria exhibited the lowest relative abundance in CCS patients. There was no change to other phyla. Compared to the other groups, the group of patients with dyslipidemia had a larger proportion of Firmicutes and Bacteroidetes (Figure 11).

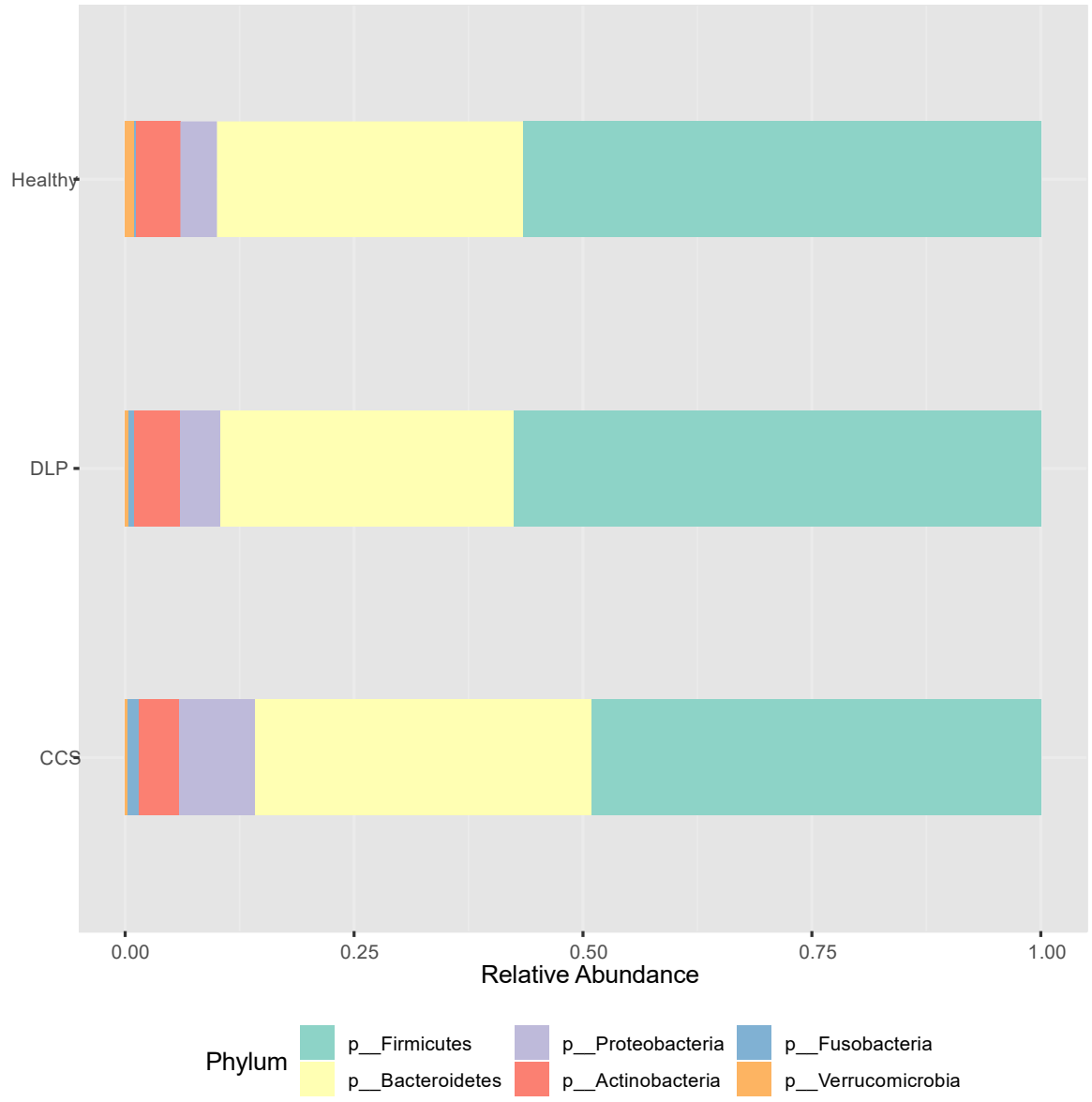


Figure 11 The relative abundance of bacterial taxonomic profile in the feces at the phylum.

CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

When the gut microbiota of the three groups was examined at the family level, the two most prevalent families were found to be Lachnospiraceae and Bacteroidaceae. Prevotellaceae, Enterobacteriaceae, and Streptococcaceae families were proportionately more common in CCS patients than in other groups (Figure 12).

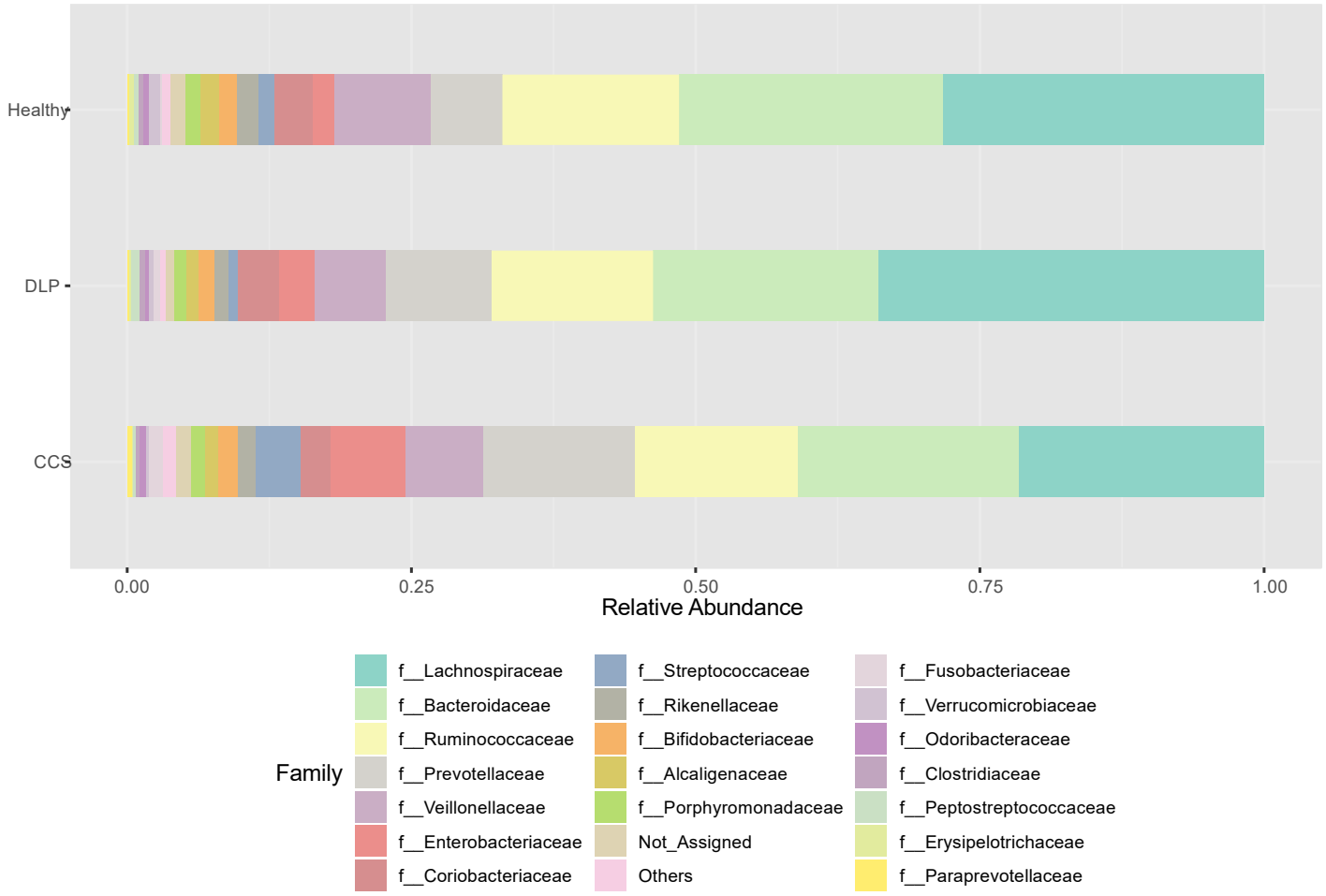


Figure 12 The relative abundance of bacterial taxonomic profile in the feces at the family level.

CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

At the genus level, *Bacteroides* had the highest abundance in all three groups (19.02, 19.15, and 21.50 percent) among patients with CCS, patients with dyslipidemia, and healthy volunteers. *Prevotella* and *Streptococcus* were more abundant in the CCS patient group than in other groups with statistical significance (Figure 13). Additionally, the relative abundance of the following genera was lowest in the CSS patients' group compared to the others: *Faecalibacterium* (the percentages of 6.20, 6.98, and 7.08 in CCS patients, dyslipidemia patients, and healthy volunteers, respectively), *Ruminococcus* (percentages of 2.40, 4.54, and 3.79 in CCS patients, dyslipidemia patients, and healthy volunteers, respectively), and *Roseburia* (percentages of 3.17, 5.67, and 4.71 in CCS patients, dyslipidemia patients, and healthy volunteers, respectively).

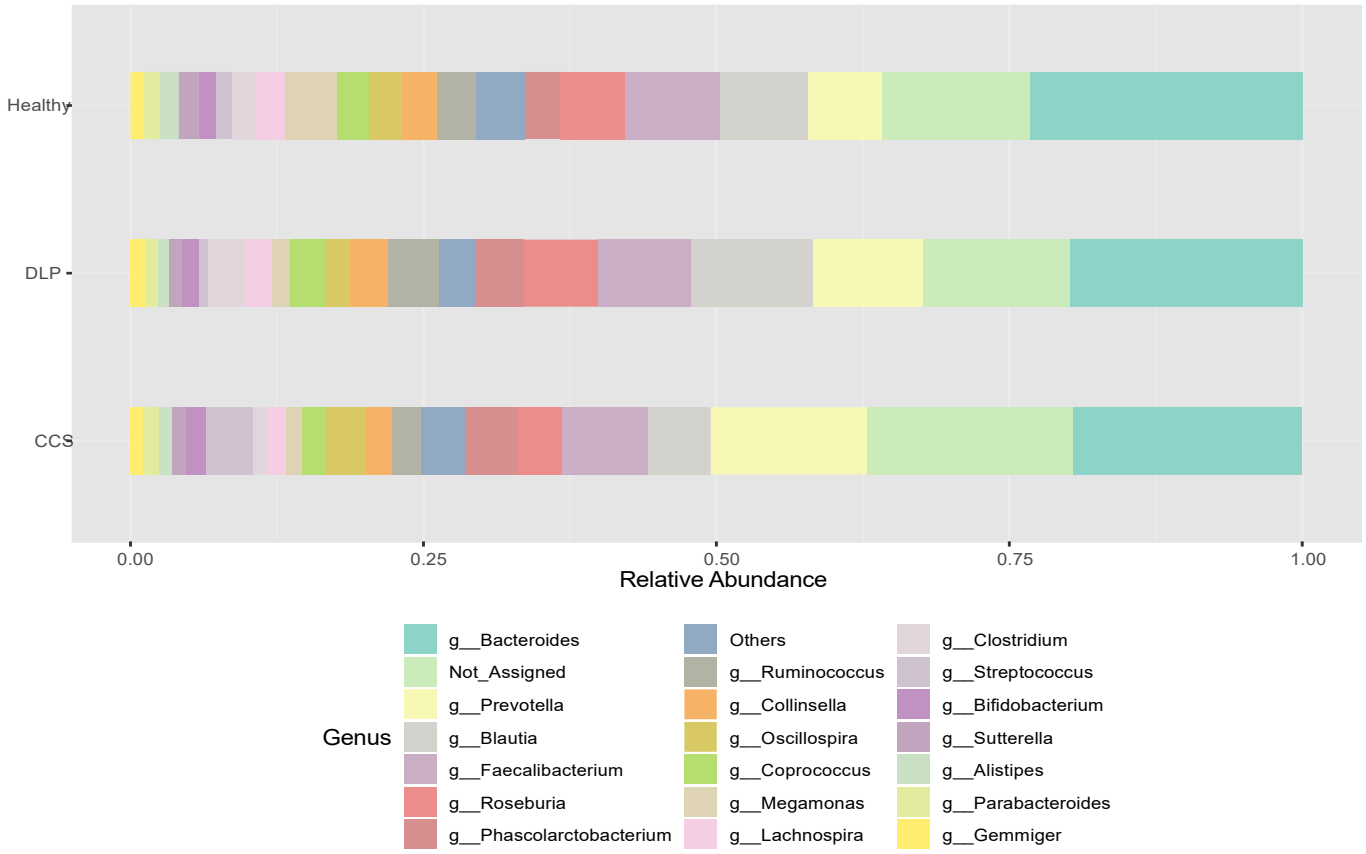


Figure 13 The relative abundance of bacterial taxonomic profile in the feces at the genus level.

CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

Based on the pairwise phylogenetic heat tree, there was a statistically significant increase in the species-level abundance of *Streptococcus, Veillonella dispar*, and *Prevotella copri* in CCS patients compared to healthy volunteers. However, in CCS patients group had lower levels of *Bifidobacterium, Anaerostipes, Subdoligranulum variabile*, and *Roseburia faecis* (Figure 14).[163]

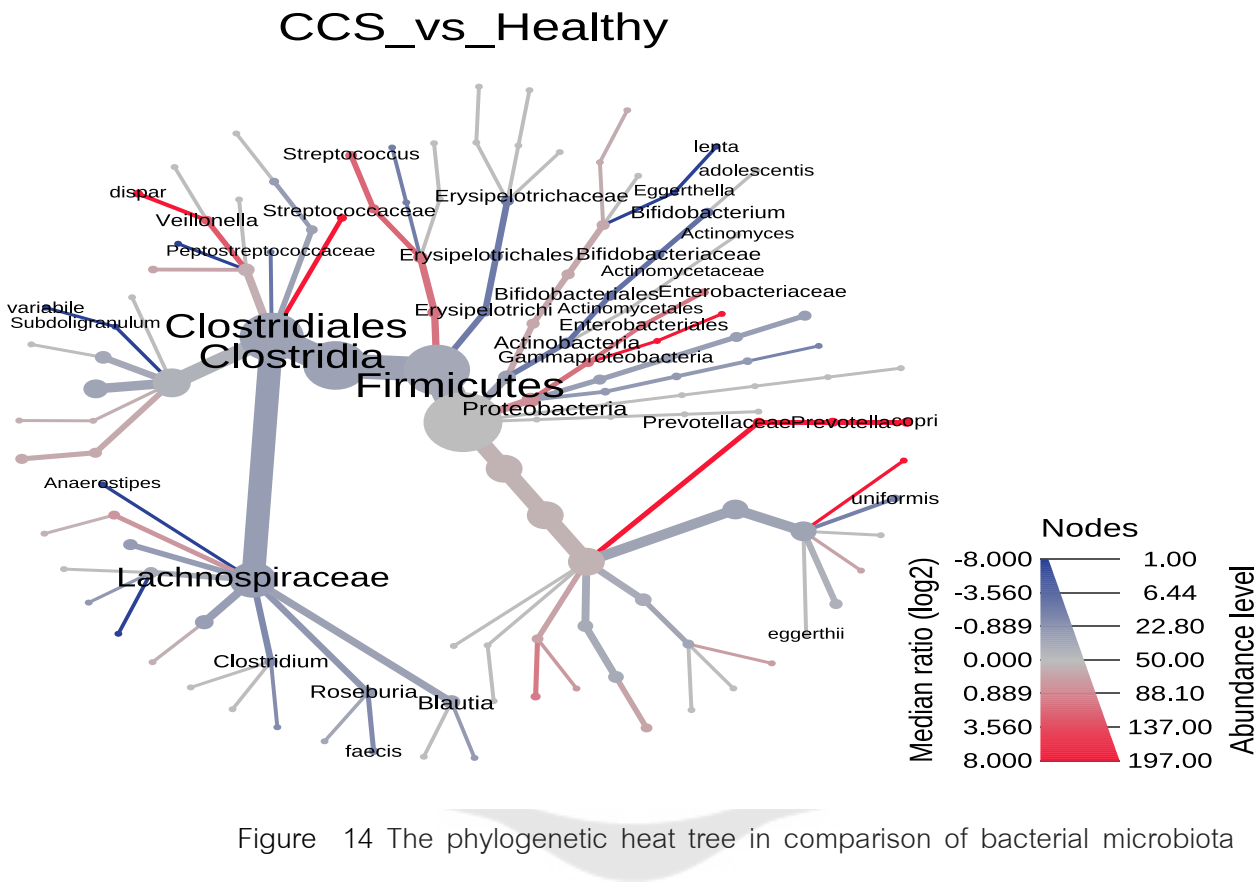


Figure 14 The phylogenetic heat tree in comparison of bacterial microbiota between CCS and healthy groups shows the bacteria composition at the species level. The color of nodes and edges represents the mean change in operational taxonomic units (OTUs) richness at each taxonomic group, with red indicating greater richness in CCS and blue in healthy, while node size represents OTUs richness of each taxonomic group across the dataset. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

3.3 Characteristics of gut microbiota

The linear discriminant analysis effect size (LEfSe) showed that, in CCS patients, the relative abundance of the families Prevotellaceae, Enterobacteriaceae, Streptococcaceae, Clostridiaceae, and Paraprevotellaceae had increased significantly compared with healthy volunteers. *Prevotella, Streptococcus, Phascolarctobacterium, Dorea, Paraprevotella,* and *Veillonella* showed significantly greater relative abundances at the genus level in CCS patients compared to healthy volunteers (Figure 15).

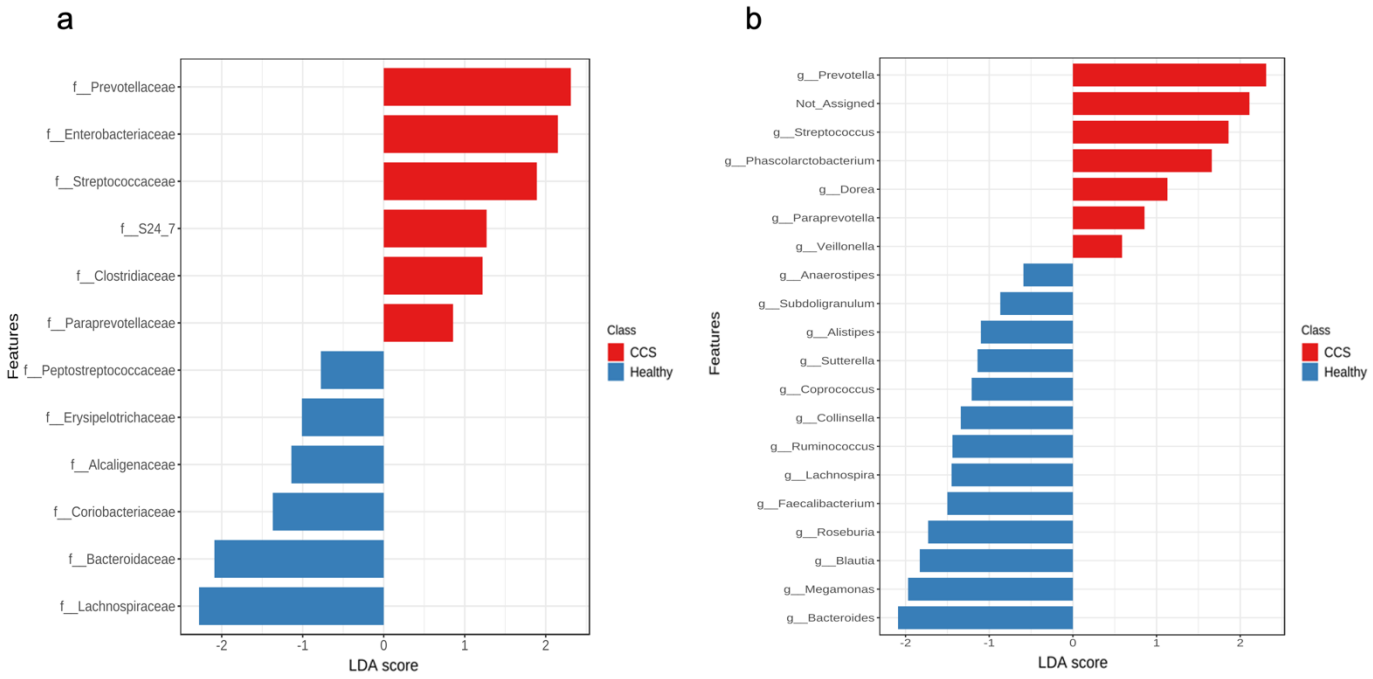


Figure 15 Linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota between CCS patients and healthy volunteers

At the family (a) and genus level (b). CCS, chronic coronary syndrome patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

According to the LEfSe, the relative abundance of the families Prevotellaceae, Enterobacteriaceae, and Fusobacteriaceae had increased significantly at the family level in individuals with dyslipidemia as compared to a group of healthy volunteers. When compared to healthy volunteers, the genus-level relative abundances of the *Prevotella, Clostridium*, and *Dorea* genera were considerably greater in dyslipidemia patients (Figure 16).

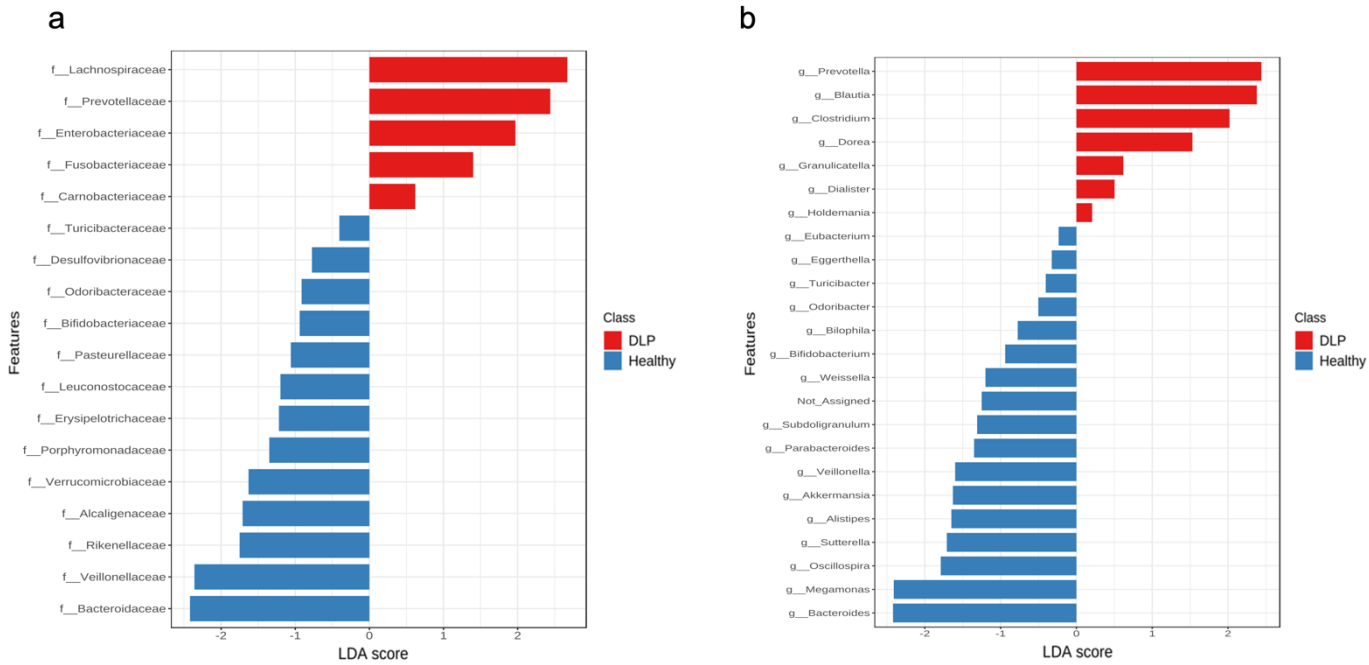


Figure 16 Linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota between dyslipidemia patients and healthy volunteers

At the family (a) and genus level (b). DLP, dyslipidemia patients; Healthy, healthy volunteers.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

The relative abundance of the Prevotellaceae, Enterobacteriaceae, Fusobacteriaceae, Clostridiaceae, Veillonellaceae, and Porphyromonadaceae families was higher in CCS patients as compared to patients with dyslipidemia (Figure 6). Upon comparing individuals with dyslipidemia and those with CCS, we discovered that the former had a greater relative abundance of the Lachnospiraceae family (Figure 17).[163]

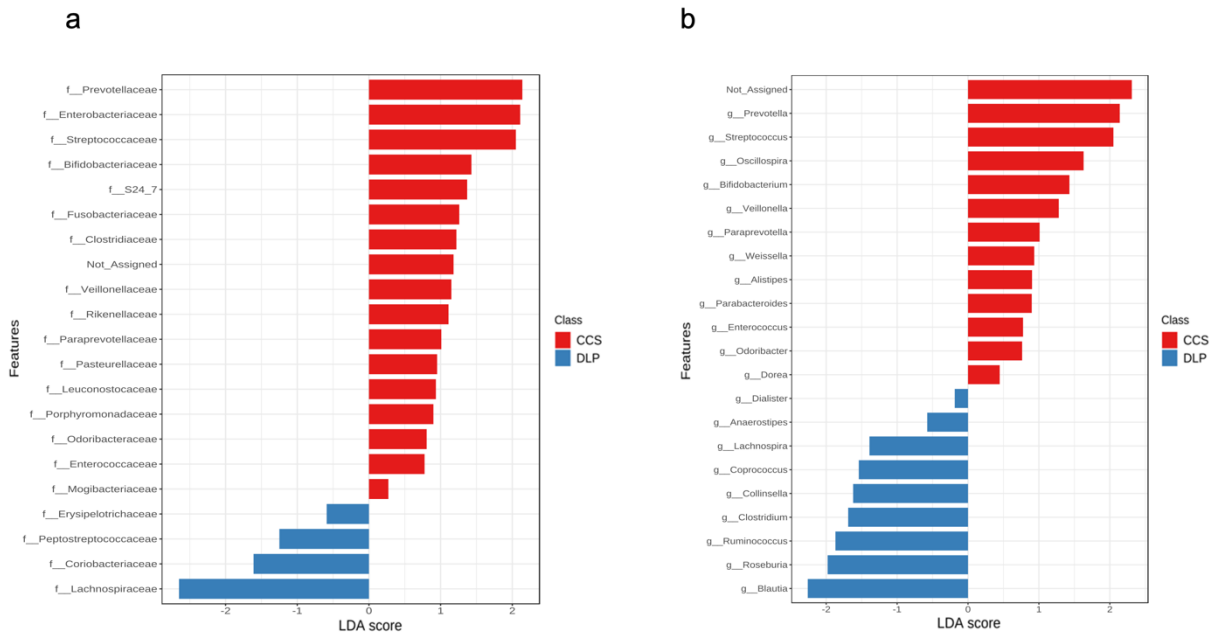


Figure 17 Linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota between CCS patients and dyslipidemia patients

At the family (a) and genus level (b). CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

3.4 Association between gut microbiota composition and related parameters in dyslipidemia and chronic coronary syndrome patients

The Spearman correlation coefficient was used to measure the parameters of CCS patients and gut microbiota (Figure 18). The result showed that Lachnospiraceae, Peptostreptococcaceae, and *Pediococcus* were positively correlated with hs-CRP. While *Sutterella* and *Roseburia* showed negative correlations with LDL-C, *Weissella* showed positive correlations with it.

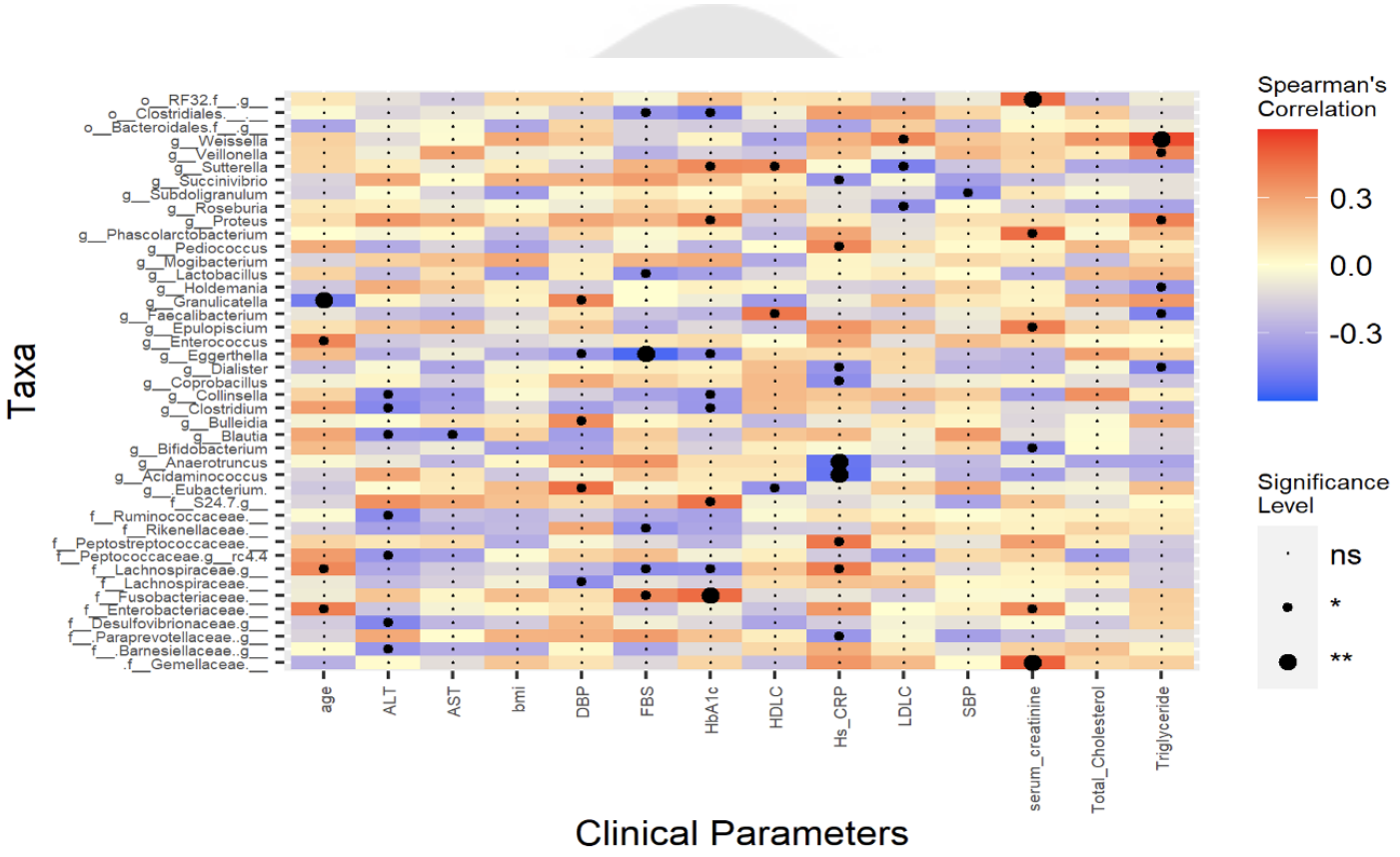


Figure 18 Spearman's correlation analysis between the clinical indexes and the microbiota in CCS patients undergoing coronary angiography group.

The colour represents positive (red) or negative (blue) correlations, and *P < 0.05, **P < 0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; F B S , fasting blood sugar; HbA1C, hemoglobin A1C; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and

chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

The BMI of individuals with dyslipidemia showed a positive correlation with the genera *Pseudobutyrivibrio, Catenibacterium, Weissella, Prevotella,* and *Anaerostipes*, while *Bacteroides* and *Bifidobacterium* showed a negative correlation. Genus *Subdoligranulum* showed a positive correlation with TC level; *Megasphaera* showed a strong positive correlation with TG level, and *Collinsella* and *Catenibacterium* also showed positive correlations with TG level; *Megasphaera* showed a negative correlation with HDL-C; *Subdoligranulum* showed a positive correlation with LDL-C level, and *Lactobacillus* and *Butyricimonas* had negative correlations with LDL-C level (Figure 19).[163]

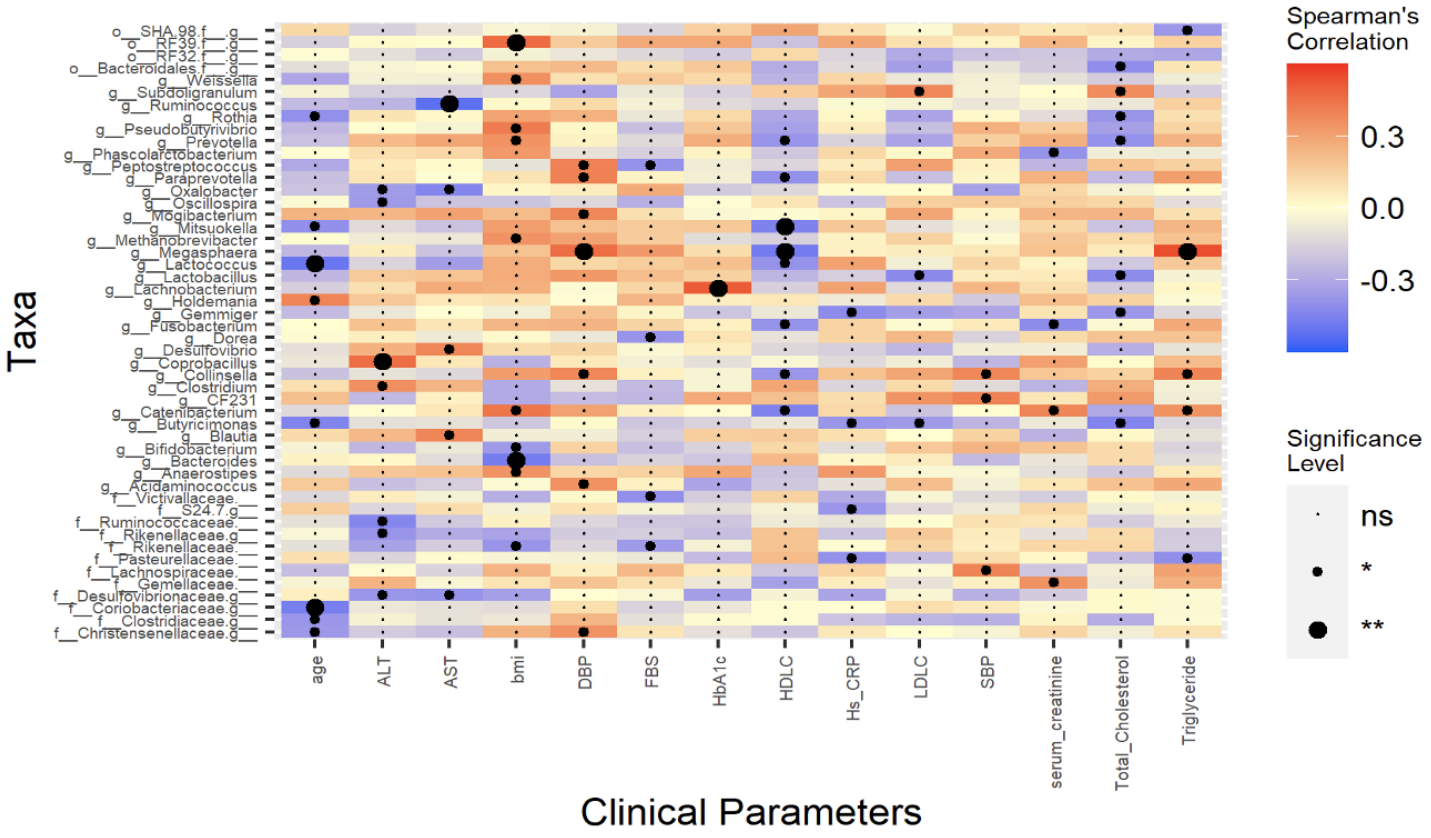


Figure 19 Spearman's correlation analysis between the clinical indexes and the microbiota in dyslipidemia patients' group.

The colour represents positive (red) or negative (blue) correlations, and *P < 0.05, **P < 0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; F B S , fasting blood sugar; HbA1C, hemoglobin A1C; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

(Reference: Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography. Research Square. 2023: 1-16.)

3.5 Association between gut microbiota composition and pro-inflammatory cytokines in CCS patients undergoing coronary angiography

According to pro-inflammatory cytokine results, 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 20).[164]

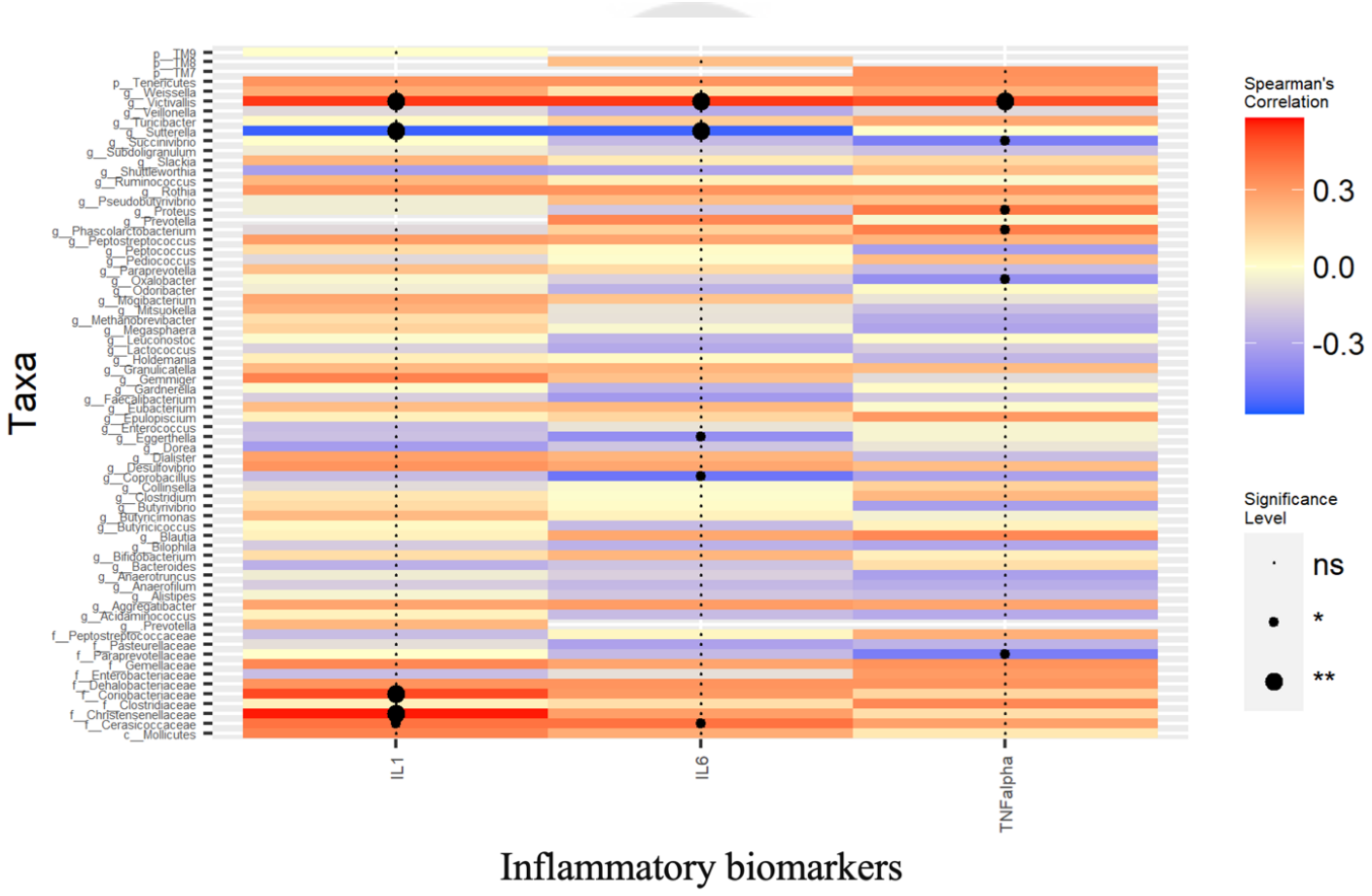


Figure 20 Spearman's correlation analysis between pro-inflammatory cytokines and the gut microbiome in CCS patients.

The color represents positive (red) or negative (blue) correlations, and *P < 0.05, **P < 0.01. IL-1, interleukin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

(Reference: Luangphiphat W, Prombutara P, Eeckhout E, Fournier S, Pradidarcheep W, Taweechotipatr M. Relationship between pro-inflammatory cytokines and gut

microbiome in chronic coronary syndrome patients undergoing coronary angiography: A cross-sectional study. J Med Assoc Thai. 2024;107(2):104-13.)

3.6 Area under the curve based on receiver operating characteristic analysis of pro-inflammatory cytokines and genera of gut microbiota

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: 0.53-0.82), 0.62 (95% CI: 0.46-0.77), 0.65 (95% CI: 0.50-0.80), and 0.66 (95% CI: 0.51-0.81, respectively (Figure 21a). The ROC analysis of the genera of gut microbiome demonstrated that the AUC values of *Phascolarctobacterium, Sutterella,* and *Prevotella* were 0.58 (95% CI: 0.42-0.74), 0.67 (95% CI: 0.52-0.81), and 0.59 (95% CI: 0.43-0.74), respectively (Figure 21b). 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: 0.56-0.85) and 0.70 (95% CI: 0.55-0.84), respectively (Figures 21c and 21d).[164]

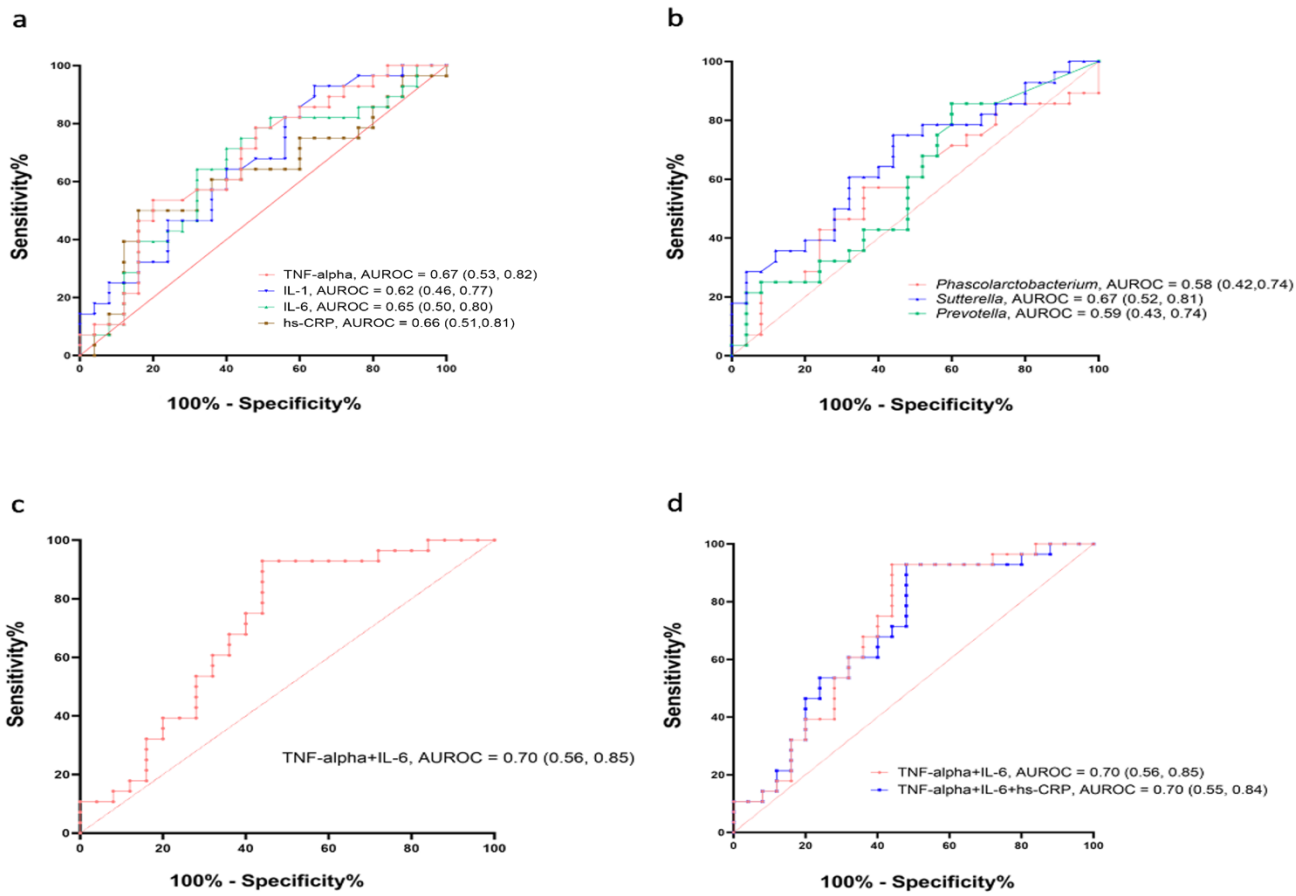


Figure 21 Gut microbiome and clinical features could effectively distinguish CCS patients from healthy participants.

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, the 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.

(Reference: Luangphiphat W, Prombutara P, Eeckhout E, Fournier S, Pradidarcheep W, Taweechotipatr M. Relationship between pro-inflammatory cytokines and gut microbiome in chronic coronary syndrome patients undergoing coronary angiography: A cross-sectional study. J Med Assoc Thai. 2024;107(2):104-13.)

3.7 Gut microbiota profile in the subgroup of CCS patients

The number of stenotic vessels and the degree of vessel stenosis have an impact on the mortality rate among CCS patients. Moreover, compared to single-vessel disease (SVD), multivessel disease (MVD) has a greater impact on clinical outcomes. In this study, 49 patients, which included 11 CCS patients with SVD, 19 CCS patients with MVD, and 19 healthy participants were analyzed.

3.7.1 Diversity of the gut microbiota in SVD and MVD patients

The Shannon and observed diversity indexes were analyzed for alpha diversity (Figures 22a and 22b). According to the Shannon index, MVD patients had the lowest diversity than SVD patients and healthy participants, however, SVD patients had slightly higher than healthy participants in the observed index.

Regarding beta diversity, the Bray-Curtis index was utilized to identify the patterns and variances in the microbial communities' compositional structures. Comparing between SVD and MVD patients, MVD patients and healthy participants were different with statistically significant, however, SVD patients and healthy participants had no statistical significance (Figure 22c).

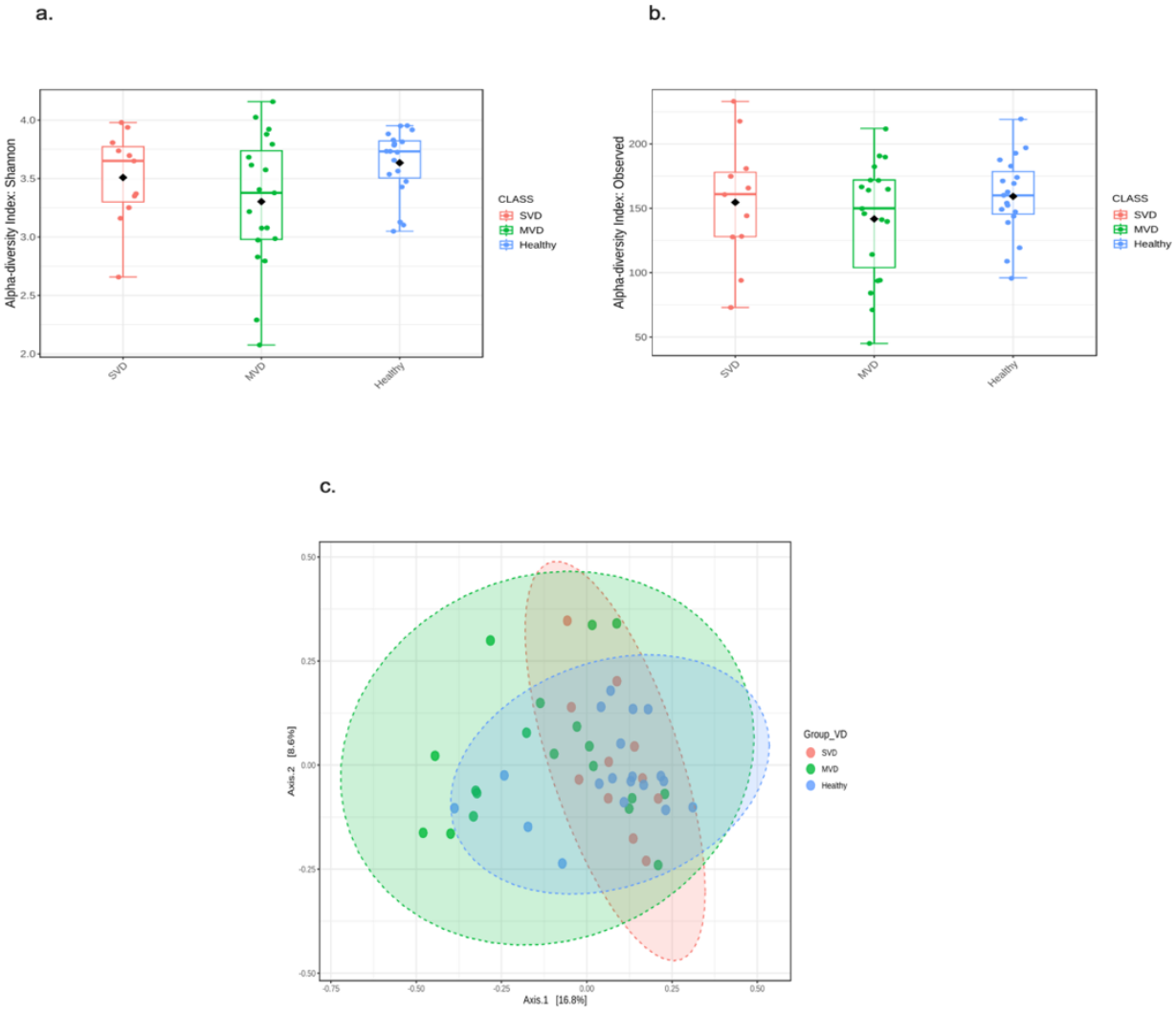


Figure 22 Analysis of alpha- and beta-diversity of microbial composition in single-vessel disease and multivessel disease patients.

Diversity within bacterial communities was measured by the Shannon diversity index (a), and the observed index (b). The principal coordinate analysis (PCoA) of beta diversity is based on the Bray-Curtis index (c). Kruskal-Wallis H test was used in the statistical test of the alpha diversity. Permanova (Permutational multivariate analysis of variance) test was used in the statistical test of the beta diversity. Healthy, healthy participants; MVD, multivessel disease patients; SVD, single-vessel disease patients.

3.7.2 Gut microbiota taxonomic composition of SVD and MVD patients

Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the four most prevalent bacterial phyla at the phylum level. Proteobacteria had a higher relative abundance in CCS patients with MVD (MVD patients had 8.35 percent Proteobacteria, SVD patients had 7.76 percent, and healthy individuals had 4.28 percent). Conversely, Verrucomicrobia and Actinobacteria exhibited the lowest relative abundance fraction in CCS patients with MVD. Compared to the other groups, SVD patients had a larger ratio of Firmicutes to Bacteroidetes (Figure 23a).

The two most prevalent families at the family level were found to be Lachnospiraceae and Bacteroidaceae. Compared to other groups, CCS patients with MVD had a proportionately higher prevalence of the Enterobacteriaceae, Streptococcaceae, and Prevotellaceae families (Figure 23b).

Prevotella and *Streptococcus* genera showed a statistically significant increase in relative abundance at the genus level in CCS patients with MVD compared to other groups (Figure 23c). In addition, CCS patients with MVD had the lowest relative abundances of the following genera: *Faecalibacterium* (the percentage in MVD patients, SVD patients, and healthy participants: 6.54, 7.70, and 8.41), *Ruminococcus* (the percentage in MVD patients, SVD patients, and healthy participants: 2.51, 2.63, and 3.40), and *Roseburia* (the percentage in MVD patients, SVD patients, and healthy participants: 2.60, 5.29, and 4.78, respectively). Moreover, the relative abundance of *Subdoligranulum* and *Collinsella* was significantly increased in healthy participants compared with SVD and MVD patients.

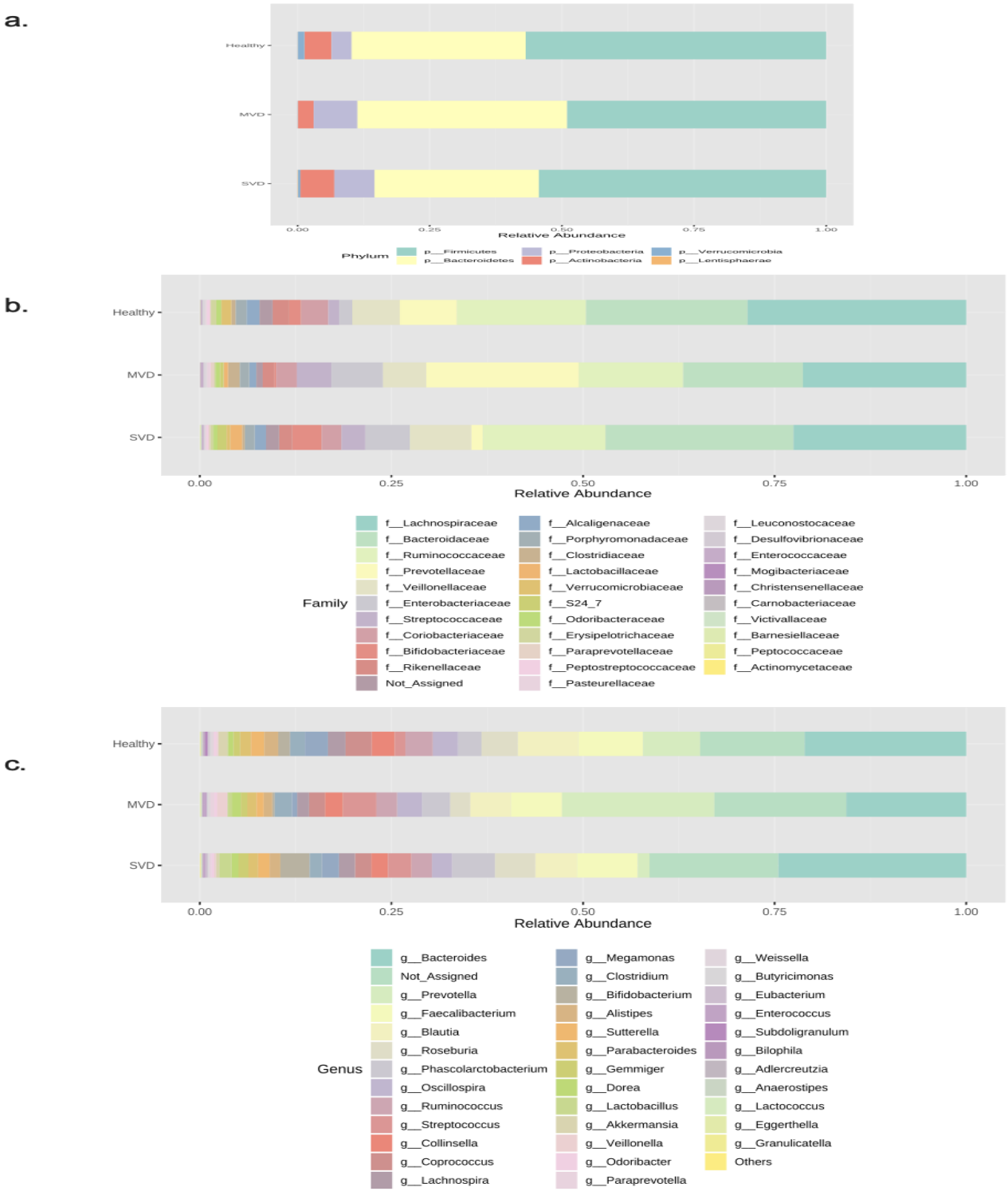


Figure 23 The relative abundance of bacterial taxonomic profile in single-vessel disease and multivessel disease patients.

At the phylum (a) family (b) and genus (c) level. Healthy, healthy participants; MVD, multivessel disease patients; SVD, single-vessel disease patients.

3.7.3 Gut microbiota characteristic of SVD and MVD patients

The LEfSe showed that in CCS patients with MVD, the relative abundance of Prevotellaceae, Enterobacteriaceae, Streptococcaceae, and f_S24_7 (in phylum Proteobacteria) had increased significantly at the family level when compared with healthy participants. When comparing CCS patients with MVD to healthy individuals, the LEfSe revealed that the *Prevotella, Veillonella*, and *Enterococcus* genera had considerably greater relative abundances at the genus level (Figure 24b).

Prevotellaceae was shown to be more comparatively frequent in MVD patients than SVD patients in this family-level examination of SVD and MVD patients (Figure 24c). *Prevotella* and *Veillonella* were found to be more abundant at the genus level in MVD patients compared to SVD patients (Figure 24c).

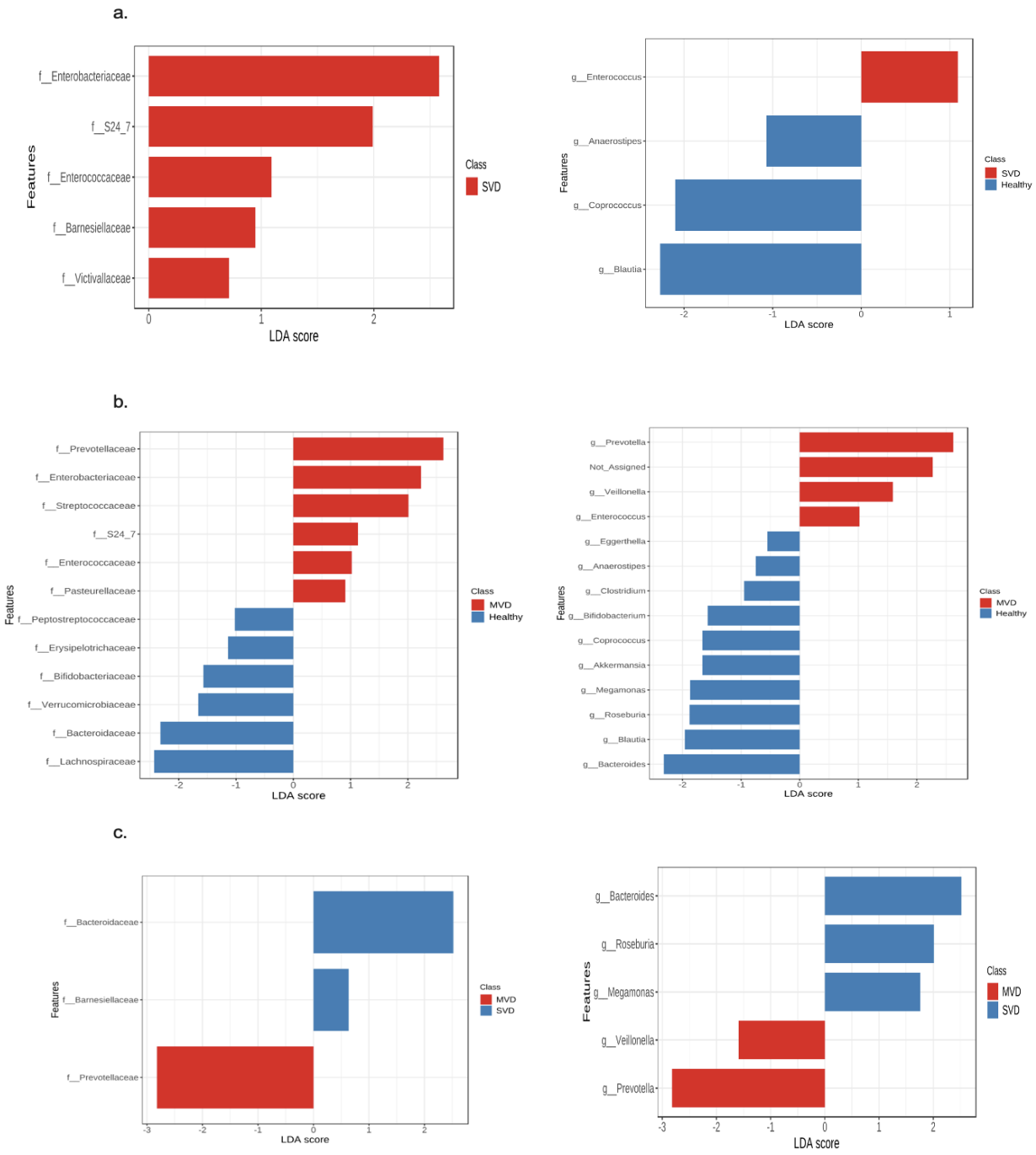


Figure 24 Linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota among the three groups.

SVD patients and healthy participants (a), MVD patients and healthy participants (b), and SVD patients and MVD patients (c). Healthy, healthy participants; MVD, multivessel disease patients; SVD, single-vessel disease patients.

3.7.4 Prediction of gut microbiota biomarkers to discriminate between SVD and MVD patients

To identify which bacteria are the crucial candidates in accounting for differences due to SVD and MVD in CCS patients, the random forest analysis was performed and demonstrated that Prevotella had the highest accuracy for discriminating MVD patients from SVD patients, followed by Veillonella, Enterococcus, Oscillospira, Catenibacterium, Lachnobacterium, Holdemania, and Streptococcus, respectively. On the other hand, Roseburia, Faecalibacterium, Bacteroides, Acidaminococcus, Bifidobacterium, Parabacteroides, and Megamonas, in that order, provided the best value for separating SVD patients from MVD patients (Figure 25).

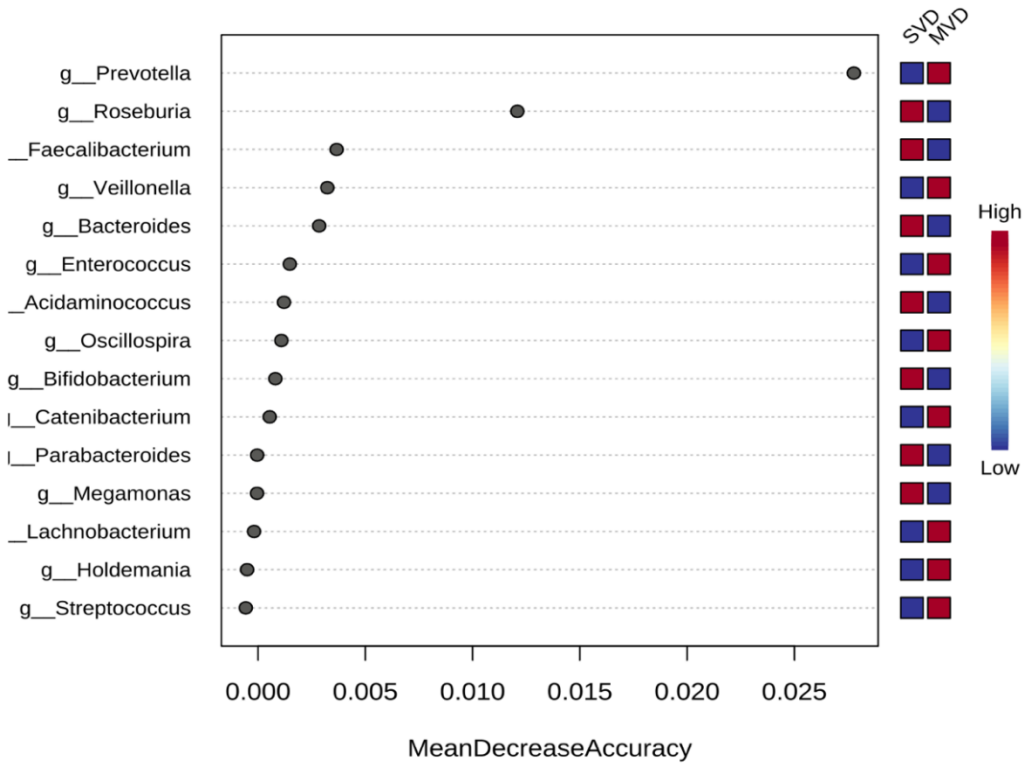


Figure 25 The Random Forest classification model

The top 15 most important microbial biomarkers to discriminate between SVD and MVD patients. Predictive attributions are ranked by their involvement in classification accuracy which is Mean Decrease Accuracy. MVD, multivessel disease patients; SVD, single-vessel disease patients.

CHAPTER V
DISCUSSION

Changes in the composition and function of the gut microbiome have been linked to a variety of chronic diseases, including metabolic syndromes, neurological disorders, gastrointestinal diseases, respiratory illnesses, and CVD such as type 2 diabetes mellitus, obesity, behavioral disorders, inflammatory bowel disease, persistent antibiotic-induced colitis, atopic asthma, and CAD.[163-164]

The three groups in this investigation have different gut microbiome. The group of patients with dyslipidemia and CCS had less diversity, which was consistent with findings from earlier investigations.[165–169] This result is in line with recent research showing that people with a variety of illnesses, such as obesity,[170] metabolic syndrome,[171] psoriatic arthritis,[172] Crohn's disease,[173] and hypertension,[174] had decreased bacterial diversity.

The bacterial microbiota's composition pattern varied considerably between the CCS patient group and the healthy volunteer group. Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the most common bacterial phyla, and these findings were consistent with previous research.[175] Changes in the major phyla Firmicutes to Bacteroidetes ratio have been suggested as a possible risk factor for CAD, implying that the development and progression of atherosclerosis and CAD may possibly be influenced by modifications in the composition of the microbiome.[176–178] Moreover, it has been demonstrated that individuals with CAD are significantly affected by a higher Firmicutes/Bacteroidetes ratio (F/B ratio).[178] The F/B ratio is a diagnostic tool for gut dysbiosis, and research has shown a correlation between it and a number of established cardiovascular risk factors, such as age, sex, diet, and BMI.[177,179–181] The group of patients with dyslipidemia in this study had the highest F/B ratio.

The phylum Proteobacteria has a large number of pathogenic taxa, such as *Salmonella, Vibrio, Escherichia,* and *Legionella*. In our investigation, the group of CCS patients had the largest relative abundance proportion of Proteobacteria.[163] A higher

relative abundance of Proteobacteria has been linked to gut dysbiosis and several human diseases, including obesity, type 2 diabetes, and cancer, according to a study by Shin et al.[182] The majority of bacteria that produce TMAO precursors are Proteobacteria, especially Enterobacteriaceae and some Firmicutes.[183]

The proportion of Fusobacteria was higher in the CCS patient group than in the other group, although Actinobacteria and Verrucomicrobia showed the lowest relative abundance proportion in the CCS patient group as compared to the other group. This result is in line with the research by Zhang et al.[184] and Cui et al.[178] *Fusobacterium nucleatum* may initially induce periodontal disease, which through inflammation and lipid metabolism eventually causes CAD.[185] Nevertheless, there is a lack of published studies on the correlation between Fusobacterium and CAD.

Furthermore, among the group of CCS patients, the genera *Streptococcus, Veillonella,* and *Prevotella* were statistically significantly more common than other groups in terms of abundance.[163] According to prior studies, *Prevotella* and *Streptococcus* were closely linked to CAD, atherosclerosis, and metabolic syndrome.[186–189] It was proposed that the bacterial microbiota of the CCS patients was characterized by alterations in the abundance of *Streptococcus, Veillonella*, and *Prevotella*. The correlation between *Streptococcus* spp. and subclinical coronary atherosclerosis was studied by Sayols-Baixeras et al. and the results showed that *S. anginosus* and *S. oralis* had the strongest relationships.[190] A study by Liu H. et al. found that the prevalence of certain gram-negative bacteria that produce LPS, such as *Escherichia, Shigella, Veillonella*, and *Klebsiella* increased with the severity of the CAD.[20] Elevated levels of LPS in the blood were linked to a tripling of the risk of incident atherosclerosis.[191] *Veillonella* is linked to cholesterol levels and can be identified in atherosclerotic plaque.[192] Furthermore, CAD patients have a significant relative abundance of Veillonella.[184,193] These results suggested that the development of CAD and lipid metabolism in patients with dyslipidemia may be related to variations in bacterial abundance.

There is debate concerning *Prevotella*'s significance to human health. *Prevotella* is a beneficial bacterium, yet it is linked to persistent inflammation. *Bacteroides* is associated with meals heavy in fat and protein, while *Prevotella* is associated with diets high in complex carbohydrates derived from plants, fruits, and vegetables.[194] *Prevotella* is a type of commensal bacteria that is present in healthy individuals. De Filippis et al. found that vegetarians had a high degree of Mediterranean diet, which was linked to these bacteria strains and increased SCFA levels.[195] In the gut microbiota of CAD patients, *Lactobacillus, Streptococcus,* and *Enterococcus* increased while *Bacteroides* and *Prevotella* decreased, according to Emoto et al.[177]

The differences in how *Prevotella* strains respond to diverse food regimens and medical situations in different patients may be explained by the strains' genetic diversity.[196] For instance, research on the gut microbiome of Italians revealed that varied dietary choices may have contributed to *P. copri* strains with distinct roles, which have implications for human health.[197] In non-Westernized individuals, *P. copri* increases the prevalence. Usually, their diets are high in fresh fruits and vegetables.[198]

Recent research, however, has linked rising *Prevotella* abundance and specific strains to low-grade systemic inflammation, obesity, insulin resistance, hypertension, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD).[186,199,200] This is because these conditions increase the immune responses of mucosal helper T-cells 17 and stimulate the production of IL-1, IL-6, IL-8, and IL-23 by epithelial cells.[201]

Our data, which is consistent with several research, showed that *P. copri* was more prevalent in CCS patients than in healthy participants. This bacterium is associated with several chronic inflammatory disorders, including CAD, heart valve calcification, rheumatoid arthritis (RA), periodontitis, HIV infection, metabolic syndrome, and inflammatory bowel disease. This immunologically important bacterium contributes to the development of RA.[202] The enrichment of *P. copri* in RA patients suggests that gut dysbiosis may play a role in the early stages of RA.[203] Furthermore, *P. copri* was linked to worsening arthritis in patients and showed a significant degree of genetic and functional variation based on the patients' lifestyles.[204] *P. copri* may be a significant risk

factor for CVD patients, especially those with calcified heart valves. The significant link between this microbe and LDL-C suggests that it may have pro-inflammatory properties. Because it plays a part in inflammation and immunity, it is a possible major pathogen linked to CVD.[205] Furthermore, periodontopathic bacteria *P. intermedia* and *P. nigrescens* were found in atherosclerotic plaques.[206]

In this study, patients with CSS had lower levels of *Faecalibacterium* than the other groups.[163] Zhu et al. found a correlation between this discovery and a decrease in *Faecalibacterium, Subdoligranulum, Roseburia*, and *Eubacterium rectale*.[207] *Faecalibacterium* has a notable anti-inflammatory function.[208]

When we compared the group of healthy volunteers to the other groups in our study, *Bacteroides* had the highest relative abundance, and *Roseburia* had a greater relative abundance in healthy volunteers. Previous studies have shown that *Bacteroides* and *Bifidobacterium* are the main protective bacteria for CCS because of their capacity to create SCFAs, which may have a protective effect on metabolism.[209–211] Furthermore, *Roseburia* has been associated with enhanced glucose intolerance and weight loss in mice and atherosclerosis patients while the normal control group has significantly higher *Roseburia* and *Eubacterium* abundances.[20,212] According to a study by Liu H. et al., as CAD progressed, the predominance of bacteria like Ruminococcaceae and Lachnospiraceae, which create butyric acid, reduced.[20] These results are consistent with our study, which found that the proportion of patients with CCS was lower in Lachnospiraceae and Ruminococcaceae than in other families.

The fermentation of complex carbohydrates produces metabolites, which are SCFAs. Acetate and butyrate are produced by members of the phylum Firmicutes, while butyrate is produced by members of the phylum Bacteroidetes. *Eubacterium rectale*, *Bacteroides,* and *Roseburia* show positive correlations with SCFAs. Colonocytes and the liver's gluconeogenic process both depend on SCFAs for energy. In addition, they enhance the host's immune response, maintain the intestinal barrier's integrity by regulating the expression of tight junction proteins, lower blood lipid levels by inhibiting

the synthesis of cholesterol, and control insulin sensitivity.[213–215] SCFAs are protective against atherosclerosis.[216]

The absence of butyrate-producing bacteria can lead to disruption to the gut barrier, which in turn can facilitate the release of microbial toxins such as LPS that bind to Toll-like receptors and cause inflammation. It has been discovered that patients with CAD have increased LPS production in their microbiomes, which is linked to both abdominal obesity and insulin resistance.[217,218]

Dietary choline, betaine, phosphatidylcholine, lecithin, and L-carnitine—found in a range of foods, including eggs, fish, red meat, soybeans, and peanuts—are necessary for the creation of TMAO, a significant risk factor for the development of CAD.[207,219] The gut bacteria contribute to the creation of TMAO by generating choline and the intermediate molecule trimethylamine (TMA). It has been revealed that the gut microbiota can synthesize choline through the enzyme phospholipase D. Hepatocytes utilize the flavin-containing monooxygenase enzyme to break down the TMA molecule produced by the microbiota into TMAO.[220] Producers of TMA include Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. Foam cell formation is induced by TMAO-dependent activation of macrophage scavenger receptors which results in impaired cholesterol metabolism in macrophages. The CAD risk increases with increasing TMAO generation.[221] Along with increased intestinal permeability, TMAO is associated with raised blood levels of the endotoxin LPS, endothelial dysfunction, and C-reactive protein. It may also result in hyperreactive platelets, which influences the advancement of CAD.[222]

Patients with CCS had the lowest LDL-C levels. The majority of patients received statin treatment. A lower prevalence of gut microbiota dysbiosis is linked to statin medication.[223] Statins have the potential to modulate gut microbiota in mice through the upregulation of *Bacteroides, Butyricimonas*, and *Mucispirillum.*[224] Furthermore, there is a correlation between the gut microbiota and the clinical outcome of statin therapy in patients with CAD. For example, decreased statin response is linked to both a notable decrease in the amount of *Lactobacillus* and *Akkermansia muciniphila* and an increase

in the number of *Holdemanella* and *Facecallibacterium*.[225] Additionally, statins have been connected to immunomodulatory and anti-inflammatory effects.[226]

The TG level showed a substantial positive correlation with *Megasphaera*, while HDL-C showed a negative correlation with it.[163] A study by Lopez-Montoya et al. supported our findings by demonstrating a strong correlation between patients with atherogenic dyslipidemia—defined as having low HDL-C and hypertriglyceridemia—and *Megasphaera* and *Escherichia-Shigella*.[227] Additionally, a study characterized *Megasphaera* as opportunistic infections and demonstrated that individuals with symptomatic stroke had a changed gut microbiota.[228] *Megasphaera* dramatically increased relative abundance and was linked with reduced physical activity in patients who were overweight and obese.[229] Megasphaera participates in a process that produces ammonia, which has negative consequences.[230]

The genera *Sutterella* and *Roseburia* showed a negative correlation with LDL-C in CCS patients. *Sutterella* is gram-negative, rod-shaped, and is associated with chronic inflammation. The potential immunomodulatory function of *Sutterella* species is suggested by their capacity to attach to intestinal epithelial cells.[231] A reduction in Firmicutes and an increase in Proteobacteria, the phylum that also includes *Sutterella* species, are the typical indicators of gut dysbiosis. An increase in Proteobacteria may increase the risk of chronic inflammatory disease in the host and contribute to non-specific mucosal inflammation brought on by LPS.[232] However, the correlation between *Sutterella* and LDL-C should be further investigated. *Roseburia* is a SCFA-producing bacteria that has a protective role in atherosclerosis and CAD.

Chronic inflammation can be identified with the usage of hs-CRP. De Oliveira et al.'s research connected periodontal disease to low-grade inflammation, elevated CRP, fibrinogen, and CAD.[233] In CCS patients, hs-CRP and the severity of CAD are correlated.[234] Major adverse cardiovascular events and death were more likely to occur in patients with myocardial infarction who had elevated hs-CRP levels (≥ 2 mg/L).[235] This study's CCS patients showed a previously unreported positive correlation between hs-CRP and the families Lachnospiraceae, Peptostreptococcaceae, and *Pediococcus*.

The association between the gut microbiota and TNF-α, IL-1 , and IL-6 in CCS patients undergoing coronary angiography was demonstrated in this study.[164] Pro-inflammatory cytokines may be related to indicate CCS patients from healthy participants in this study. According to our study, TNF-α was statistically significantly greater in CCS patients than in the other group, which is consistent with other research. 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.[236] The study of mice showed that TNF-α had a significant role in the development of atherosclerosis.[237] On the other hand, TNF-α inhibition resulted in reduced atherosclerosis.[238,239]

In the present study, CCS patients had greater levels of IL-6 than the control, which is consistent with many other studies. In the early stages of CAD development, the inflammatory response is important. One of the key inflammatory cytokines that control the inflammatory response in CAD is IL-6.[240] IL-6 is the acute-phase response and its effects include activating the endothelium via von Willebrand factor (VWF), tissue plasminogen activator antigen (t-PA), activating coagulation via factor VIII, fibrin D-dimer and releasing white blood cells, platelets from bone marrow as well as stimulating the production of CRP and fibrinogen in the liver. Adipocytes and white blood cells release plasma IL-6, which has been linked to obesity, insulin resistance, and dyslipidemia.[241,242] According to reports, the IL-6 gene has an impact on IL-6 levels in the blood, which is closely related to the development of CAD and type 2 diabetes.[243–245]

IL-6 does not only contribute to the development of CAD but is also involved in the prognosis and a potential maker of CAD. For CCS patients' cardiovascular prognosis, IL-6 level was an inflammatory marker and was linked to atherosclerosis and several clinical outcomes, including myocardial infarction, heart failure, cardiovascular death, all-cause death, and cancer death. As a result, this is helpful for CAD therapy and prevention.[191,246] Moreover, plasma IL-6 has a potential role as a biomarker in ACS. In an optical coherence tomography (OCT) study' Koyama et al. showed a correlation between thin-cap fibroatheroma (TCFA), a lesion with a high potential for adverse

cardiac events, and both IL-6 and hs-CRP levels. Furthermore, IL-6 is more likely to accurately predict TCFA than hs-CRP.[247]

There was no difference in IL-1 levels between the CCS patients and the control group in this investigation. However, some studies indicated that IL-1 is a pro-inflammatory cytokine that has a role in the development of atherosclerosis. Chronic stress is related to trimethylamine N-oxide (TMAO), mediated by IL-1.[248] IL-1, a pro-inflammatory cytokine that regulates endothelial cell proliferation and the expression of adhesion molecules on the artery wall, stimulates the production of IL-6.[249] IL-1 was associated with the pathogenesis of atherosclerosis vascular calcification.[250] In the Anti-inflammatory Therapy with Canakinumab for Atherosclerotic Disease (CANTOS) trial, in patients who already had a heart attack, treatment with the monoclonal IL-1 neutralizing antibody canakinumab may decrease the risk of further cardiovascular events.[251]

Although hs-CRP is a nonspecific inflammatory marker, increased levels of hs-CRP are strongly correlated with inflammation and CAD, as the result of this study.[252] A significant acute phase reactant and systemic inflammatory mediator, CRP is primarily generated by hepatocytes in response to cytokines including IL-1, IL-6, and TNF-α. Nitric oxide level decreased as a result of CRP's inhibition of an endothelial nitric oxide synthase. According to several clinical studies, endothelial dysfunction and different CAD stages are consistently linked to high CRP levels. Recently, it was discovered that high hs-CRP levels correlated favorably with IL-6 and LDL-C, raised the risk of CAD, and increased mortality. In CCS patients, there is a connection between hs-CRP and the severity of the CAD.[234]

Specific bacterial genera, such as *Sutterella, Prevotella, and Phascolarctobacterium* may be helpful to better distinguish CCS patients from healthy participants, this finding has not been reported before.[164] *Prevotella*'s impact on human health is debatable. Although *Prevotella* is helpful, chronic inflammation is linked to it. Recent studies have linked increased *Prevotella* abundance and specific strains to obesity, insulin resistance, and low-grade systemic inflammation due to the stimulation of epithelial cells to produce IL-1, IL-6, IL-8, and IL-23 and augmentation of mucosal

helper T-cell 17 immune responses.[201] *Prevotella copri* and LDL-C are positively correlated, which suggests that they may have pro-inflammatory properties. Because of its functions in inflammation and immunology, it is a potential major pathogen linked to CAD.[205]

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

Gram-negative bacteria that generate LPS, including *Escherichia coli, Shigella, Proteus, Veillonella,* and *Klebsiella*, increased the severity of CAD.[20] 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.[253] 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.[254–256] Even though *Veillonella parvula* LPS is less effective than Enterobacteriaceae LPS, it may still cause the production of cytokines (TNF-α, IL-1, IL-6, and IL-10) via Toll-like receptor (TLR) pathways in humans and mice.[257]

Prior studies suggested that *Bifidobacterium* (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.[211,258,259] 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.[260] Moreover, *B. lactis can* lower cholesterol levels, TNF-α, IL-6, and BMI, which may lower the risk of CVD in metabolic syndrome patients.[259] *B. breve* and *B. longum* may be effective in treating TMAO-related diseases.[261] CAD mice and patients have a lower

relative abundance of *Bifidobacterium*.[262] However, this bacterium did not find a statistically significant association in this study.

This analysis encourages that gut microbiome in specific genera and pro-inflammatory cytokines may be related to indicate significant CAD in CCS patients from healthy participants. Moreover, the correlation between pro-inflammatory cytokines and the gut microbiome in CCS patients was demonstrated.

After analysis of characteristics different in gut microbial community diversity and composition of 49 patients, including 11 CCS patients with SVD, 19 CCS patients with MVD, and 19 healthy participants. In CCS patients with MVD had differences in the diversity and composition of the gut microbiome compared to CCS patients with SVD and healthy participants. The reduction in diversity in MVD patients compared to the other two groups, this finding in more complex diseases is consistent with other studies, for example, there was a trend to less diversity in CAD with nonalcoholic fatty liver disease (NAFLD) patients comparing CAD patients.[184]

This study indicated that CCS patients with MVD increased in Proteobacteria, *Prevotella,* and *Streptococcus*. Proteobacteria is linked to gut dysbiosis and a high concentration of bacteria-producing trimethylamine-*N*-oxide (TMAO), a gut metabolite linked to a high risk of developing CAD.[263] The direct effects of these microorganisms on CCS patients with MVD have not been studied. CAD and atherosclerosis were closely connected with the *Prevotella* and *Streptococcus* genera.[186]

On the other hand, beneficial microorganisms, such as *Roseburia, Ruminococcus,* and *Faecalibacterium* genera had the lowest relative abundance in CCS patients with MVD. *Roseburia* has been connected to mice's enhanced glucose tolerance and weight reduction, as well as atherosclerosis patients. *Ruminococcus* is reduced with the progression of CAD.[20] In addition, *Faecalibacterium* has an anti-inflammatory property.[208]

The development of CAD is significantly affected by the gut microbiome. In Yu et al.'s study, the abundance of *Escherichia-Shigella* was significantly increased in the MVD and SVD groups and positively correlated with LDL-C, while the abundance of

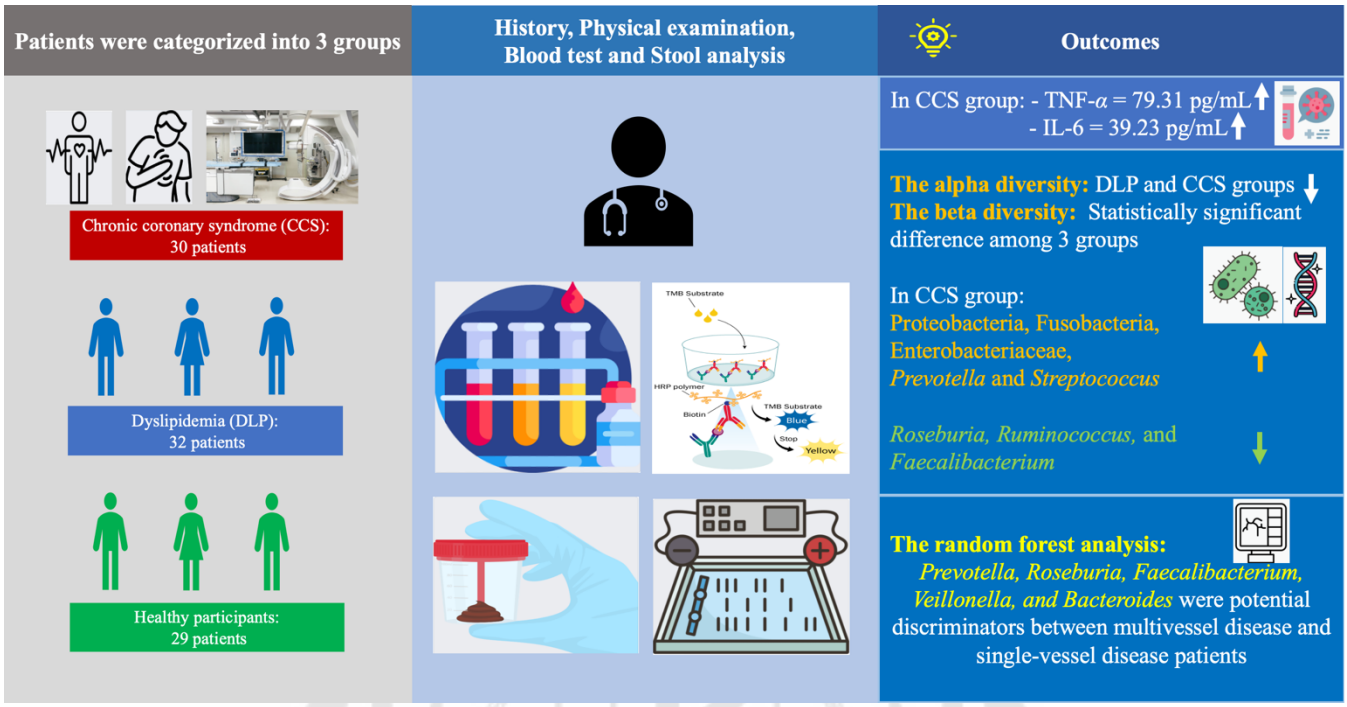
Subdoligranulum and *Collinsella* was significantly decreased compared with the control group.[264] These findings are correlated with this study, the Proteobacteria phylum and the Enterobacteriaceae family were more common in CCS patients with MVD than in others. Most bacteria generating TMAO precursors are Proteobacteria, particularly those in the Enterobacteriaceae family and some Firmicutes.[183]

Prevotella's influence on human health is controversial. There was no study about *Prevotella* in varying numbers of coronary stenotic vessels in CCS patients. *Prevotella* has been associated with diets high in complex carbohydrates from plants, fruits, and vegetables and can be found in healthy humans.[194] *Bacteroides* and *Prevotella were* decreased in CAD patients.[177] The genetic variety of *Prevotella* strains may explain the variances in how it responds to dietary and health conditions in different persons.[196] On the other hand, *Prevotella* abundance is increased in metabolic syndrome, insulin resistance, and low-grade systemic inflammation due to the stimulation of epithelial cells to produce IL-1, IL-6, IL-8, and IL-23 and the stimulation of mucosal helper T-cell 17 immune responses.[201] For instance, *P. copri* may be a significant risk factor for CAD patients due to a positive connection between this bacterium and LDL-C and it may have pro-inflammatory properties.[205] The latter finding supports our investigation that *Prevotella* was higher in CCS patients with MVD than in the other groups.

To better understand the roles of *Prevotella* in the pathophysiology of CCS and potentially develop a set of diagnostic biomarkers for early detection and additional therapy of the disease, we should investigate the genetic diversity and function of *Prevotella* in CCS in a larger and more diverse population.

Conclusion

This investigation showed that the gut microbiota compositions of patients with CCS, patients with dyslipidemia, and healthy volunteers differed significantly. Compared to healthy volunteers, patients with CCS and dyslipidemia had reduced alpha diversity. In contrast to *Roseburia, Ruminococcus*, and *Faecalibacterium*, which were much lower in CCS patients, the relative abundance of Proteobacteria, Fusobacteria, *Prevotella,* and *Streptococcus* was significantly higher. In CCS patients, the genera *Sutterella* and *Roseburia* were negatively correlated with LDL-C. *Megasphaera* showed a high positive correlation with TG level and a negative correlation with HDL-C in patients with dyslipidemia. Gut microbiota profile may be associated with parameters involved in the development of CAD in CCS patients. There is a potential relationship between gut microbiome composition and inflammatory biomarkers 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. Moreover, MVD patients had differences in the diversity and composition of the gut microbiome compared to SVD patients. *Prevotella* and *Veillonella* were more enriched in MVD patients compared to SVD patients. As a result, the development of SVD and MVD is associated with changes in the gut microbiome.


Patients were categorized into 3 groups
Chronic coronary syndrome (CCS): 30 patients
Dyslipidemia (DLP): 32 patients
Healthy participants: 29 patients
History, Physical examination, Blood test and Stool analysis
TMB Substrate
HRP polymer
Biotin
Blue
Stop
Yellow
Outcomes
In CCS group: - TNF-α = 79.31 pg/mL
- IL-6 = 39.23 pg/mL
The alpha diversity: DLP and CCS groups
The beta diversity: Statistically significant difference among 3 groups
In CCS group:
Proteobacteria, Fusobacteria, Enterobacteriaceae, Prevotella and Streptococcus
Roseburia, Ruminococcus, and Faecalibacterium
The random forest analysis:
Prevotella, Roseburia, Faecalibacterium, Veillonella, and Bacteroides were potential discriminators between multivessel disease and single-vessel disease patients

REFERENCE

1. National Statistical Office [Internet]. Thailand: Death rates per 100,000 population by leading cause of death and sex whole kingdom 2007-2014. 2017 [Cited 2017 Jul 5]. Available from: http://www.nso.go.th/sites/2014en. National statistical office. Death rates per 100,000 population by leading cause of death and sex, whole kingdom 2007 - 2014. 2014.

2. Vaduganathan M, Mensah GA, Turco JV, Fuster V, Roth GA. The global burden of cardiovascular diseases and risk. J Am Coll Cardiol. 2022;80(25):2361–71.

3. Abdul-Ghani M, DeFronzo RA, Del Prato S, Chilton R, Singh R, Ryder REJ. Cardiovascular disease and type 2 diabetes: Has the dawn of a new era arrived? Diabetes Care. 2017;40(7):813–20.

4. Du Z, Qin Y. Dyslipidemia and cardiovascular disease: Current knowledge, existing challenges, and new opportunities for management strategies. J Clin Med. 2023;12(1):363.

5. Dustan HP. Hypertension. J Am Coll Cardiol. 2000;35(5):10B-12B.

6. Powell-Wiley TM, Poirier P, Burke LE, Després JP, Gordon-Larsen P, Lavie CJ, et al. Obesity and cardiovascular disease: A scientific statement from the American Heart Association Circulation [Internet]. 2021 [cited 2023 Dec 12];143(21). Available from: https://www.ahajournals.org/doi/10.1161/CIR.0000000000000973

7. Adeva-Andany MM, Martínez-Rodríguez J, González-Lucán M, Fernández-Fernández C, Castro-Quintela E. Insulin resistance is a cardiovascular risk factor in humans. Diabetes Metab Syndr. 2019;13(2):1449–55.

8. Tune JD, Goodwill AG, Sassoon DJ, Mather KJ. Cardiovascular consequences of metabolic syndrome. Transl Res. 2017;183:57–70.

9. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. The Lancet. 2004;364(9438):937–52.

10. Knuuti J, Wijns W, Saraste A, Capodanno D, Barbato E, Funck-Brentano C, et al. 2019 ESC guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J. 2020;41(3):407–77.

11. Rahman M, Islam F, Rashid H, Mamun AA, Rahaman MdS, Islam M, et al. The gut microbiota (microbiome) in cardiovascular disease and its therapeutic regulation. Front Cell Infect Microbiol. 2022;12:903570.

12. Hiergeist A, Gläsner J, Reischl U, Gessner A. Analyses of intestinal microbiota: culture versus sequencing. ILAR J. 2015;56(2):228–40.

13. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017;474(11):1823–36.

14. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol. 2021;19(1):55–71.

15. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506–14.

16. Carlson JL, Erickson JM, Lloyd BB, Slavin JL. Health effects and sources of prebiotic dietary fiber. Current Developments in Nutrition [Internet]. 2018 [cited 2022 Jun 8];2(3). Available from: https://academic.oup.com/cdn/article/doi/10.1093/cdn/nzy005/4828321

17. Tenorio-Jiménez C, Martínez-Ramírez MJ, Gil Á, Gómez-Llorente C. Effects of probiotics on metabolic syndrome: A systematic review of randomized clinical trials. Nutrients. 2020;12(1):124.

18. Office of policy and strategy ministry of health. Public health statistics report number and death rate per 100,000 population, classified by major causes, 2014-2018 [Internet]. 2017 [cited 2020 Nov 20]. Available from: https://bps.moph.go.th/new_bps/sites/default/files/.

19. Liu H, Zhuang J, Tang P, Li J, Xiong X, Deng H. The role of the gut microbiota in coronary heart disease. Curr Atheroscler Rep. 2020;22(12):77.

20. Liu H, Chen X, Hu X, Niu H, Tian R, Wang H, et al. Alterations in the gut microbiome and metabolism with coronary artery disease severity. Microbiome. 2019;7(1):68.

21. Jonsson AL, Bäckhed F. Role of gut microbiota in atherosclerosis. Nat Rev Cardiol. 2017;14(2):79–87.

22. Petersen JW, Pepine CJ. Microvascular coronary dysfunction and ischemic heart disease: Where are we in 2014? Trends Cardiovas Med. 2015;25(2):98–103.

23. Ellis SG, Tamai H, Nobuyoshi M, Kosuga K, Colombo A, Holmes DR, et al. Contemporary percutaneous treatment of unprotected left main coronary stenoses: Initial results from a multicenter registry analysis 1994–1996. Circulation. 1997;96(11):3867–72.

24. Knuuti J, Wijns W, Saraste A, Capodanno D, Barbato E, Funck-Brentano C, et al. 2019 ESC guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J. 2020;41(3):407–77.

25. Ralapanawa U, Sivakanesan R. Epidemiology and the magnitude of coronary artery disease and acute coronary syndrome: A Narrative Review. J Epidemiol Glob Health. 2021;11(2):169–77.

26. Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A. Epidemiology of coronary heart disease and acute coronary syndrome. Ann Transl Med. 2016;4(13):256.

27. Centers for Disease Control and Prevention. Underlying cause of death, 1999–2018. CDC WONDER online database. Atlanta, GA: Centers for Disease Control and Prevention; 2018. Accessed March 12, 2020.

28. Fryar CD, Chen TC, Li X. Prevalence of uncontrolled risk factors for cardiovascular disease: United States, 1999-2010. NCHS Data Brief. 2012;(103):1–8.

29. Tatsanavivat P. Prevalence of coronary heart disease and major cardiovascular risk factors in Thailand. Int J Epidemiol. 1998;27(3):405–9.

30. Fox KAA, Metra M, Morais J, Atar D. The myth of “stable” coronary artery disease. Nat Rev Cardiol. 2020;17(1):9–21.

31. Perdoncin E, Duvernoy C. Treatment of coronary artery disease in women. Methodist Debakey Cardiovasc J. 2017;13(4):201–8.

32. Malakar AKr, Choudhury D, Halder B, Paul P, Uddin A, Chakraborty S. A review on coronary artery disease, its risk factors, and therapeutics. J Cell Physiol. 2019;234(10):16812–23.

33. Shrestha N, Shrestha N, Bhusal S, Neupane A, Pandey R, Lohala N, et al. Prevalence of smoking among medical students in a tertiary care teaching hospital. J Nepal Med Assoc [Internet]. 2020 [cited 2022 Sep 15];58(226). Available from: https://www.jnma.com.np/jnma/index.php/jnma/article/view/5006

34. Yusuf S, Hawken S, Ôunpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. The Lancet. 2004;364(9438):937–52.

35. Linton MF, Yancey PG, Davies SS, Jerome WG, Linton EF, Song WL, et al. The role of lipids and lipoproteins in atherosclerosis. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 [cited 2022 Sep 15]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK343489/

36. Castelli WP. The new pathophysiology of coronary artery disease. Am J Card. 1998;82(10):60–5.

37. Jinnouchi H, Kolodgie FD, Romero M, Virmani R, Finn AV. Pathophysiology of coronary artery disease. In: Yuan C, Hatsukami TS, Mossa-Basha M, editors. Vessel Based Imaging Techniques [Internet]. Cham: Springer International Publishing; 2020 [cited 2022 Jun 22]. p. 211–27. Available from: http://link.springer.com/10.1007/978-3-030-25249-6_11

38. Libby P, Theroux P. Pathophysiology of coronary artery disease. Circulation. 2005;111(25):3481–8.

39. Malmberg M, Gunn J, Rautava P, Sipilä J, Kytö V. Outcome of acute myocardial infarction versus stable coronary artery disease patients treated with coronary bypass surgery. Ann Med. 2021;53(1):70–7.

40. Farkouh ME, Boden WE, Bittner V, Muratov V, Hartigan P, Ogdie M, et al. Risk Factor control for coronary artery disease secondary prevention in large randomized trials. J Am Coll Cardiol. 2013;61(15):1607–15.

41. Lukkarinen H, Hentinen M. Treatments of coronary artery disease improve quality of life in the long term. Nursing Research [Internet]. 2006;55(1). Available from: https://journals.lww.com/nursingresearchonline/Fulltext/2006/01000/Treatments_of_Coronary_Artery_Disease_Improve.4.aspx

42. Kandaswamy E, Zuo L. Recent advances in treatment of coronary artery disease: Role of science and technology. IJMS. 2018;19(2):424.

43. Neumann FJ, Sousa-Uva M, Ahlsson A, Alfonso F, Banning AP, Benedetto U, et al. 2018 ESC/EACTS guidelines on myocardial revascularization. EuroIntervention. 2019;14(14):1435–534.

44. Gu D, Qu J, Zhang H, Zheng Z. Revascularization for coronary artery disease: Principle and challenges. In: Wang M, editor. Coronary artery disease: Therapeutics and drug discovery [Internet]. Singapore: Springer Singapore; 2020 [cited 2022 Jun 22]. p. 75–100. (Advances in Experimental Medicine and Biology; vol. 1177). Available from: http://link.springer.com/10.1007/978-981-15-2517-9_3

45. Hayward RA. Optimizing statin treatment for primary prevention of coronary artery disease. Ann Intern Med. 2010;152(2):69.

46. Ambrose JA, Najafi A. Strategies for the prevention of coronary artery disease complications: Can we do better? Am J Med. 2018;131(9):1003–9.

47. Winzer EB, Woitek F, Linke A. Physical activity in the prevention and treatment of coronary artery disease. JAHA. 2018;7(4):e007725.

48. Steg PG, Ducrocq G. Future of the prevention and treatment of coronary artery disease. Circ J. 2016;80(5):1067–72.

49. Akyuz A. Exercise and coronary heart disease. In: Xiao J, editor. Physical Exercise for Human Health [Internet]. Singapore: Springer Singapore; 2020 [cited 2022 Jun 29]. p. 169–79. (Advances in Experimental Medicine and Biology; vol. 1228). Available from: http://link.springer.com/10.1007/978-981-15-1792-1_11

50. Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, et al. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce

atherosclerotic cardiovascular risk in adults. J Am Coll Cardiol. 2014;63(25):2889–934.

51. The AIM-HIGH Investigators. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N Engl J Med. 2011;365(24):2255–67.

52. Lin CF, Chang YH, Chien SC, Lin YH, Yeh HY. Epidemiology of dyslipidemia in the Asia Pacific region. Int J Gerontol. 2018;12(1):2–6.

53. Aekplakorn W, Taneepanichskul S, Kessomboon P, Chongsuvivatwong V, Putwatana P, Sritara P, et al. Prevalence of dyslipidemia and management in the Thai population, national health examination survey IV, 2009. J Lipids. 2014;2014:1–13.

54. Pirillo A, Casula M, Olmastroni E, Norata GD, Catapano AL. Global epidemiology of dyslipidaemias. Nat Rev Cardiol. 2021;18(10):689–700.

55. Amarenco P, Labreuche J. Lipid management in the prevention of stroke: review and updated meta-analysis of statins for stroke prevention. Lancet Neurol. 2009;8(5):453–63.

56. Feingold KR. Introduction to lipids and lipoproteins. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 [cited 2022 Jun 28]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK305896/

57. Galis ZS. Vulnerable plaque: The devil is in the details. Circulation. 2004;110(3):244–6.

58. Horton J, Cohen J, Hobbs H. Molecular biology of PCSK9: its role in LDL metabolism. Trends Biochem Sci. 2007;32(2):71–7.

59. Goldstein JL, Brown MS. A century of cholesterol and coronaries: From plaques to genes to statins. Cell. 2015;161(1):161–72.

60. Lipid-related markers and cardiovascular disease prediction. JAMA [Internet]. 2012 [cited 2022 Jun 29];307(23). Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2012.6571

61. Khera AV, Won HH, Peloso GM, Lawson KS, Bartz TM, Deng X, et al. Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. J Am Coll Cardiol. 2016;67(22):2578–89.

62. Linton MF, Farese RV, Young SG. Familial hypobetalipoproteinemia. J Lipid Res. 1993;34(4):521–41.

63. Tybjarghansen A, Humphries S. Familial defective apolipoprotein B-100: a single mutation that causes hypercholesterolemia and premature coronary artery disease. Atherosclerosis. 1992;96(2–3):91–107.

64. Cholesterol Treatment Trialists' (CTT) Collaboration. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170000 participants in 26 randomised trials. The Lancet. 2010;376(9753):1670–81.

65. Jellinger PS, Handelsman Y, Rosenblit PD, Bloomgarden ZT, Fonseca VA, Garber AJ, et al. American Association of Clinical Endocrinologists and American College of Endocrinology Guidelines for management of dyslipidemia and prevention of cardiovascular disease. Endocr Pract. 2017;23:1–87.

66. Phrommintikul A, Chinwong D, Patumanond J, Chinwong S, Siriwattana K, Gunaparn S, et al. Low-density lipoprotein cholesterol of less than 70 mg/dL is associated with fewer cardiovascular events in acute coronary syndrome patients: a real-life cohort in Thailand. TCRM. 2015;659.

67. Liao JK. Safety and efficacy of statins in Asians. Am J Cardiol. 2007;99(3):410–4.

68. Grundy SM, Stone NJ, Bailey AL, Beam C, Birtcher KK, Blumenthal RS, et al. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the management of blood cholesterol: A report of the American College of Cardiology/American Heart Association Task Force on clinical practice guidelines. Circulation [Internet]. 2019 [cited 2022 Jun 29];139(25). Available from: https://www.ahajournals.org/doi/10.1161/CIR.0000000000000625

69. Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, W Neil HA, Livingstone SJ, et al. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. The Lancet. 2004;364(9435):685–96.

70. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, et al. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. N Engl J Med. 2004;350(15):1495–504.

71. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med. 1995;333(20):1301–7.

72. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet. 1994;344(8934):1383–9.

73. de Lemos JA, Blazing MA, Wiviott SD, Lewis EF, Fox KAA, White HD, et al. Early intensive vs a delayed conservative simvastatin strategy in patients with acute coronary syndromes: Phase Z of the A to Z trial. JAMA. 2004;292(11):1307.

74. Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, et al. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: The ASTEROID Trial. JAMA. 2006;295(13):1556.

75. Oyama K, Giugliano RP, Blazing MA, Park JG, Tershakovec AM, Sabatine MS, et al. Baseline low-density lipoprotein cholesterol and clinical outcomes of combining ezetimibe with statin therapy in IMPROVE-IT. J Am Coll Cardiol. 2021;78(15):1499–507.

76. Cannon CP, Blazing MA, Giugliano RP, McCagg A, White JA, Theroux P, et al. Ezetimibe added to statin therapy after acute coronary syndromes. N Engl J Med. 2015;372(25):2387–97.

77. Gencer B, Mach F, Murphy SA, De Ferrari GM, Huber K, Lewis BS, et al. Efficacy of evolocumab on cardiovascular outcomes in patients with recent myocardial infarction: A prespecified secondary analysis from the FOURIER Trial. JAMA Cardiol. 2020;5(8):952.

78. Ridker PM, Tardif JC, Amarenco P, Duggan W, Glynn RJ, Jukema JW, et al. Lipid-reduction variability and antidrug-antibody formation with Bococizumab. N Engl J Med. 2017;376(16):1517–26.

79. Wong ND, Rosenblit PD, Greenfield RS. Advances in dyslipidemia management for prevention of atherosclerosis: PCSK9 monoclonal antibody therapy and beyond. Cardiovasc Diagn Ther. 2017;67(1):S11–20.

80. Raal FJ, Honarpour N, Blom DJ, Hovingh GK, Xu F, Scott R, et al. Inhibition of PCSK9 with evolocumab in homozygous familial hypercholesterolaemia (TESLA Part B): a randomised, double-blind, placebo-controlled trial. The Lancet. 2015;385(9965):341–50.

81. Novakovic M, Rout A, Kingsley T, Kirchoff R, Singh A, Verma V, et al. Role of gut microbiota in cardiovascular diseases. WJC. 2020;12(4):110–22.

82. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804–10.

83. MetaHIT Consortium, Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59–65.

84. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiol Rev. 2001;81(3):1031–64.

85. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009;9(1):123.

86. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci USA. 2011;108(supplement_1):4680–7.

87. Spanogiannopoulos P, Bess EN, Carmody RN, Turnbaugh PJ. The microbial pharmacists within us: a metagenomic view of xenobiotic metabolism. Nat Rev Microbiol. 2016;14(5):273–87.

88. Ahmad AF, Dwivedi G, O'Gara F, Caparros-Martin J, Ward NC. The gut microbiome and cardiovascular disease: current knowledge and clinical potential. Am J Physiol Heart Circ Physiol. 2019;317(5):H923–38.

89. Lindskog Jonsson A, Hållenius FF, Akrami R, Johansson E, Wester P, Arnerlöv C, et al. Bacterial profile in human atherosclerotic plaques. Atherosclerosis. 2017;263:177–83.

90. Epstein SE, Zhu J, Burnett MS, Zhou YF, Vercellotti G, Hajjar D. Infection and atherosclerosis: Potential roles of pathogen burden and molecular mimicry. ATVB. 2000;20(6):1417–20.

91. Muzio M, Polntarutti N, Bosisio D, Prahladan MK, Mantovani A. Toll like receptor family (TLT) and signalling pathway. Eur Cytokine Netw. 2000;11(3):489–90.

92. Scudamore CL, Jepson MA, Hirst BH, Hugh R, Miller P. The rat mucosal mast cell chymase, RMCP-11, alters epithelial cell monolayer permeability in association with altered distribution of the tight junction proteins ZO-1 and occludin. Eur J Cell Biol. 1998;75(4):321–30.

93. Moreira APB, Texeira TFS, Ferreira AB, do Carmo Gouveia Peluzio M, de Cássia Gonçalves Alfenas R. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. Br J Nutr. 2012;108(5):801–9.

94. Karlsson FH, Fåk F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012;3(1):1245.

95. Jie Z, Xia H, Zhong SL, Feng Q, Li S, Liang S, et al. The gut microbiome in atherosclerotic cardiovascular disease. Nat Commun. 2017;8(1):845.

96. Tomaro-Duchesneau C, Saha S, Malhotra M, Jones ML, Rodes L, Prakash S. *Lactobacillus fermentum* NCIMB 5221 and NCIMB 2797 as cholesterol-lowering probiotic biotherapeutics: *in vitro* analysis. Benef Microbes. 2015;6(6):861–9.

97. Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. *Akkermansia Muciniphila* protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in *Apoe* $^{-/-}$ mice. Circulation. 2016;133(24):2434–46.

98. Tunick MH, Van Hekken DL. Dairy products and health: Recent insights. J Agric Food Chem. 2015;63(43):9381–8.

99. Palaniyandi SA, Damodharan K, Suh JW, Yang SH. Probiotic characterization of cholesterol-lowering Lactobacillus fermentum MJM60397. Probiotics Antimicrob Proteins. 2020;12(3):1161–72.

100. Lim PS, Loke CF, Ho YW, Tan HY. Cholesterol homeostasis associated with probiotic supplementation in vivo. J Appl Microbiol. 2020;129(5):1374–88.

101. Jonsson AL, Bäckhed F. Role of gut microbiota in atherosclerosis. Nat Rev Cardiol. 2017;14(2):79–87.

102. Hansson GK, Jonasson L. The discovery of cellular immunity in the atherosclerotic plaque. ATVB. 2009;29(11):1714–7.

103. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci USA. 2011;108(supplement_1):4592–8.

104. Frostegård J. Immunity, atherosclerosis and cardiovascular disease. BMC Med. 2013;11(1):117.

105. Elkind MSV, Luna JM, Moon YP, Boden-Albala B, Liu KM, Spitalnik S, et al. Infectious burden and carotid plaque thickness: The northern Manhattan study. Stroke [Internet]. 2010 [cited 2022 Jun 23];41(3). Available from: https://www.ahajournals.org/doi/10.1161/STROKEAHA.109.571299

106. Yamashita T. Intestinal immunity and gut microbiota in atherogenesis. J Atheroscler Thromb. 2017;24(2):110–9.

107. Nie J, Zhang L, Zhao G, Du X. Quercetin reduces atherosclerotic lesions by altering the gut microbiota and reducing atherogenic lipid metabolites. J Appl Microbiol. 2019;127(6):1824–34.

108. Chistiakov DA, Bobryshev YV, Kozarov E, Sobenin IA, Orekhov AN. Role of gut microbiota in the modulation of atherosclerosis-associated immune response. Front Microbiol [Internet]. 2015 [cited 2022 Jun 23];6. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00671/abstract

109. Wick G. Atherosclerosis – An autoimmune disease due to an immune reaction against heat-shock protein 60. Herz. 2000;25(2):87–90.

110. Xu Q. Role of heat shock proteins in atherosclerosis. ATVB. 2002;22(10):1547–59.

111. Ford PJ, Gemmell E, Chan A, Carter CL, Walker PJ, Bird PS, et al. Inflammation, heat shock proteins and periodontal pathogens in atherosclerosis: an immunohistologic study. Oral Microbiol Immunol. 2006;21(4):206–11.

112. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. BBRC. 2009;388(4):621–5.

113. O'Neill LAJ, Golenbock D, Bowie AG. The history of toll-like receptors — redefining innate immunity. Nat Rev Immunol. 2013;13(6):453–60.

114. Satoh T, Akira S. Toll-like receptor signaling and its inducible proteins. Gordon S, editor. Microbiol Spectr. 2016;4(6):4.6.41.

115. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat Immunol. 2010;11(5):373–84.

116. Kuzmich N, Sivak K, Chubarev V, Porozov Y, Savateeva-Lyubimova T, Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines. 2017;5(4):34.

117. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, et al. Reciprocal modulation of toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-

kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem. 2013;278(39):37041–51.

118. Wong SW, Kwon MJ, Choi AMK, Kim HP, Nakahira K, Hwang DH. Fatty acids modulate toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. J Biol Chem. 2009;284(40):27384–92.

119. Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, et al. Lack of toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci USA. 2004;101(29):10679–84.

120. Zhang S, Zou J, Li P, Zheng X, Feng D. Curcumin protects against atherosclerosis in apolipoprotein E-knockout mice by inhibiting toll-like receptor 4 expression. J Agric Food Chem. 2018;66(2):449–56.

121. Edfeldt K, Swedenborg J, Hansson GK, Yan Z qun. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation. 2002;105(10):1158–61.

122. Yoo JY, Sniffen S, McGill Percy KC, Pallaval VB, Chidipi B. Gut dysbiosis and immune system in atherosclerotic cardiovascular disease (ACVD). Microorganisms. 2022;10(1):108.

123. Yeh CF, Chen YH, Liu SF, Kao HL, Wu MS, Yang KC, et al. Mutual interplay of host immune system and gut microbiota in the immunopathology of atherosclerosis. IJMS. 2020;21(22):8729.

124. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.

125. Subramaniam S, Fletcher C. Trimethylamine N-oxide: Breathe new life. Br J Pharmacol. 2018;175(8):1344–53.

126. Kaysen GA, Johansen KL, Chertow GM, Dalrymple LS, Kornak J, Grimes B, et al. Associations of trimethylamine N-oxide with nutritional and inflammatory biomarkers and cardiovascular outcomes in patients new to dialysis. J Ren Nutr. 2015;25(4):351–6.

127. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. Cell. 2016;165(1):111–24.

128. Seldin MM, Meng Y, Qi H, Zhu W, Wang Z, Hazen SL, et al. Trimethylamine N-oxide promotes vascular inflammation through signaling of mitogen-activated protein kinase and nuclear factor-KB. JAHA. 2016;5(2):e002767.

129. Senthong V, Kiatchoosakun S, Wongvipaporn C, Phetcharaburanin J, Tatsanavivat P, Sritara P, et al. Gut microbiota-generated metabolite, trimethylamine-N-oxide, and subclinical myocardial damage: a multicenter study from Thailand. Sci Rep. 2021;11(1):14963.

130. Omland T, de Lemos JA, Sabatine MS, Christophi CA, Rice MM, Jablonski KA, et al. A sensitive cardiac troponin T assay in stable coronary artery disease. N Engl J Med. 2009;361(26):2538–47.

131. Yoo J, Kim S. Probiotics and prebiotics: Present status and future perspectives on metabolic disorders. Nutrients. 2016;8(3):173.

132. Dixon A, Robertson K, Yung A, Que M, Randall H, Wellalagodage D, et al. Efficacy of probiotics in patients of cardiovascular disease risk: a systematic review and meta-analysis. Curr Hypertens Rep. 2020;22(9):74.

133. Tascanov MB. Comparisons of microbiota-generated metabolites in patients with young and elderly acute coronary syndrome. Anatol J Cardiol [Internet]. 2020 [cited 2022 Jun 8]; Available from: https://www.anatoljcardiol.com//en/comparisons-of-microbiota-generated-metabolites-in-patients-with-young-and-elderly-acute-coronary-syndrome-133799

134. Yoshida N, Emoto T, Yamashita T, Watanabe H, Hayashi T, Tabata T, et al. *Bacteroides vulgatus* and *Bacteroides dorei* reduce gut microbial lipopolysaccharide production and inhibit atherosclerosis. Circulation. 2018;138(22):2486–98.

135. De Preter V, Coopmans T, Rutgeerts P, Verbeke K. Influence of long-term administration of lactulose and saccharomyces boulardii on the colonic generation of phenolic compounds in healthy human subjects. J Am Coll Nutr. 2006;25(6):541–9.

136. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc. 2003;62(1):67–72.

137. Piccioni A, de Cunzo T, Valletta F, Covino M, Rinninella E, Raoul P, et al. Gut microbiota and environment in coronary artery disease. IJERPH. 2021;18(8):4242.

138. Vinolo MAR, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. JNB. 2011;22(9):849–55.

139. Stubbs JR, House JA, Ocque AJ, Zhang S, Johnson C, Kimber C, et al. Serum trimethylamine-N-oxide is elevated in CKD and correlates with coronary atherosclerosis burden. JASN. 2016;27(1):305–13.

140. Zhu Q, Gao R, Zhang Y, Pan D, Zhu Y, Zhang X, et al. Dysbiosis signatures of gut microbiota in coronary artery disease. Physiol Genomics. 2018;50(10):893–903.

141. Ostos MA, Recalde D, Zakin MM, Scott-Algara D. Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation. FEBS Letters. 2002;519(1–3):23–9.

142. Ettinger G, MacDonald K, Reid G, Burton JP. The influence of the human microbiome and probiotics on cardiovascular health. Gut Microbes. 2014;5(6):719–28.

143. Ramji DP, Davies TS. Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. Cytokine Growth Factor Rev. 2015;26(6):673–85.

144. Kadomoto S, Izumi K, Mizokami A. Macrophage polarity and disease control. IJMS. 2021;23(1):144.

145. Ross R. Atherosclerosis an inflammatory disease. N Engl J Med. 1999;340(2):115–26.

146. Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, et al. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am J Cardiol. 2000;85(2):140–6.

147. Ishizaka N, Ishizaka Y, Yamkado M. Atherosclerosis as a possible extrahepatic manifestation of chronic hepatitis C virus infection. Clin Med Insights Cardiol. 2014;8s3:CMC.S17069.

148. Rudd JHF, Myers KS, Bansilal S, Machac J, Woodward M, Fuster V, et al. Relationships among regional arterial inflammation, calcification, risk factors, and biomarkers: A prospective fluorodeoxyglucose positron-emission tomography/computed tomography imaging study. Circ Cardiovasc Imaging. 2009;2(2):107–15.

149. Al-Sadi R, Ye D, Boivin M, Guo S, Hashimi M, Ereifej L, et al. Interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by JNK pathway activation of claudin-2 gene. PLoS ONE. 2014;9(3):e85345.

150. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci U S A. 1985;82(20):6955–9.

151. Wensel CR, Pluznick JL, Salzberg SL, Sears CL. Next-generation sequencing: insights to advance clinical investigations of the microbiome. J Clin Invest. 2022;132(7):e154944.

152. La Rosa PS, Brooks JP, Deych E, Boone EL, Edwards DJ, Wang Q, et al. Hypothesis testing and power calculations for taxonomic-based human microbiome data. White EP, editor. PLoS ONE. 2012;7(12):e52078.

153. Zhao D. Epidemiological features of cardiovascular disease in Asia. JACC: Asia. 2021;1(1):1–13.

154. Lin CF, Chang YH, Chien SC, Lin YH, Yeh HY. Epidemiology of dyslipidemia in the Asia Pacific region. Int J Gerontol. 2018;12(1):2–6.

155. Williams B, Mancia G, Spiering W, Rosei EA, Azizi M, Burnier M, et al. 2018 ESC/ESH guidelines for the management of arterial hypertension. Eur Heart J. 2019;77(2):71–159.

156. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014;37 Suppl 1:S81-90.

157. Huang PL. A comprehensive definition for metabolic syndrome. Dis Model Mech. 2009;2(5–6):231–7.

158. Engin A. The definition and prevalence of obesity and metabolic syndrome. Adv Exp Med Biol. 2017;960:1–17.

159. Marinescu MA, Löffler AI, Ouellette M, Smith L, Kramer CM, Bourque JM. Coronary microvascular dysfunction, microvascular angina, and treatment strategies. JACC Cardiovasc Imaging. 2015;8(2):210–20.

160. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852–7.

161. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

162. Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc. 2020;15(3):799–821.

163. Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography [Internet]. In Review; 2023 [cited 2024 Mar 18]. Available from: https://www.researchsquare.com/article/rs-3231426/v1

164. Luangphiphat W, Prombutara P, Eeckhout E, Fournier S, Pradidarcheep W, Taweechotipatr M. Relationship between pro-inflammatory cytokines and gut microbiome in chronic coronary syndrome patients undergoing coronary angiography: A cross-sectional study. J Med Assoc Thai. 2024;107(2):104–13.

165. Bull MJ, Plummer NT. Part 1: The human gut microbiome in health and disease. Integr Med (Encinitas). 2014;13(6):17–22.

166. Durack J, Lynch SV. The gut microbiome: Relationships with disease and opportunities for therapy. JEM. 2019;216(1):20–40.

167. Sawicka-Smiarowska E, Bondarczuk K, Bauer W, Niemira M, Szalkowska A, Raczkowska J, et al. Gut microbiome in chronic coronary syndrome patients. J Clin Med. 2021;10(21):5074.

168. Fu J, Bonder MJ, Cenit MC, Tigchelaar EF, Maatman A, Dekens JAM, et al. The gut microbiome contributes to a substantial proportion of the variation in blood lipids. Cir Res. 2015;117(9):817–24.

169. Huttenhower C, Gevers D, Knight R, Abubucker S, Jonathan HB, T. Chinwalla A, et al. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207–14.

170. Pascale A, Marchesi N, Govoni S, Coppola A, Gazzaruso C. The role of gut microbiota in obesity, diabetes mellitus, and effect of metformin: new insights into old diseases. Curr Opin Pharmacol. 2019;49:1–5.

171. Arora T, Bäckhed F. The gut microbiota and metabolic disease: current understanding and future perspectives. J Intern Med. 2016;280(4):339–49.

172. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM, et al. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease: Gut microbiota in PsA. Arthritis Rheumatol. 2015;67(1):128–39.

173. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, et al. Microbiota of de-novo pediatric IBD: Increased Faecalibacterium Prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. Am J Gastroenterol. 2012;107(12):1913–22.

174. Yang T, Santisteban MM, Rodriguez V, Li E, Ahmari N, Carvajal JM, et al. Gut dysbiosis is linked to hypertension. Hypertension. 2015;65(6):1331–40.

175. Zheng YY, Wu TT, Liu ZQ, Li A, Guo QQ, Ma YY, et al. Gut microbiome-based diagnostic model to predict coronary artery disease. J Agric Food Chem. 2020;68(11):3548–57.

176. Emoto T, Yamashita T, Kobayashi T, Sasaki N, Hirota Y, Hayashi T, et al. Characterization of gut microbiota profiles in coronary artery disease patients using data mining analysis of terminal restriction fragment length polymorphism: gut microbiota could be a diagnostic marker of coronary artery disease. Heart Vessels. 2017;32(1):39–46.

177. Emoto T, Yamashita T, Sasaki N, Hirota Y, Hayashi T, So A, et al. Analysis of gut microbiota in coronary artery disease patients: a possible link between gut microbiota and coronary artery disease. J Atheroscler Thromb. 2016;23(8):908–21.

178. Cui L, Zhao T, Hu H, Zhang W, Hua X. Association study of gut flora in coronary heart disease through high-throughput sequencing. Biomed Res Int. 2017;217:1–10.

179. Canfora EE, Van Der Beek CM, Hermes GDA, Goossens GH, Jocken JWE, Holst JJ, et al. Supplementation of diet with galacto-oligosaccharides increases Bifidobacteria, but not insulin sensitivity, in obese prediabetic individuals. Gastroenterology. 2017;153(1):87-97.e3.

180. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009;9(1):123.

181. Holscher HD, Caporaso JG, Hooda S, Brulc JM, Fahey GC, Swanson KS. Fiber supplementation influences phylogenetic structure and functional capacity of the

human intestinal microbiome: follow-up of a randomized controlled trial. Am J Clin Nutr. 2015;101(1):55–64.

182. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015;33(9):496–503.

183. Dalla Via A, Gargari G, Taverniti V, Rondini G, Velardi I, Gambaro V, et al. Urinary TMAO levels are associated with the taxonomic composition of the gut microbiota and with the choline tma-lyase gene (cutC) harbored by Enterobacteriaceae. Nutrients. 2019;12(1):62.

184. Zhang Y, Xu J, Wang X, Ren X, Liu Y. Changes of intestinal bacterial microbiota in coronary heart disease complicated with nonalcoholic fatty liver disease. BMC Genom. 2019;20(1):862.

185. Zhou J, Liu L, Wu P, Zhao L, Wu Y. Fusobacterium nucleatum accelerates atherosclerosis via macrophage-driven aberrant proinflammatory response and lipid metabolism. Front Microbiol. 2022;13:798685.

186. Madhogaria B, Bhowmik P, Kundu A. Correlation between human gut microbiome and diseases. Infectious Medicine. 2022;1(3):180–91.

187. Yakob M, Söder B, Meurman JH, Jogestrand T, Nowak J, Söder PÖ. Prevotella nigrescens and Porphyromonas gingivalis are associated with signs of carotid atherosclerosis in subjects with and without periodontitis: Periodontal microorganisms and atherosclerosis. J Periodontal Res. 2011;46(6):749–55.

188. Abranches J, Zeng L, Bélanger M, Rodrigues PH, Simpson-Haidaris PJ, Akin D, et al. Invasion of human coronary artery endothelial cells by Streptococcus mutans OMZ175: S. mutans endothelial cell invasion. Oral Microbiol Immunol. 2009;24(2):141–5.

189. Bazaz R, Marriott HM, Wright C, Chamberlain J, West LE, Gelsthorpe C, et al. Transient increase in atherosclerotic plaque macrophage content following Streptococcus pneumoniae pneumonia in ApoE-deficient mice. Front Cell Infect Microbiol. 2023;13:1090550.

190. Sayols-Baixeras S, Dekkers KF, Baldanzi G, Jönsson D, Hammar U, Lin YT, et al. Streptococcus species abundance in the gut is linked to subclinical coronary atherosclerosis in 8973 participants from the SCAPIS cohort. Circulation. 2023;1–14.

191. Mayr M, Kiechl S, Tsimikas S, Miller E, Sheldon J, Willeit J, et al. Oxidized low-density lipoprotein autoantibodies, chronic Infections, and carotid atherosclerosis in a population-based study. J Am Coll Cardiol. 2006;47(12):2436–43.

192. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci USA. 2011;108(supplement_1):4592–8.

193. Bouzid F, Gtif I, Alfadhli S, Charfeddine S, Ghorbel W, Abdelhédi R, et al. A potential oral microbiome signature associated with coronary artery disease in Tunisia. Biosci Rep. 2022;42(7):BSR20220583.

194. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105–8.

195. De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut. 2016;65(11):1812–21.

196. Ley RE. Prevotella in the gut: choose carefully. Nat Rev Gastroenterol Hepatol. 2016;13(2):69–70.

197. De Filippis F, Pasolli E, Tett A, Tarallo S, Naccarati A, De Angelis M, et al. Distinct genetic and functional traits of human intestinal Prevotella copri strains are associated with different habitual diets. Cell Host Microbe. 2019;25(3):444-453.e3.

198. Tett A, Huang KD, Asnicar F, Fehlner-Peach H, Pasolli E, Karcher N, et al. The Prevotella copri complex comprises four distinct clades underrepresented in Westernized populations. Cell Host Microbe. 2019;26(5):666-679.e7.

199. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BAH, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016;535(7612):376–81.

200. Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: A connection between endogenous alcohol and NASH. Hepatol. 2013;57(2):601–9.

201. Larsen JM. The immune response to Prevotella bacteria in chronic inflammatory disease. Immunol. 2017;151(4):363–74.

202. Pianta A, Arvikar S, Strle K, Drouin EE, Wang Q, Costello CE, et al. Evidence of the immune relevance of *Prevotella copri*, a gut microbe, in patients with rheumatoid arthritis. Arthritis Rheumatol. 2017;69(5):964–75.

203. Alpizar-Rodriguez D, Lesker TR, Gronow A, Gilbert B, Raemy E, Lamacchia C, et al. *Prevotella copri* in individuals at risk for rheumatoid arthritis. Ann Rheum Dis. 2019;78(5):590–3.

204. Nii T, Maeda Y, Motooka D, Naito M, Matsumoto Y, Ogawa T, et al. Genomic repertoires linked with pathogenic potency of arthritogenic *Prevotella copri* isolated from the gut of patients with rheumatoid arthritis. Ann Rheum Dis. 2023;82(5):621–9.

205. Liu Z, Li J, Liu H, Tang Y, Zhan Q, Lai W, et al. The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease. Atherosclerosis. 2019;284:121–8.

206. Gaetti-Jardim E, Marcelino SL, Feitosa ACR, Romito GA, Avila-Campos MJ. Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. J Med Microbiol. 2009;58(12):1568–75.

207. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. Cell. 2016;165(1):111–24.

208. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V. A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut. 2014;63(8):1275–83.

209. Hoving LR, Katiraei S, Heijink M, Pronk A, Van Der Wee-Pals L, Streefland T, et al. Dietary mannan oligosaccharides modulate gut microbiota, increase fecal bile acid excretion, and decrease plasma cholesterol and atherosclerosis development. Mol Nutr Food Res. 2018;62(10):1700942.

210. Kazemian N, Mahmoudi M, Halperin F, Wu JC, Pakpour S. Gut microbiota and cardiovascular disease: opportunities and challenges. Microbiome. 2020;8(1):36.

211. Abdi M, Esmaeili GG H, Ranjbar R. Lactobacilli and Bifidobacterium as anti-atherosclerotic agents. Iran J Basic Med Sci. 2022;25(8):934–46.

212. Zhu Q, Gao R, Zhang Y, Pan D, Zhu Y, Zhang X, et al. Dysbiosis signatures of gut microbiota in coronary artery disease. Physiol Genomics. 2018;50(10):893–903.

213. Chambers ES, Preston T, Frost G, Morrison DJ. Role of gut microbiota-generated short-chain fatty acids in metabolic and cardiovascular health. Curr Nutr Rep. 2018;7(4):198–206.

214. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559–63.

215. Krishnan S, Alden N, Lee K. Pathways and functions of gut microbiota metabolism impacting host physiology. Curr Opin Biotechnol. 2015;36:137–45.

216. Vinolo MAR, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem. 2011;22(9):849–55.

217. Troseid M, Nestvold TK, Rudi K, Thoresen H, Nielsen EW, Lappegård KT. Plasma lipopolysaccharide is closely associated with glycemic control and abdominal obesity. Diabetes Care. 2013;36(11):3627–32.

218. Brandsma E, Kloosterhuis NJ, Koster M, Dekker DC, Gijbels MJJ, Van Der Velden S, et al. A proinflammatory gut microbiota increases systemic inflammation and accelerates atherosclerosis. Circ Res. 2019;124(1):94–100.

219. Tang WHW, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.

220. Phillips IR, Dolphin CT, Clair P, Hadley MR, Hutt AJ, McCombie RR, et al. The molecular biology of the flavin-containing monooxygenases of man. Chem Biol Interact. 1995;96(1):17–32.

221. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.

222. Al-Obaide M, Singh R, Datta P, Rewers-Felkins K, Salguero M, Al-Obaidi I, et al. Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. J Clin Med. 2017;6(9):86.

223. Vieira-Silva S, Falony G, Belda E, Nielsen T, Aron-Wisnewsky J, Chakaroun R, et al. Statin therapy is associated with lower prevalence of gut microbiota dysbiosis. Nature. 2020;581(7808):310–5.

224. Kim J, Lee H, An J, Song Y, Lee CK, Kim K, et al. Alterations in gut microbiota by statin therapy and possible intermediate effects on hyperglycemia and hyperlipidemia. Front Microbiol. 2019;10:1947.

225. Wang L, Zhou W, Guo M, Hua Y, Zhou B, Li X, et al. The gut microbiota is associated with clinical response to statin treatment in patients with coronary artery disease. Atherosclerosis. 2021;325:16–23.

226. Lee S, Lee Y, Kim J, An J, Kim K, Lee H, et al. Atorvastatin and rosuvastatin improve physiological parameters and alleviate immune dysfunction in metabolic disorders. Biochem Biophys Res Commun. 2016;478(3):1242–7.

227. López-Montoya P, Cerqueda-García D, Rodríguez-Flores M, López-Contreras B, Villamil-Ramírez H, Morán-Ramos S, et al. Association of gut microbiota with atherogenic dyslipidemia, and its impact on serum lipid levels after bariatric surgery. Nutrients. 2022;14(17):3545.

228. Zeng X, Gao X, Peng Y, Wu Q, Zhu J, Tan C, et al. Higher risk of stroke is correlated with increased opportunistic pathogen load and reduced levels of butyrate-producing bacteria in the gut. Front Cell Infect Microbiol. 2019;9:4.

229. Palmas V, Pisanu S, Madau V, Casula E, Deledda A, Cusano R, et al. Gut microbiota markers associated with obesity and overweight in Italian adults. Sci Rep. 2021;11(1):5532.

230. Anand S, Kaur H, Mande SS. Comparative in silico analysis of butyrate production pathways in gut commensals and pathogens. Front Microbiol [Internet]. 2016 Dec 2 [cited 2023 Jul 12];7. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01945/full

231. Hiippala K, Kainulainen V, Kalliomäki M, Arkkila P, Satokari R. Mucosal prevalence and interactions with the epithelium indicate commensalism of Sutterella spp. Front Microbiol [Internet]. 2016 [cited 2024 Mar 12];7. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01706/full

232. Mukhopadhya I, Hansen R, El-Omar EM, Hold GL. IBD—what role do Proteobacteria play? Nat Rev Gastroenterol Hepatol. 2012;9(4):219–30.

233. De Oliveira C, Watt R, Hamer M. Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish health survey. Br Med J. 2010;340:c2451–c2451.

234. Habib SS, Al Masri AA. Relationship of high sensitivity C-reactive protein with presence and severity of coronary artery disease. Pak J Med Sci [Internet]. 2013 [cited 2023 Jul 12];29(6). Available from: http://pjms.com.pk/index.php/pjms/article/view/3302

235. Carrero JJ, Andersson Franko M, Obergfell A, Gabrielsen A, Jernberg T. Hs-CRP level and the risk of death or recurrent cardiovascular events in patients with myocardial infarction: A healthcare-based study. J Am Heart Assoc. 2019;8(11):e012638.

236. Bruunsgaard H, Skinhøj P, Pedersen AN, Schroll M, Pedersen BK. Ageing, tumour necrosis factor-alpha and atherosclerosis. Clin Exp Immunol. 2008;121(2):255–60.

237. Boesten L, Zadelaar A, Vannieuwkoop A, Gijbels M, Dewinther M, Havekes L, et al. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-leiden transgenic mice. Cardiovasc Res. 2005;66(1):179–85.

238. Brånén L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of tumor necrosis factor-α reduces atherosclerosis in apolipoprotein E knockout mice. ATVB. 2004;24(11):2137–42.

239. Kim M, Huda MN, Bennett BJ. Sequence meets function microbiota and cardiovascular disease. Cardiovasc Res. 2022;118(2):399–412.

240. Mahalle N, Garg M, Kulkarni M, Naik S. Association of inflammatory cytokines with traditional and nontraditional cardiovascular risk factors in Indians with known coronary artery disease. Ann Med Health Sci Res. 2014;4(5):706.

241. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol - Endocrinol Metab. 2001;280(5):E745–51.

242. Zuliani G, Volpato S, Blè A, Bandinelli S, Corsi AM, Lauretani F, et al. High interleukin-6 plasma levels are associated with low HDL-C levels in community-dwelling older adults: The InChianti study. Atherosclerosis. 2007;192(2):384–90.

243. Pradhan AD. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA. 2001;286(3):327.

244. Wei Y sheng, Lan Y, Liu Y guang, Tang R guang, Lan J sheng. Relationship between interleukin-6 gene polymorphism and coronary heart disease and its effect on plasma lipid levels. Chinese Crit Care Med. 2006;18(4):233–6.

245. Wannamethee SG, Whincup PH, Rumley A, Lowe GDO. Inter-relationships of interleukin-6, cardiovascular risk factors and the metabolic syndrome among older men. JTH. 2007;5(8):1637–43.

246. Feng Y, Ye D, Wang Z, Pan H, Lu X, Wang M, et al. The role of interleukin-6 family members in cardiovascular diseases. Front Cardiovasc Med. 2022;9:818890.

247. Koyama K, Yoneyama K, Mitarai T, Ishibashi Y, Takahashi E, Kongoji K, et al. Association between inflammatory biomarkers and thin-cap fibroatheroma detected by optical coherence tomography in patients with coronary heart disease. Arch Med Sci. 2015;3:505–12.

248. Cintron M, Ortiz-Whittingham L, Baumer Y, Farmer N, Collins B, Wallen G, et al. Abstract P264: Interleukin-1β mediation of chronic stress-related neural activity and trimethylamine-N-oxide data from the Washington DC cardiovascular health and needs assessment. Circulation [Internet]. 2023 [cited 2023 Jul 24];147(Suppl_1). Available from: https://www.ahajournals.org/doi/10.1161/circ.147.suppl_1.P264

249. Andreotti F. Inflammatory gene polymorphisms and ischaemic heart disease: review of population association studies. Heart. 2002;87(2):107–12.

250. Shen J, Zhao M, Zhang C, Sun X. IL-1β in atherosclerotic vascular calcification: From bench to bedside. Int J Biol Sci. 2021;17(15):4353–64.

251. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377(12):1119–31.

252. Bucova M, Bernadic M, Buckingham T. C-reactive protein, cytokines and inflammation in cardiovascular diseases. Bratisl Lek Listy. 2008;109(8):333–40.

253. Zhang H, Peterson JW, Niesel DW, Klimpel GR. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. J Immunol. 1997;159(10):4868–78.

254. Maes M, Kubera M, Leunis JC. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. Neuro Endocrinol Lett. 2008;29(1):117–24.

255. Lim SG, Menzies IS, Lee CA, Johnson MA, Pounder RE. Intestinal permeability and function in patients infected with Human Immunodeficiency Virus: A comparison with Coeliac disease. Scand J Gastroenterol. 1993;28(7):573–80.

256. Sundqvist T, Lindström F, Magnusson KE, Sköldstam L, Stjernström I, Tagesson C. Influence of fasting on intestinal permeability and disease activity in patients with rheumatoid arthritis. Scand J Rheumatol. 1982;11(1):33–8.

257. Matera G, Muto V, Vinci M, Zicca E, Abdollahi-Roodsaz S, Van De Veerdonk FL, et al. Receptor recognition of and immune intracellular pathways for Veillonella parvula lipopolysaccharide. Clin Vaccine Immunol. 2009;16(12):1804–9.

258. Nie N, Bai C, Song S, Zhang Y, Wang B, Li Z. Bifidobacterium plays a protective role in TNF-**α**-induced inflammatory response in Caco-2 cell through NF-**K**B and p38MAPK pathways. Mol Cell Biochem. 2020;464(1–2):83–91.

259. Bernini LJ, Simão ANC, Alfieri DF, Lozovoy MAB, Mari NL, De Souza CHB, et al. Beneficial effects of Bifidobacterium lactis on lipid profile and cytokines in patients with metabolic syndrome: A randomized trial. Effects of probiotics on metabolic syndrome. Nutrition. 2016;32(6):716–9.

260. Sun B, Ma T, Li Y, Yang N, Li B, Zhou X, et al. Bifidobacterium lactis probio-M8 adjuvant treatment confers added benefits to patients with coronary artery disease

via target modulation of the gut-heart/-brain axes. Gibbons SM, editor. mSystems. 2022;7(2):e00100-22.

261. Wang Q, Guo M, Liu Y, Xu M, Shi L, Li X, et al. Bifidobacterium breve and Bifidobacterium longum attenuate choline-induced plasma trimethylamine N-oxide production by modulating gut microbiota in mice. Nutrients. 2022;14(6):1222.

262. Peng Y, Zhang N, Li WJ, Tan K, Zhou Y, She C, et al. Correlations of changes in inflammatory factors, glucose and lipid metabolism indicators and adiponectin with alterations in intestinal flora in rats with coronary heart disease. Eur Rev Med Pharmacol Sci. 2020;24(19):10118–25.

263. Romano KA, Vivas EI, Amador-Noguez D, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-*N*-oxide. Blaser MJ, editor. mBio. 2015;6(2):e02481-14.

264. Yu H, Li L, Deng Y, Zhang G, Jiang M, Huang H, et al. The relationship between the number of stenotic coronary arteries and the gut microbiome in coronary heart disease patients. Front Cell Infect Microbiol. 2022;12:903828.

Appendix

Two articles of our research (one original article and one preprint) have been published:

1. Luangphiphat W, Prombutara P, Eeckhout E, Fournier S, Pradidarcheep W, Taweechotipatr M. Relationship between pro-inflammatory cytokines and gut microbiome in chronic coronary syndrome patients undergoing coronary angiography: A cross-sectional study. J Med Assoc Thai. 2024;107(2):104-13.
2. Luangphiphat W, Prombutara P, Muangsillapasart V, Sukitpunyaroj D, Eeckhout E, Taweechotipatr M. Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography . Research Square. 2023: 1-16.

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 lipopolysaccharides (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

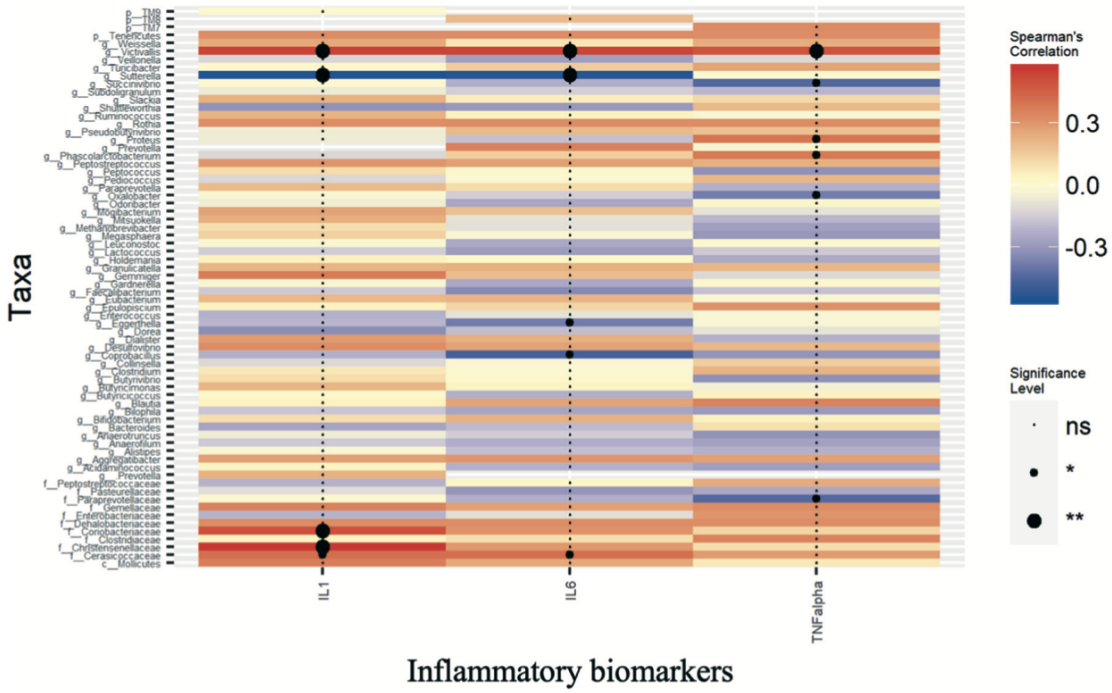

Figure 1. Spearman's correlation analysis between pro-inflammatory cytokines and the gut microbiome in CCS patients (n=28). The color represents positive (red) or negative (blue) correlations.

* 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

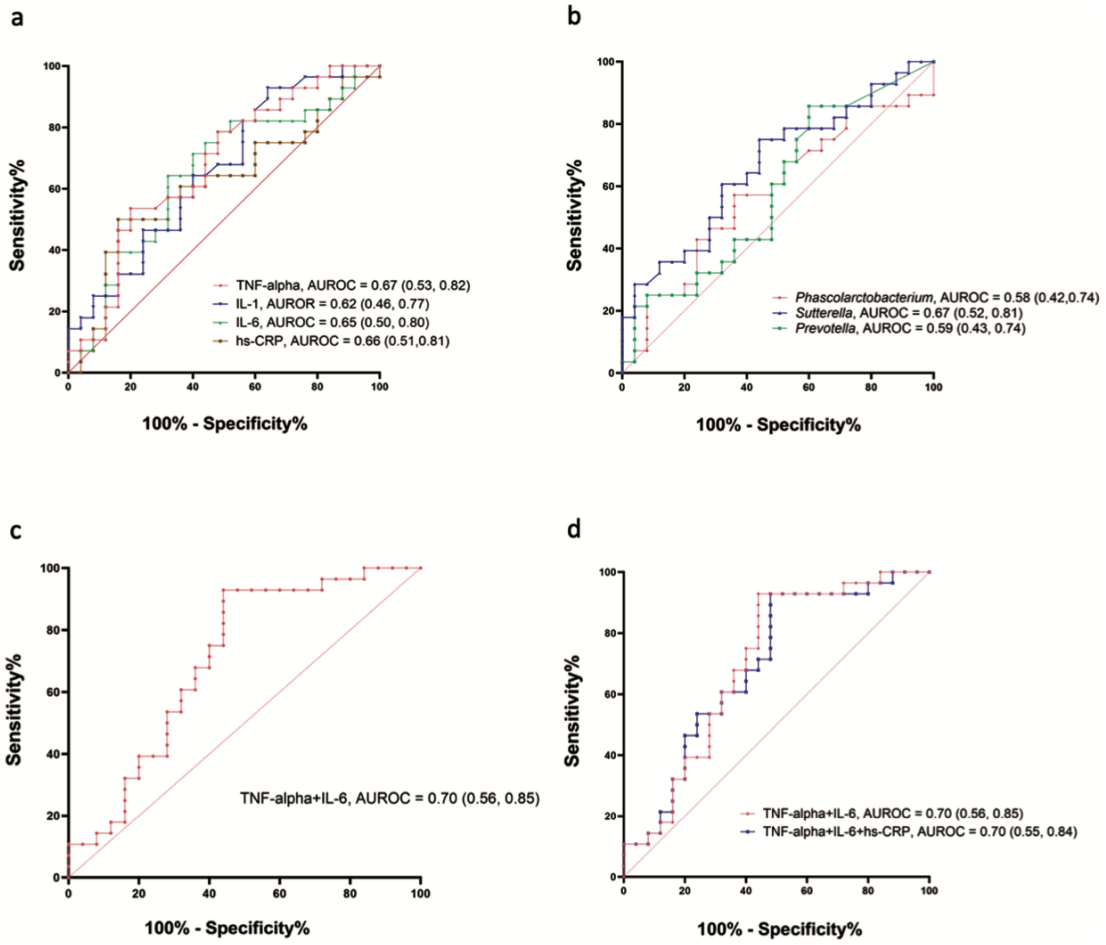

Figure 2. Gut microbiome and clinical features could effectively distinguish CCS patients from healthy participants. TNF-α, IL-1, IL-6, and hs-CRP (a), gut microbiome features (b), the combination of TNF-α and IL-6 (c), the combination of TNF-α and IL-6 and TNF-α, IL-6, and hs-CRP (d) to build the prediction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

References

1. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis. Nat Rev Dis Primers 2019;5:56.
2. Knuuti J, Wijns W, Saraste A, Capodanno D, Barbato E, Funck-Brentano C, et al. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J 2020;41:407-77.
3. Zhao D. Epidemiological features of cardiovascular disease in Asia. JACC Asia 2021;1:1-13.
4. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
5. Ishizaka N, Ishizaka Y, Yamkado M. Atherosclerosis as a possible extrahepatic manifestation of chronic hepatitis C virus infection. Clin Med Insights Cardiol 2014;8:1-5.
6. Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, et al. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am J Cardiol 2000;85:140-6.
7. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. N Engl J Med 2016;375:2369-79.
8. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol 2021;19:55-71.
9. Toya T, Corban MT, Marrietta E, Horwath IE, Lerman LO, Murray JA, et al. Coronary artery disease is associated with an altered gut microbiome composition. PLoS One 2020;15:e0227147.
10. Liu H, Chen X, Hu X, Niu H, Tian R, Wang H, et al. Alterations in the gut microbiome and metabolism with coronary artery disease severity. Microbiome 2019;7:68.
11. Al-Sadi R, Ye D, Boivin M, Guo S, Hashimi M, Ereifej L, et al. Interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by JNK pathway activation of claudin-2 gene. PLoS One 2014;9:e85345.

12. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010;328:228-31.
13. Mann DL. Innate immunity and the failing heart: the cytokine hypothesis revisited. Circ Res 2015;116:1254-68.
14. La Rosa PS, Brooks JP, Deych E, Boone EL, Edwards DJ, Wang Q, et al. Hypothesis testing and power calculations for taxonomic-based human microbiome data. PLoS One 2012;7:e52078.
15. Bruunsgaard H, Skinhøj P, Pedersen AN, Schroll M, Pedersen BK. Ageing, tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. Clin Exp Immunol 2000;121:255-60.
16. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, et al. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. Cardiovasc Res 2005;66:179-85.
17. Brånén L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 2004;24:2137-42.
18. Kim M, Huda MN, Bennett BJ. Sequence meets function-microbiota and cardiovascular disease. Cardiovasc Res 2022;118:399-412.
19. Chakaroun RM, Olsson LM, Bäckhed F. The potential of tailoring the gut microbiome to prevent and treat cardiometabolic disease. Nat Rev Cardiol 2023;20:217-35.
20. Masenga SK, Hamooya B, Hangoma J, Hayumbu V, Ertuglu LA, Ishimwe J, et al. Recent advances in modulation of cardiovascular diseases by the gut microbiota. J Hum Hypertens 2022;36:952-9.
21. Zhu Q, Gao R, Zhang Y, Pan D, Zhu Y, Zhang X, et al. Dysbiosis signatures of gut microbiota in coronary artery disease. Physiol Genomics 2018;50:893-903.
22. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J 2017;474:1823-36.
23. Sinsuebchuea J, Paenkaew P, Wutthiin M, Nantanaranon T, Laeman K, Kittichotirat W, et al. Characterization of the gut microbiota in Urban Thai individuals reveals enterotype-specific signature. Microorganisms 2023;11:136.
24. Wutthi-In M, Cheevadhanarak S, Yasom S, Kerdphoo S, Thiennimitr P, Phrommintikul A, et al. Gut microbiota profiles of treated metabolic syndrome patients and their relationship with metabolic health. Sci Rep 2020;10:10085.
25. Piccioni A, de Cunzo T, Valletta F, Covino M, Rinninella E, Raoul P, et al. Gut microbiota and environment in coronary artery disease. Int J Environ Res Public Health 2021;18:4242.
26. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. Cell 2016;165:111-24.
27. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575-84.
28. Dalla Via A, Gargari G, Taverniti V, Rondini G, Velardi I, Gambaro V, et al. Urinary TMAO levels are associated with the taxonomic composition of the gut microbiota and with the choline TMA-lyase gene (cutC) Harbored by Enterobacteriaceae. Nutrients 2019;12:62.
29. Al-Obaide MAI, Singh R, Datta P, Rewers-Felkins KA, Salguero MV, Al-Obaidi I, et al. Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. J Clin Med 2017;6:86.
30. Zheng YY, Wu TT, Liu ZQ, Li A, Guo QQ, Ma YY, et al. Gut microbiome-based diagnostic model to predict coronary artery disease. J Agric Food Chem 2020;68:3548-57.
31. Madhogaria B, Bhowmik P, Kundu A. Correlation between human gut microbiome and diseases. Infect Med (Beijing) 2022;1:180-91.
32. Yakob M, Söder B, Meurman JH, Jogestrand T, Nowak J, Söder P. Prevotella nigrescens and Porphyromonas gingivalis are associated with signs of carotid atherosclerosis in subjects with and without periodontitis. J Periodontal Res 2011;46:749-55.
33. Abranches J, Zeng L, Bélanger M, Rodrigues PH, Simpson-Haidaris PJ, Akin D, et al. Invasion of human coronary artery endothelial cells by Streptococcus mutans OMZ175. Oral Microbiol Immunol 2009;24:141-5.
34. Bazaz R, Marriott HM, Wright C, Chamberlain J, West LE, Gelsthorpe C, et al. Transient increase in atherosclerotic plaque macrophage content following Streptococcus pneumoniae pneumonia in ApoE-deficient mice. Front Cell Infect Microbiol 2023;13:1090550.
35. Sayols-Baixeras S, Dekkers KF, Baldanzi G, Jönsson D, Hammar U, Lin YT, et al. Streptococcus species abundance in the gut is linked to subclinical coronary atherosclerosis in 8973 participants from the SCAPIS Cohort. Circulation 2023;148:459-72.
36. Larsen JM. The immune response to Prevotella bacteria in chronic inflammatory disease. Immunology 2017;151:363-74.
37. Liu Z, Li J, Liu H, Tang Y, Zhan Q, Lai W, et al. The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease. Atherosclerosis 2019;284:121-8.
38. Zhang H, Peterson JW, Niesel DW, Klimpel GR. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. J Immunol 1997;159:4868-78.
39. Maes M, Kubera M, Leunis JC. The gut-brain barrier in major depression: intestinal mucosal dysfunction

with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. Neuro Endocrinol Lett 2008;29:117-24.
40. Lim SG, Menzies IS, Lee CA, Johnson MA, Pounder RE. Intestinal permeability and function in patients infected with human immunodeficiency virus. A comparison with coeliac disease. Scand J Gastroenterol 1993;28:573-80.
41. Sundqvist T, Lindström F, Magnusson KE, Sköldstam L, Stjernström I, Tagesson C. Influence of fasting on intestinal permeability and disease activity in patients with rheumatoid arthritis. Scand J Rheumatol 1982;11:33-8.
42. Matera G, Muto V, Vinci M, Zicca E, Abdollahi-Roodsaz S, van de Veerdonk FL, et al. Receptor recognition of and immune intracellular pathways for Veillonella parvula lipopolysaccharide. Clin Vaccine Immunol 2009;16:1804-9.
43. Nie N, Bai C, Song S, Zhang Y, Wang B, Li Z. Bifidobacterium plays a protective role in TNF-α-induced inflammatory response in Caco-2 cell through NF-κB and p38MAPK pathways. Mol Cell Biochem 2020;464:83-91.
44. Bernini LJ, Simão AN, Alfieri DF, Lozovoy MA, Mari NL, de Souza CH, et al. Beneficial effects of Bifidobacterium lactis on lipid profile and cytokines in patients with metabolic syndrome: A randomized trial. Effects of probiotics on metabolic syndrome. Nutrition 2016;32:716-9.
45. Abdi M, Esmaeili Gouvarchin Ghaleh H, Ranjbar R. Lactobacilli and Bifidobacterium as anti-atherosclerotic agents. Iran J Basic Med Sci 2022;25:934-46.
46. Sun B, Ma T, Li Y, Yang N, Li B, Zhou X, et al. Bifidobacterium lactis Probio-M8 Adjuvant Treatment Confers Added Benefits to Patients with Coronary Artery Disease via Target Modulation of the Gut-Heart/-Brain Axes. mSystems 2022;7:e0010022.
47. Wang Q, Guo M, Liu Y, Xu M, Shi L, Li X, et al. Bifidobacterium breve and Bifidobacterium longum Attenuate Choline-Induced Plasma Trimethylamine N-Oxide Production by Modulating Gut Microbiota in Mice. Nutrients 2022;14:1222.
48. Peng Y, Zhang N, Li WJ, Tan K, Zhou Y, She C, et al. Correlations of changes in inflammatory factors, glucose and lipid metabolism indicators and adiponectin with alterations in intestinal flora in rats with coronary heart disease. Eur Rev Med Pharmacol Sci 2020;24:10118-25.

Association of gut microbiota with dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography

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Abstract

Chronic coronary syndrome (CCS) has a high mortality rate, and dyslipidemia is a major risk factor. Atherosclerosis, a cause of CCS, is influenced by gut microbiota dysbiosis and its metabolites. We aimed to study the diversity and composition of gut microbiota and related clinical parameters among CCS patients undergoing coronary angiography and dyslipidemia patients in comparison to healthy volunteers in Thailand. We reported that CCS patients had more risk factors and higher inflammatory marker, high-sensitivity C-reactive protein (hs-CRP) than others. We showed that the alpha diversity was lower in dyslipidemia and CCS patients than in the healthy group. A significant difference in the composition of gut microbiota was observed among the three groups. The relative abundance of Proteobacteria, Fusobacteria, Enterobacteriaceae, *Prevotella* and *Streptococcus* were significantly increased while *Roseburia*, *Ruminococcus* and *Faecalibacterium* were lower in CCS patients. In CCS patients, Lachnospiraceae, Peptostreptococcaceae and *Pediococcus* were positively correlated with hs-CRP. In dyslipidemia patients, *Megasphaera* was strongly positively correlated with triglyceride (TG) level and negatively correlated with high-density lipoprotein cholesterol (HDL-C). The modification of gut microbiota was associated with changes in clinical parameters involved in the development of coronary artery disease (CAD) in CCS patients.

Introduction

Cardiovascular disease (CVD) is the world's leading cause of mortality and one of Thailand's significant health issues. This disease has a number of risk factors, including dyslipidemia [1], diabetes mellitus [2], hypertension [3], obesity, insulin resistance, and metabolic syndrome [4]. Currently, lifestyle changes like exercising, eating healthily, and consuming less salt to lower blood pressure can help to reduce the risk factors of CVD. These changes can be combined with blood-sugar-lowering drugs, statins, and antihypertensive drugs. However, morbidity and mortality rates, which are still very high all over the world, including Thailand, are impacted by CVD.

Despite the fact that there are other coronary artery disease (CAD) risk factors, dyslipidemia is a major one [5]. Acute and chronic coronary syndromes are two subtypes of CAD that are distinguished by the timing of the onset of the signs and symptoms. A phrase used to describe CAD as a chronic, progressive condition that can be stabilized is chronic coronary syndrome (CCS) [6].

More than 1,100 different bacterial species have been linked to numerous symptoms and diseases, including cancer, diabetes mellitus, obesity, and CVD [7], according to earlier research. Since metabolic syndrome is a cardiovascular condition, eating the proper amount of healthful food can both prevent and treat it. The majority of gastrointestinal tract microorganisms cannot be cultured. The genes of the microorganism, also known as the microbiome, must therefore be examined in order to determine the type and quantity of these microorganisms.

Gut microbiota is a microorganism that lives in the human digestive tract. The quantity and type of microorganisms in the gastrointestinal tract are determined and influenced by a number of external factors such as geographic origin, age, genetics, diet, and use of prebiotics and antibiotics [8,9]. The gastrointestinal tract contains a variety of microorganisms such as Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria, in which their mechanisms of action and habitats are different. Crucial gastrointestinal bacteria that have been presented since birth include *Lactobacillus* and *Bifidobacterium* [10]. Nowadays people pay attention to the diversity of gut microbiota, which affects the immune system, nutrient metabolism, and the stabilization of the lining of the gastrointestinal tract (gut epithelium). The gut microbiota is also responsible for digesting certain nutrients that cannot be digested in the stomach and also help to synthesize vitamins and hormones in the body. The body and the gut microbiome have a complicated interaction through metabolites, including short-chain fatty acids (SCFAs), bile acid (BA), and trimethylamine-*N*-oxide (TMAO). Several organs may be affected by these metabolites' effects. Atherosclerosis, a cause of CVD, is influenced by gut microbiota and metabolites [11].

Recent studies have highlighted the role of gut dysbiosis in several diseases, including atherosclerosis and many CVD. These microorganisms are inherited from the mother at birth, as well as environment, food and medicine [12]. The changing intake of macronutrients led to metabolic syndrome, if there is an imbalance of microorganisms (dysbiosis) in the gastrointestinal tract. Alterations in gut microbiota will lead to an increase in various diseases and consuming a low fiber, high fat and high sugar diet affects the digestive system including the gut ecosystem. The increase of abundance of lipopolysaccharides (LPS)-producing gram-negative bacteria, such as *Escherichia, Shigella, Veillonella, Haemophilus*, and *Klebsiella*, increased with the severity of CAD in contrast to the bacteria that produce butyric acid, such as Ruminococcaceae and Lachnospiraceae, decreased the onset of CAD. Moreover, several types of gut microbiota, including those from Bacteroidetes phylum, *Roseburia intestinalis, Faecalibacterium prausnitzii*, and *Eubacterium rectale*, are also protective factors against CAD [13–15].

Sequencing of the 16S rRNA gene is a clustering independent widely used technique in microbiome studies to analyze the composition and diversity of bacterial communities. This technique provides insights into the diversity, distribution, and relative abundance of bacteria, which are essential for understanding the role of microbiota in various environments, including the human body, soil, water, and other ecological systems [16,17].

To the best of our knowledge, no any study has been conducted in Thailand to determine if the gut microbiome is associated with the parameters of dyslipidemia and CCS patients undergoing coronary angiography. The purpose of this exploratory study is to identify potential relationships among relative gut microbiota composition and related parameters in patients with dyslipidemia and CCS patients undergoing coronary angiography in Thailand.

Results

Clinical characteristics

Ninety-one patients were included and divided into three groups: CCS patients undergoing coronary angiography, dyslipidemia patients, and healthy volunteers, with 30, 32, and 29 patients in each group, respectively. To be mentioned, 96 patients were recruited but the microbiota data of one dyslipidemia patient and four CCS patients were missed because stool samples could not be collected. So, we examined 91 samples of the gut microbiota. The patients were 43.96% female, with the mean age of 57.57 ± 8.35 years and 26.37% had hypertension. There was no statistically significant difference in age or gender among the groups. Baseline characteristics is shown in Table 1.

Table 1

Clinical characteristics of the patients (N = 91) in this study. Categorical variables are expressed as frequency and percentage. Continuous variables are expressed as mean ± standard deviation. [a] One-way ANOVA, post hoc test analysis using Scheffe, significant when p-value < 0.05, [b] Kruskal-Wallis test, post hoc test analysis using Mann-Whitney U test, significant when p-value < 0.017 (0.05/3), [c] Chi square test, significant when p-value < 0.05, [d] Fisher's exact test, significant when p-value < 0.05, [+]Obesity, BMI ≥ 25 kg/m^2, [*]Paunchy = waist circumference > 90 cm for male, waist circumference > 80 cm for female, [**]Hypertriglyceridemia = triglyceride ≥ 150 mg/dl, [#]Low HDL-C = HDL-C < 40 mg/dL for male, HDL-C < 50 mg/dL for female, [@]Impaired fasting glucose = FBS ≥ 100 mg/dL, ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; FBS, fasting blood sugar; HbA1C, hemoglobin A1C; healthy, healthy volunteers; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; IU/L, international units per liter; LDL-C, low-density lipoprotein cholesterol; mmHg, millimeters of mercury; mg/dL, milligrams per deciliter, ng/L, nanogram per liter; PAD, peripheral artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Parameters	Total (n = 91)	CCS (n = 30)	DLP (n = 32)	Healthy (n = 29)	p-value
Age (years)	57.57 ± 8.35	59.60 ± 9.04	57.44 ± 8.88	55.62 ± 6.60	0.066[b]
Male (%)	51 (56.04)	20 (66.67)	16 (50.00)	15 (51.72)	0.356[c]
BMI (kg/m^2)	24.10 ± 4.07	24.63 ± 3.47	25.19 ± 5.45	22.37 ± 1.78	0.003[b]
Waist circumference (cm)	82.32 ± 9.76	88.20 ± 8.92	81.25 ± 10.59	77.43 ± 6.04	< 0.001[b]
History of CAD (%)	17 (18.68)	17 (56.67)	0 (0.00)	0 (0.00)	< 0.001[c]
Medication					
Antiplatelets	30 (32.97)	30 (100.00)	0 (0.00)	0 (0.00)	< 0.001[c]
Antihypertensive drugs	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	< 0.001[c]
Oral antidiabetic drugs	7 (7.69)	7 (23.33)	0 (0.00)	0 (0.00)	< 0.001[d]
Statins	58 (63.74)	29 (96.67)	29 (90.63)	0 (0.00)	< 0.001[d]
Statin intensity					< 0.001[d]
Low intensity	2 (3.45)	0 (0.00)	2 (6.90)	0 (0.00)	
Moderate intensity	18 (31.03)	2 (6.90)	16 (55.17)	0 (0.00)	
High intensity	38 (65.52)	27 (93.10)	11 (37.93)	0 (0.00)	
Duration ≥ 3 months	54 (93.10)	29 (100.00)	25 (86.21)	0 (0.00)	0.112[d]

Parameters	Total (n = 91)	CCS (n = 30)	DLP (n = 32)	Healthy (n = 29)	p-value
Obesity (%)	19 (21.11)	13 (43.33)	6 (19.35)	0 (0.00)	< 0.001[c]
Paunchy (%)*	21 (23.08)	13 (43.33)	5 (15.63)	3 (10.34)	0.005[c]
Hypertriglyceridemia (%)**	18 (19.78)	8 (26.67)	10 (31.25)	0 (0.00)	0.005[c]
Low HDL-C (%)#	18 (19.78)	13 (43.33)	5 (15.63)	0 (0.00)	< 0.001[c]
Impaired fasting glucose (%)@	28 (30.77)	21 (70.00)	3 (9.38)	4 (13.79)	< 0.001[c]
Metabolic syndrome (%)	10 (10.99)	10 (33.33)	0 (0.00)	0 (0.00)	< 0.001[d]
Hypertension (%)	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	< 0.001[c]
Diabetes mellitus (%)	9 (9.89)	9 (30.00)	0 (0.00)	0 (0.00)	< 0.001[d]
Dyslipidemia (%)	49 (53.85)	17 (56.67)	32 (100.00)	0 (0.00)	< 0.001[c]
Heart failure (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
Stroke (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.000[d]
PAD (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
SBP (mmHg)	121.65 ± 11.88	124.23 ± 14.34	122.41 ± 10.12	118.14 ± 10.35	0.174[b]
DBP (mmHg)	73.99 ± 9.64	72.27 ± 7.76	73.59 ± 10.57	76.21 ± 10.20	0.283[a]
Laboratory data					
FBS (mg/dl)	99.74 ± 18.81	114.31 ± 23.98	95.59 ± 11.00	89.76 ± 8.53	< 0.001[b]
HbA1C (mg/dL)	5.69 ± 0.75	6.04 ± 0.97	5.74 ± 0.50	5.28 ± 0.47	< 0.001[b]
Total Cholesterol (mg/dL)	175.76 ± 44.19	144.33 ± 41.24	202.93 ± 43.67	178.28 ± 22.28	< 0.001[b]
Triglyceride (mg/dL)	119.26 ± 74.86	135.83 ± 92.69	136.44 ± 76.38	83.17 ± 25.98	0.003[b]
LDL-C (mg/dL)	106.72 ± 39.97	77.11 ± 34.44	129.96 ± 40.78	111.70 ± 21.97	< 0.001[b]
HDL-C (mg/dL)	56.31 ± 17.89	45.90 ± 15.08	59.84 ± 18.09	63.17 ± 15.88	< 0.001[b]

Parameters	Total (n = 91)	CCS (n = 30)	DLP (n = 32)	Healthy (n = 29)	p-value
Serum creatinine (mg/dL)	0.86 ± 0.22	0.98 ± 0.20	0.83 ± 0.21	0.77 ± 0.19	< 0.001[b]
AST (IU/L)	22.90 ± 11.23	24.50 ± 11.96	25.19 ± 10.60	18.72 ± 10.29	< 0.001[b]
ALT (IU/L)	23.22 ± 15.45	26.10 ± 12.79	25.59 ± 10.94	17.62 ± 20.43	< 0.001[b]
hs-CRP (mg/dL)	2.36 ± 2.99	3.46 ± 4.26	2.03 ± 2.05	1.58 ± 1.80	0.043[b]

The average age of the CCS patients was 59.60 ± 9.04 years and 56.67% of them had history of CAD. In comparison to other groups, more patients had obesity, low high-density lipoprotein cholesterol (HDL-C) level, metabolic syndrome, hypertension, and diabetes mellitus at 43.33%, 43.33%, 33.33%, 80% and 30% with statistically significant, respectively. Additionally, the level of fasting blood glucose (FBS), hemoglobin A1C (HbA1C) and high-sensitivity C-reactive protein (hs-CRP) were higher in this group than others with statistical significance.

In dyslipidemia patients, the mean age was 57.44 ± 8.88 years. No patients with hypertension and diabetes mellitus. There were 9.38% of the patients with impaired fasting glucose in this group however the average of FBS and HbA1C was in the normal range. Total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) were higher than others with statistical significance.

Unexpectedly, TC and LDL-C were found to be lowest in CCS patients compared to patients with dyslipidemia and healthy volunteers at 144.33 ± 41.24 and 77.11 ± 34.44 vs 202.93 ± 43.67 and 129.96 ± 40.78 vs 178.28 ± 22.28 and 111.70 ± 21.97, respectively. This study found that CCS patients used statins at a rate of 96.67% with high-intensity statin of 93.10% and every patient took statins for at least three months, while patients with dyslipidemia used statins at a rate of 90.63% with only high-intensity statin of 37.93% with statistical significance.

Diversity of the gut microbiota

The alpha diversity analysis was used to measure the Chao1 index and Shannon diversity index. (see Fig. 1a, and 1b). From both Chao1 and Shannon indexes, there was a trend that showed lower diversity in dyslipidemia patients and CCS patients than in healthy volunteers.

The beta diversity was used to determine the similarities and differences in the composition structure of microbial communities using weighted and un-weighted UniFac distance. Principal coordinates analysis illustrated statistically significant difference (PERMANOVA, $p < 0.01$) in clustering of fecal samples between gut microbiome of the dyslipidemia patients, CCS patients, and healthy volunteers (see Fig. 1c and 1d).

Assessment of bacterial taxonomic composition of CCS and dyslipidemia patients and healthy volunteers

At the phylum level, among all the three groups, the most common bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. The relative abundance of Proteobacteria was increased in CCS patients' group (the percentage of Proteobacteria in CCS patients, dyslipidemia patients and healthy volunteers: 8.22, 4.79 and 4.16, respectively). Moreover, the relative abundance of Fusobacteria was also increased in CCS patients' group (the percentage of Fusobacteria in CCS patients, dyslipidemia patients and healthy volunteers: 1.48, 1.07 and 0.89, respectively). On the other hand, in CCS patients, Actinobacteria and Verrucomicrobia had the lowest relative abundances proportion among the three groups. In terms of other phyla, there was no difference. The proportion of Firmicutes and Bacteroidetes was higher in the group of dyslipidemia patients than in the other groups (see Fig. 2a).

At the family level, when the gut microbiota of the three groups was analyzed at this level, Lachnospiraceae and the Bacteroidaceae families were shown to be the two most common families. Compared to other groups, Prevotellaceae, Enterobacteriaceae and Streptococcaceae families were more prevalent proportionately in CCS patients (see Fig. 2b).

At the genus level, the highest abundance in each group belonged to genus *Bacteroides* (the percentage of *Bacteroides* in CCS patients, dyslipidemia patients and healthy volunteers: 19.02, 19.15 and 21.50, respectively). In the group of CCS patients, *Prevotella* and *Streptococcus* genera were more prevalent abundance than other groups, with statistical significance following from *Bacteroides* genus (see Fig. 2c). Moreover, the relative abundance of *Roseburia* (the percentage of *Roseburia* in CCS patients, dyslipidemia patients and healthy volunteers: 3.17, 5.67 and 4.71, respectively), *Ruminococcus* (the percentage of *Ruminococcus* in CCS patients, dyslipidemia patients and healthy volunteers: 2.40, 4.54 and 3.79, respectively) and *Faecalibacterium* genera (the percentage of *Faecalibacterium* in CCS patients, dyslipidemia patients and healthy volunteers: 6.20, 6.98 and 7.08, respectively) were the lowest in CSS patients' group than in the others.

At the specie level, from the pairwise phylogenetic heat tree, the abundance of *Streptococcus, Veillonella dispar* and *Prevotella copri* were higher in CCS patients than healthy volunteers with statistically significant. On the other side, fewer *Roseburia faecis*, *Subdoligranulum variabile, Bifidobacterium* and *Anaerostipes* were detected in this group (see Fig. 2d).

The characteristic of bacterial microbiota of CCS patients undergoing coronary angiography

In CCS patients' group, the relative abundance of Prevotellaceae, Enterobacteriaceae, Streptococcaceae, Clostridiaceae and Paraprevotellaceae families had significantly risen at the family level compared with healthy volunteers, according to the linear discriminant analysis effect size (LEfSe). At the genus level, the LEfSe revealed that *Prevotella, Streptococcus, Phascolarctobacterium, Dorea, Paraprevotella* and

Veillonella genera had significantly higher relative abundances in CCS patients compare to healthy volunteers (see Fig. 3a).

When compared CCS patients with dyslipidemia patients, The result showed that the relative abundance of Prevotellaceae, Enterobacteriaceae, Streptococcaceae, Fusobacteriaceae, Clostridiaceae, Veillonellaceae and Porphyromonadaceae families were higher in CCS patients (see Fig. 3c).

The characteristic of bacterial microbiota of dyslipidemia patients

In dyslipidemia patients' group, the relative abundance of Prevotellaceae, Enterobacteriaceae and Fusobacteriaceae families had significantly risen at the family level compared with healthy volunteers' group, according to the LEfSe. At the genus level, *Prevotella, Clostridium* and *Dorea* genera had significantly higher relative abundances in dyslipidemia patients compare to healthy volunteers (see Fig. 3b).

When we compared dyslipidemia patients with CCS patients, we found that the relative abundance of Lachnospiraceae family was higher in dyslipidemia patients (see Fig. 3c).

Associations of risk factors with gut microbiome composition in CCS patients undergoing coronary angiography

We calculated the Spearman correlation coefficient between a range of clinical indicators that may be associated with the risk factors of CCS patients (Table 1) and gut microbiota at genus level (see Fig. 4a). We found that Lachnospiraceae, Peptostreptococcaceae families and *Pediococcus* genus was positively correlated with hs-CRP. *Weissella* genus was positively correlated with LDL-C while *Sutterella, Roseburia* genera were negatively correlated with LDL-C.

Associations of risk factors with gut microbiome composition in dyslipidemia patients

In dyslipidemia patients, *Pseudobutyrivibrio, Catenibacterium, Weissella, Prevotella* and *Anaerostipes* genera were positively correlated with body mass index (BMI) while *Bacteroides* and *Bifidobacterium* genera were negatively correlated with BMI. For this group's lipid profile, genus *Subdoligranulum* was positively correlated with TC level; genus *Megasphaera* was strongly positively correlated and genera *Collinsella* and *Catenibacterium* were also positively correlated with triglyceride (TG) level; genus *Megasphaera* was negatively correlated with HDL-C; genus *Subdoligranulum* was positively correlated with LDL-C level and genera *Lactobacillus* and *Butyricimonas* were negatively correlated with LDL-C level (see Fig. 4b).

Discussion

This is the first investigation on gut microbiota and associated factors in CCS patients undergoing coronary angiography in Thailand. In this study, the gut microbiome differed among the three groups. There was a reduction in diversity in dyslipidemia patients' group and CCS patients' group, which correlated with other studies [18–20]. This finding is in consistent with other studies that patients suffering with varies diseases have also reduced bacterial diversity, for instance, hypertension [21], Crohn's disease [22], psoriatic arthritis [23], metabolic syndrome[24], diabetes mellitus and obesity [25].

In this study, the composition pattern of the bacterial microbiota differed significantly between the group of CCS patients and healthy volunteers. The most prevalent bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria, which correlated with other studies [26]. Alterations in the ratio of the major phyla Firmicutes to Bacteroidetes have been proposed as a potential CAD risk factor, suggesting that changes in the microbiome's composition may also contribute to the onset and progression of atherosclerosis and CAD [27–29]. Furthermore, it was established that a greater Firmicutes/Bacteroidetes ratio (F/B ratio) plays a significant impact in CAD patients [29]. F/B ratio is used to diagnose gut dysbiosis and the relationship between this ratio and numerous well-known cardiovascular risk factors, including age, sex, food, and BMI, has been demonstrated [28,30–32]. However, in this study, dyslipidemia patients' group had the greatest F/B ratio whereas the CCS patients' group did not.

Numerous pathogenic genera, including *Escherichia, Salmonella, Vibrio, Yersinia*, and *Legionella*, are members of the phylum Proteobacteria. In our study, the relative abundance proportion of Proteobacteria was the highest in CCS patients' group. According to Shin et al. study, a higher relative abundance of Proteobacteria is associated with gut dysbiosis and many diseases (obesity, T2DM, and cancers) in human [33]. Mainly Proteobacteria, particularly by *Enterobacteriaceae* and some Firmicutes is the abundance of bacteria producing TMAO precursor [34].

Fusobacteria proportion was increased in the group of CCS patients, however, Actinobacteria and Verrucomicrobia exhibited the lowest relative abundances proportion in CCS patients in comparison with others. This finding is consistent with the study by Zhang et al [35] and Cui et al [29]. *Fusobacterium nucleatum* may initially cause periodontal disease, which then cause CAD due to atherosclerosis via inflammation and lipid metabolism [36]. However, no research on the association between *Fusobacterium* and CAD has been published.

In addition, *Streptococcus, Veillonella* and *Prevotella* genera were more common abundance than other groups in the group of CCS patients, with statistical significance following from the *Bacteroides* genus. Previous studies have reported that *Prevotella* and *Streptococcus* genera had a close relationship with metabolic syndrome, atherosclerosis and CAD [37–40]. It suggested that the changes in the abundance of *Streptococcus, Veillonella, Prevotella* was 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 [41]. The prevalence of various LPS-producing gram-negative bacteria, including *Escherichia, Shigella, Veillonella, Klebsiella* and *Haemophilus* increased with the severity of the CAD, according to a study by Liu H. et al [14]. High blood LPS level were associated with a threefold increased risk of incident atherosclerosis [42]. *Veillonella* could be found in atherosclerosis plaque and associated with cholesterol levels [43]. In addition, *Veillonella* is high relative abundance in CAD patients [35,44]. These findings suggested that the differences in bacterial abundance between the three groups may be linked to the progression of CAD and lipid metabolism in dyslipidemia patients.

The role of *Prevotella* in human health is controversial. *Prevotella* is a beneficial microbe, however it is associated to chronic inflammation. *Prevotella* is linked to high in complex carbohydrates diets from plants, fruits and vegetables, whereas *Bacteroides* is connected to fat and protein diets [45]. *Prevotella* can be found in healthy human and is considered as commensal bacteria. According to De Filippis et al.'s research, vegetarians had the Mediterranean diet at a high level, that was connected with this bacteria strains and higher levels of SCFAs [46]. According to Emoto et, *Lactobacillus, Streptococcus*, and *Enterococcus* increased in the gut microbiota of CAD patients whereas *Bacteroides* and *Prevotella* was decreased [28].

Prevotella strains' genetic diversity may help to explain the variations in how it reacts to dietary and health conditions in different patients [47]. For example, in the study of Italian people's gut microbiome showed that varying dietary choices could be responsible for *P. copri* strains with different functions, which resulted in different way for human health [48]. *P. copri* increases the prevalence in non-Westernized people. They typically consume diets rich in fresh vegetables and fruits [49].

However, recent studies have connected increasing *Prevotella* abundance and certain strains to metabolic syndrome, obesity, hypertension, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and low-grade systemic inflammation [37,50,51] due to augmentation mucosal helper T-cell (Th17) immune responses and stimulation epithelial cells to produce interleukin-1 (IL-1), IL-8, IL-6 and IL-23 [52].

P. copri was higher in CCS patients than healthy volunteers in our study, which is correlated with many studies. Numerous diseases are linked with this microorganism to chronic inflammatory process; for example, rheumatoid arthritis, periodontitis, HIV infection, metabolic syndrome, inflammatory bowel disease, CAD and cardiac valve calcification. This bacterium has a role in the development of rheumatoid arthritis (RA) and is immune-relevant [53]. In RA patients, gut dysbiosis may have a role in the early stage of RA as shown by the enrichment of *P. copri* [54]. Moreover, *P. copri* showed a high degree of genetic and functional diversity depending on the lifestyle of the patients and associated with worse arthritis in these patients [55]. In CVD patients, *P. copri* may be a major risk factor, particularly in cardiac valve calcification patients. This microorganism and LDL-C have a positive correlation, which likely supports its pro-inflammatory effects. It is a potential key pathogen implicated in CVDs because of its roles in immunity and inflammation [56]. Moreover, *P. intermedia, P. nigrescens* were periodontopathic bacteria in atherosclerotic plaques [57].

In this investigation, *Faecalibacterium* genera were lower in CSS patients' group than in the others. This finding is correlated with Zhu et al, *Faecalibacterium, Subdoligranulum, Roseburia*, and *Eubacterium rectale* were decreased [58]. There is a significant anti-inflammatory property of *Faecalibacterium* [59].

The *Bacteroides* genus had the highest relative abundance when we compared the healthy volunteers' group to the other groups, and the relative abundance of the *Roseburia* genus was higher in the healthy volunteers' group than in the other groups in our study. Due to their ability to produce SCFAs, prior research indicated that *Bacteroides* and *Bifidobacterium* have a certain protective effect on metabolism and are primarily protective bacteria for CCS [60–62]. In addition, *Roseburia* has been linked to improved glucose intolerance and weight loss in mice and in patients with atherosclerosis comparatively high amounts of *Collinsella*, whereas the normal control group has a substantially larger abundance of *Roseburia* and *Eubacterium* [14,63]. A study by Liu H. et al. revealed that the prevalence of bacteria that produced butyric acid, such as *Lachnospiraceae* and *Ruminococcaceae*, decreased with the progression of CAD [14], this findings is correlated with our study that *Lachnospiraceae* and *Ruminococcacea* families were less proportion in CCS patients when compare to others.

SCFAs are metabolites of the fermentation of complex carbohydrates. Members of phylum Bacteroidetes can produce butyrate and acetate, whereas phylum Firmicutes can produce butyrate. SCFAs have a favorable correlation with *Roseburia, Bacteroides* spp., and *Eubacterium rectale*. They have been proposed as a protective effect of CCS patients and SCFAs producers have decreased in some CCS patients. Additionally, they improve health by boosting the immunological response of the host, preserving the integrity of the intestinal barrier by controlling the expression of tight junction proteins, and reducing blood lipid levels by preventing the production of cholesterol [64–66]. SCFAs plays a protective role in atherosclerosis, while LPS stimulates body inflammation and accelerates the formation of atherosclerosis [67].

When butyrate-producing bacteria disappear, the gut barrier may become damaged, making it easier for microbial toxins like LPS to leak and cause inflammation by binding to Toll-like receptors. Patients with CAD have been found to have a higher LPS biosynthesis in the microbiome, which has connected to insulin resistance and abdominal obesity [68,69].

The synthesis of TMAO, a strong risk factor for the development of CAD, involves dietary choline, betaine, phosphatidylcholine, L-carnitine and lecithin which are obtained from a variety of sources, including egg, fish, red meat, soybean, peanuts [58,70]. By producing choline and the intermediary molecule trimethylamine (TMA), the gut bacteria also contribute to the synthesis of TMAO. The capacity of the gut microbiota to synthesise choline via the phospholipase D (PLD) enzyme has just recently been discovered. The flavin-containing monooxygenase (FMO) enzyme metabolizes the microbiome-derived TMA molecule into TMAO in hepatocytes [71]. Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes are the TMA producers. The production of foam cells is stimulated by the impairment of cholesterol metabolism in macrophages caused by TMAO-dependent activation of macrophage scavenger receptors

and CD36 expression. The higher TMAO production, the higher CAD risk [72]. In the context of increased intestinal permeability, TMAO is also linked to C-reactive protein (CRP), endothelial dysfunction, and elevated blood level of the LPS endotoxin. It can also cause platelet hyperreactivity, which have an impact on CAD progression [73].

The LDL-C level was lowest in CCS patients. The reasons for the differences in LDL-C levels was that the majority of patients (96.67%) were treated with statins. The treatment with statins is associated with lower prevalence of gut microbiota dysbiosis [74]. In mice study, statins could moderate gut microbiota by increasing the abundance of *Bacteroides, Butyricimonas* and *Mucispirillum* [75]. Moreover, the clinical response to statin therapy in individuals with CAD is associated with the gut microbiota. For instance, poor statin response is associated with a significant reduction in the number of beneficial bacteria (*Akkermansia muciniphila* and *Lactobacillus*) and an increase in the number of bacteria (Holdemanella and Facecallibacterium) [76]. Statins have also been linked to anti-inflammatory and immunomodulatory properties [77].

The genus *Megasphaera* was strongly positively correlated with TG level and negatively correlated with HDL-C. Lopez-Montoya et al. investigation confirmed our results, showing *Megasphaera* and *Escherichia-Shigella* were highly associated with atherogenic dyslipidemia patients, defined as both hypertriglyceridemia and low HDL-C [78]. Moreover, one study has shown that patients with symptomatic stroke had an altered gut microbiota and defined *Megasphaera* as opportunistic pathogens [79]. In obesity and overweight patients, *Megasphaera* was significantly increased relative abundance and correlated with low physical activity [80]. *Megasphaera* is involved in a mechanism that generates ammonia, which has negative consequences [81].

Hs-CRP can help in identifying chronic inflammation. Periodontal disease was linked to CAD and low grade inflammation, increased CRP and fibrinogen, according to de Oliveira et al.'s research [82]. There is correlation between hs-CRP and CAD severity in CCS patients [83]. Patients with myocardial infarction with elevated hs-CRP level (≥ 2 mg/L) were at higher risk of major adverse cardiovascular events and death [84]. In CCS patients of our study, the Lachnospiraceae, Peptostreptococcaceae families and *Pediococcus* genus was positively correlated with hs-CRP, a finding that has not been reported before. There are some limitations in our study, the specific function and the metabolites of the gut microbiota were not examined.

In conclusion, this study demonstrated a significant difference in the composition of gut microbiota between CCS patients, dyslipidemia patients and healthy volunteers. The alpha diversity was lower in CCS and dyslipidemia patients than in healthy volunteers. The relative abundance of Proteobacteria, Fusobacteria, *Prevotella* and *Streptococcus* were significantly increased while *Roseburia*, *Ruminococcus* and *Faecalibacterium* were lower in CCS patients. In dyslipidemia patients, *Megasphaera* was strongly positively correlated with TG level and negatively correlated with HDL-C. Modification of gut microbiota is associated with changes in clinical parameters involved in development of CAD in CCS patients.

Methods

Subject enrollment

From 25 February 2023 to 25 June 2023, patients between the ages of 35 and 70 were admitted to Chulabhorn Hospital's cardiovascular center were recruited. In patients with CCS undergoing coronary angiography group, they must meet the following criteria: (1) patients with stable anginal symptoms (2) patients < 1 year after an acute coronary syndrome (ACS) with stabilized symptoms after revascularization (3) CCS patients > 1 year after initial diagnosis or revascularization (4) asymptomatic patients in whom CAD is found after screening [6]. Coronary angiography was used to confirm the diagnosis of CCS in those with ≥ 70% stenosis in coronary arteries larger than 2.5 mm in one view; ≥ 50% stenosis in coronary arteries in two views; and ≥ 50% stenosis in the left main coronary artery [85]; and they must assent by signing the consent forms. In the group of dyslipidemia patients, they must only have one risk factor for CVD, dyslipidemia (TC ≥ 200 mg/dL or TG ≥ 150 mg/dL or LDL-C ≥ 160 mg/dL or patients on treatment) [86], without type 2 diabetes mellitus (T2DM) (FBS ≥ 126 mg/dL for 2 times separately or patients on treatment) [87], hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg at least 2 different days or patients on treatment) [88], obesity (body mass index ≥ 30 kg/m2) [89] and metabolic syndrome (at least 3 of the following risk factors: waist circumference ≥ 90 cm for men and ≥ 80 cm for women, blood pressure ≥ 130/85 mmHg or on antihypertensive drugs, TG ≥ 150 mg/dL or on medication, HDL-C ≤ 40 mg/dL for men and ≤ 50 mg/dL for women and FBS ≥ 100 mg/dL or on medication) [90].

Additionally, none of the aforementioned diseases must exist among the group of healthy volunteers. Patients who met the following criteria were excluded: (1) Kidney disease (estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m2 for more than 3 months), liver disease (Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) > 5 times normal or jaundice), cancer, intestinal diseases such as inflammatory bowel disease, thyroid dysfunction, immunodeficiency such as human immunodeficiency virus (HIV); (2) use antibiotics, immunosuppressants, probiotics supplement, synbiotics, herbal supplements, antacids or laxatives within 4 weeks before participating in the study; (3) History of gastrointestinal disease and other infections within 4 weeks; (4) Smoking; (5) Alcoholism; (6) Pregnant or lactating; (7) Covid-19 infection within 4 weeks.

By using statistical matching techniques with age and sex, participants were separated into three groups: CCS patients undergoing coronary angiography, patients with cardiovascular risk factors, dyslipidemia and healthy volunteers. The study received approval from Chulabhorn Hospital and Srinakharinwirot University research ethics committees (IEC No: 174/2564, dated: 22/07/2022 and IEC No: SWUEC/E/M-100/2565E, dated: 22/02/2023, respectively), and the study was conducted in accession with the Good Practices for Clinical Research in Thailand. The study was approved by Thai Clinical Trails Registry (TCTR) (TCTR20230428002). Written informed consent was obtained from all study participants.

The sample size calculation is based on “Hypothesis testing and power calculations for taxonomic-based human microbiome data, in this research, the value of Alpha (α) = 5%, power = 80% and function of the number of sequence reads is equal to 50,000 with three groups. A total of 25 volunteers were obtained and it was determined that the dropout rate of 20%, resulting in a total of 30 volunteers per group, or 90 volunteers in total [91].

Sampling and sequencing

Each patient’s blood was collected to evaluate FBS, HbA1C, TC, TG, HDL-C, LDL-C, AST, ALT, creatinine and hs-CRP. Fresh stool was collected in DNA/RNA shield fecal collection tubes (Zymo Research, CA, USA) 1 day before an appointment and immediately frozen at -20°C for 48 hours before further analysis. Using the QIAamp Stool Mini kit (Qiagen, USA), DNA was extracted from stool samples. Nanodrop and electrophoresis were used to evaluate the quantity and quality of DNA. Using 515 F and 806R primers and 2X KAPA hot-start ready mix, the V4 hypervariable region of the 16S rRNA gene was amplified by PCR. The PCR conditions included an initial denaturation at 94°C for 3 min, followed by 25 cycles of 98°C for 20 s., 55°C for 30 s., 72°C for 30 s., and a final extension step at 72°C for 5 min. The 16S amplicons were purified using AMPure XP beads and indexed using Nextera XT index kit, followed by 8 cycles of the forementioned PCR condition. Finally, the PCR products were cleaned and pooled for cluster generation and 250-bp paired-end read sequencing on the Illumina® MiSeq™.

Sequencing data analysis

QIIME 2 2019.10 was used for microbiome bioinformatics [92]. The raw sequence data was demultiplexed using the q2-demux plugin, and reads with expected errors (maxEE) higher than 3.0 was discarded by denoising software, DADA2 (via q2-dada2). A phylogeny was constructed using the SEPP q2-plugin, placing short sequences into sepp-refs-gg-13-8.qza reference phylogenetic tree. Alpha-diversity metric, beta-diversity metric, and Principle Coordinate Analysis (PCoA) were estimated using q2- diversity after samples were rarefied (subsampled without replacement) to a minimum read. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier against the Greengenes 13_8 99% operational taxonomic units (OTUs) reference sequences. Statistical tests of alpha and beta diversity were performed using Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) (number of permutations = 999), respectively. Moreover, the significantly differential abundance analysis of microbiota was conducted using LEfSe [93] via the algorithm module on the Galaxy platform at http://huttenhower.sph.harvard.edu/galaxy. First, nonparametric factorial Kruskal-Wallissum-rank tests was applied to choose features differentially distributed among classes ($p < 0.05$). The linear discriminant analysis (LDA) model was used to estimate their effect sizes and supported by 30-fold bootstrapping (cutoff = logarithmic LDA score of ≥ 2.0). In addition, significant p-values associated with the microbial significantly differential features by LEfSe were corrected for multiple hypothesis testing using the Benjamini and Hochberg false discovery rate correction.

Statistical analysis and visualization

Stata/SE 16.1 software (StataCorp LP, College Station, TX, USA) was used to analyze the statistical data. Statistics were considered significant when p-value < 0.05. All study variables were subjected to descriptive statistics analysis, which was provided as frequency (%) for categorical data and mean ± standard deviation (SD) or median for nonnormal quantitative data. One-way analysis of variance (ANOVA) statistic and post hoc analysis using the Scheffe test with p-value < 0.05 were utilized if the distribution of the quantitative data, such as age and laboratory results, was normal. The Kruskal-Wallis test and post hoc Mann-Whitney U test with p-value < 0.01 were used if the data distribution was not normal.

The following Spearman's correlation coefficient was analyzed; (1) anthropometric measurements, including BMI and waist circumference (2) physical examination; blood pressure and (3) blood tests; FBS, HbA1C, TC, TG, LDL-C, HDL-C, AST, ALT, and hs-CRP. Correlation heat map visualization was performed using the ggplot2 R package. A p-value < 0.05 was considered statistically significant and was labeled in the figure. In addition, the phylogenetic heat tree was visualized using the metacoder R package

Declarations

Data availability

The raw sequence data are available under BioProject PRJNA1000984, and Biosample SAMN36786011.

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Author contributions

W.L., P.P., and M.T. performed the statistical analysis and wrote the main manuscript. W.L., P.P., and M.T. performed gut microbiota statistical analysis. W.L., M.T., V.M., and D.S. provided clinical data and samples. P.P., M.T., and E.E. provided expert review and editing. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

References

1. Hedayatnia, M. *et al.* Dyslipidemia and cardiovascular disease risk among the MASHAD study population. *Lipids Health Dis.* **19**, 42 (2020).
2. Abdul-Ghani, M. *et al.* Cardiovascular disease and type 2 diabetes: Has the dawn of a new era arrived? *Diabetes Care* **40**, 813–820 (2017).
3. Dustan, H. P. Hypertension. *J. Am. Coll. Cardiol.* **35**, 10B-12B (2000).

4. Tune, J. D., Goodwill, A. G., Sassoon, D. J. & Mather, K. J. Cardiovascular consequences of metabolic syndrome. *Transl. Res.* **183**, 57–70 (2017).
5. Yusuf, S. *et al.* Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *The Lancet* **364**, 937–952 (2004).
6. Knuuti, J. *et al.* 2019 ESC guidelines for the diagnosis and management of chronic coronary syndromes. *Eur. Heart J.* **41**, 407–477 (2020).
7. Afzaal, M. *et al.* Human gut microbiota in health and disease: Unveiling the relationship. *Front. Microbiol.* **13**, 1–14 (2022).
8. Thursby, E. & Juge, N. Introduction to the human gut microbiota. *Biochem J* **474**, 1823–1836 (2017).
9. Fan, Y. & Pedersen, O. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* **19**, 55–71 (2021).
10. Yang, B. *et al.* Bifidobacterium and Lactobacillus composition at species level and gut microbiota diversity in Infants before 6 weeks. *Int. J. Mol. Sci.* **20**, 3306 (2019).
11. Pieczynska, M. D. *et al.* Gut microbiota and its metabolites in atherosclerosis development. *Molecules* **25**, 594 (2020).
12. Santacroce, L., Man, A., Alexandros Charitos, I., Haxhirexha, K. & Topi, S. Current knowledge about the connection between health status and gut microbiota from birth to elderly. *Landmark* **26**, 135 (2021).
13. Liu, H. *et al.* The role of the gut microbiota in coronary heart disease. *Curr. Atheroscler. Rep.* **22**, 77 (2020).
14. Liu, H. *et al.* Alterations in the gut microbiome and metabolism with coronary artery disease severity. *Microbiome* **7**, 68 (2019).
15. Jonsson, A. L. & Bäckhed, F. Role of gut microbiota in atherosclerosis. *Nat. Rev. Cardiol.* **14**, 79–87 (2017).
16. Lane, D. J. *et al.* Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6955–6959 (1985).
17. Wensel, C. R., Pluznick, J. L., Salzberg, S. L. & Sears, C. L. Next-generation sequencing: insights to advance clinical investigations of the microbiome. *J. Clin. Investig.* **132**, e154944 (2022).
18. Sawicka-Smiarowska, E. *et al.* Gut microbiome in chronic coronary syndrome patients. *J. Clin. Med.* **10**, 5074 (2021).
19. Fu, J. *et al.* The gut microbiome contributes to a substantial proportion of the variation in blood lipids. *Cir. Res.* **117**, 817–824 (2015).
20. Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
21. Yang, T. *et al.* Gut dysbiosis is linked to hypertension. *Hypertension* **65**, 1331–1340 (2015).
22. Hansen, R. *et al.* Microbiota of de-novo pediatric IBD: Increased Faecalibacterium Prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *Am. J. Gastroenterol.* **107**, 1913–1922 (2012).

23. Scher, J. U. *et al.* Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease: Gut microbiota in PsA. *Arthritis Rheumatol.* **67**, 128–139 (2015).
24. Arora, T. & Bäckhed, F. The gut microbiota and metabolic disease: current understanding and future perspectives. *J. Intern. Med.* **280**, 339–349 (2016).
25. Pascale, A., Marchesi, N., Govoni, S., Coppola, A. & Gazzaruso, C. The role of gut microbiota in obesity, diabetes mellitus, and effect of metformin: new insights into old diseases. *Curr. Opin. Pharmacol.* **49**, 1–5 (2019).
26. Zheng, Y.-Y. *et al.* Gut microbiome-based diagnostic model to predict coronary artery disease. *J. Agric. Food Chem.* **68**, 3548–3557 (2020).
27. Emoto, T. *et al.* Characterization of gut microbiota profiles in coronary artery disease patients using data mining analysis of terminal restriction fragment length polymorphism: gut microbiota could be a diagnostic marker of coronary artery disease. *Heart Vessels.* **32**, 39–46 (2017).
28. Emoto, T. *et al.* Analysis of gut microbiota in coronary artery disease patients: a possible link between gut microbiota and coronary artery disease. *J. Atheroscler. Thromb.* **23**, 908–921 (2016).
29. Cui, L., Zhao, T., Hu, H., Zhang, W. & Hua, X. Association study of gut flora in coronary heart disease through high-throughput sequencing. *Biomed Res. Int.* **2017**, 1–10 (2017).
30. Canfora, E. E. *et al.* Supplementation of diet with galacto-oligosaccharides increases Bifidobacteria, but not insulin sensitivity, in obese prediabetic individuals. *Gastroenterology.* **153**, 87-97.e3 (2017).
31. Mariat, D. *et al.* The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* **9**, 123 (2009).
32. Holscher, H. D. *et al.* Fiber supplementation influences phylogenetic structure and functional capacity of the human intestinal microbiome: follow-up of a randomized controlled trial. *Am. J. Clin. Nutr.* **101**, 55–64 (2015).
33. Shin, N.-R., Whon, T. W. & Bae, J.-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* **33**, 496–503 (2015).
34. Dalla Via, A. *et al.* Urinary TMAO levels are associated with the taxonomic composition of the gut microbiota and with the choline tma-lyase gene (cutC) harbored by Enterobacteriaceae. *Nutrients* **12**, 62 (2019).
35. Zhang, Y., Xu, J., Wang, X., Ren, X. & Liu, Y. Changes of intestinal bacterial microbiota in coronary heart disease complicated with nonalcoholic fatty liver disease. *BMC Genom.* **20**, 862 (2019).
36. Zhou, J., Liu, L., Wu, P., Zhao, L. & Wu, Y. Fusobacterium nucleatum accelerates atherosclerosis via macrophage-driven aberrant proinflammatory response and lipid metabolism. *Front. Microbiol.* **13**, 798685 (2022).
37. Madhogaria, B., Bhowmik, P. & Kundu, A. Correlation between human gut microbiome and diseases. *Infectious Medicine* **1**, 180–191 (2022).

38. Yakob, M. *et al.* Prevotella nigrescens and Porphyromonas gingivalis are associated with signs of carotid atherosclerosis in subjects with and without periodontitis: Periodontal microorganisms and atherosclerosis. *J. Periodontal Res.* **46**, 749–755 (2011).
39. Abranches, J. *et al.* Invasion of human coronary artery endothelial cells by Streptococcus mutans OMZ175: S. mutans endothelial cell invasion. *Oral Microbiol. Immunol.* **24**, 141–145 (2009).
40. Bazaz, R. *et al.* Transient increase in atherosclerotic plaque macrophage content following Streptococcus pneumoniae pneumonia in ApoE-deficient mice. *Front. Cell. Infect. Microbiol.* **13**, 1090550 (2023).
41. Sayols-Baixeras, S. *et al. Streptococcus* species abundance in the gut is linked to subclinical coronary atherosclerosis in 8973 participants from the SCAPIS cohort. *Circulation* 1–14 (2023) doi:10.1161/CIRCULATIONAHA.123.063914.
42. Mayr, M. *et al.* Oxidized low-density lipoprotein autoantibodies, chronic Infections, and carotid atherosclerosis in a population-based study. *J. Am. Coll. Cardiol.* **47**, 2436–2443 (2006).
43. Koren, O. *et al.* Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 4592–4598 (2011).
44. Bouzid, F. *et al.* A potential oral microbiome signature associated with coronary artery disease in Tunisia. *Biosci. Rep.* **42**, BSR20220583 (2022).
45. Wu, G. D. *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**, 105–108 (2011).
46. De Filippis, F. *et al.* High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut* **65**, 1812–1821 (2016).
47. Ley, R. E. Prevotella in the gut: choose carefully. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 69–70 (2016).
48. De Filippis, F. *et al.* Distinct genetic and functional traits of human intestinal Prevotella copri strains are associated with different habitual diets. *Cell Host Microbe.* **25**, 444-453.e3 (2019).
49. Tett, A. *et al.* The Prevotella copri complex comprises four distinct clades underrepresented in Westernized populations. *Cell Host Microbe.* **26**, 666-679.e7 (2019).
50. Pedersen, H. K. *et al.* Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* **535**, 376–381 (2016).
51. Zhu, L. *et al.* Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: A connection between endogenous alcohol and NASH. *Hepatol.* **57**, 601–609 (2013).
52. Larsen, J. M. The immune response to *Prevotella* bacteria in chronic inflammatory disease. *Immunol.* **151**, 363–374 (2017).
53. Pianta, A. *et al.* Evidence of the immune relevance of *Prevotella copri*, a gut microbe, in patients with rheumatoid arthritis. *Arthritis Rheumatol.* **69**, 964–975 (2017).
54. Alpizar-Rodriguez, D. *et al. Prevotella copri* in individuals at risk for rheumatoid arthritis. *Ann. Rheum. Dis.* **78**, 590–593 (2019).

55. Nii, T. *et al.* Genomic repertoires linked with pathogenic potency of arthritogenic *Prevotella copri* isolated from the gut of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **82**, 621–629 (2023).
56. Liu, Z. *et al.* The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease. *Atherosclerosis* **284**, 121–128 (2019).
57. Gaetti-Jardim, E., Marcelino, S. L., Feitosa, A. C. R., Romito, G. A. & Avila-Campos, M. J. Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. *J. Med. Microbiol.* **58**, 1568–1575 (2009).
58. Zhu, W. *et al.* Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell* **165**, 111–124 (2016).
59. Machiels, K. *et al.* A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* **63**, 1275–1283 (2014).
60. Hoving, L. R. *et al.* Dietary mannan oligosaccharides modulate gut microbiota, increase fecal bile acid excretion, and decrease plasma cholesterol and atherosclerosis development. *Mol. Nutr. Food Res.* **62**, 1700942 (2018).
61. Kazemian, N., Mahmoudi, M., Halperin, F., Wu, J. C. & Pakpour, S. Gut microbiota and cardiovascular disease: opportunities and challenges. *Microbiome* **8**, 36 (2020).
62. Abdi, M., Esmaeili GG, H. & Ranjbar, R. Lactobacilli and Bifidobacterium as anti-atherosclerotic agents. *Iran. J. Basic. Med. Sci.* **25**, 934–46 (2022).
63. Zhu, Q. *et al.* Dysbiosis signatures of gut microbiota in coronary artery disease. *Physiol. Genomics* **50**, 893–903 (2018).
64. Chambers, E. S., Preston, T., Frost, G. & Morrison, D. J. Role of gut microbiota-generated short-chain fatty acids in metabolic and cardiovascular health. *Curr. Nutr. Rep.* **7**, 198–206 (2018).
65. David, L. A. *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559–563 (2014).
66. Krishnan, S., Alden, N. & Lee, K. Pathways and functions of gut microbiota metabolism impacting host physiology. *Curr. Opin. Biotechnol.* **36**, 137–145 (2015).
67. Vinolo, M. A. R. *et al.* Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J. Nutr. Biochem.* **22**, 849–855 (2011).
68. Trøseid, M. *et al.* Plasma lipopolysaccharide is closely associated with glycemic control and abdominal obesity. *Diabetes Care* **36**, 3627–3632 (2013).
69. Brandsma, E. *et al.* A proinflammatory gut microbiota increases systemic inflammation and accelerates atherosclerosis. *Circ. Res.* **124**, 94–100 (2019).
70. Tang, W. H. W. *et al.* Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* **368**, 1575–1584 (2013).
71. Phillips, I. R. *et al.* The molecular biology of the flavin-containing monooxygenases of man. *Chem. Biol. Interact.* **96**, 17–32 (1995).

72. Wang, Z. *et al.* Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **472**, 57–63 (2011).
73. Al-Obaide, M. *et al.* Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. *J. Clin. Med.* **6**, 86 (2017).
74. Vieira-Silva, S. *et al.* Statin therapy is associated with lower prevalence of gut microbiota dysbiosis. *Nature* **581**, 310–315 (2020).
75. Kim, J. *et al.* Alterations in gut microbiota by statin therapy and possible intermediate effects on hyperglycemia and hyperlipidemia. *Front. Microbiol.* **10**, 1947 (2019).
76. Wang, L. *et al.* The gut microbiota is associated with clinical response to statin treatment in patients with coronary artery disease. *Atherosclerosis* **325**, 16–23 (2021).
77. Lee, S. *et al.* Atorvastatin and rosuvastatin improve physiological parameters and alleviate immune dysfunction in metabolic disorders. *Biochem. Biophys. Res. Commun.* **478**, 1242–1247 (2016).
78. López-Montoya, P. *et al.* Association of gut microbiota with atherogenic dyslipidemia, and its impact on serum lipid levels after bariatric surgery. *Nutrients* **14**, 3545 (2022).
79. Zeng, X. *et al.* Higher risk of stroke is correlated with increased opportunistic pathogen load and reduced levels of butyrate-producing bacteria in the gut. *Front. Cell. Infect. Microbiol.* **9**, 4 (2019).
80. Palmas, V. *et al.* Gut microbiota markers associated with obesity and overweight in Italian adults. *Sci. Rep.* **11**, 5532 (2021).
81. Anand, S., Kaur, H. & Mande, S. S. Comparative in silico analysis of butyrate production pathways in gut commensals and pathogens. *Front. Microbiol.* **7**, (2016).
82. De Oliveira, C., Watt, R. & Hamer, M. Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish health survey. *Br. Med. J.* **340**, c2451–c2451 (2010).
83. Habib, S. S. & Al Masri, A. A. Relationship of high sensitivity C-reactive protein with presence and severity of coronary artery disease. *Pak. J. Med. Sci.* **29**, (2013).
84. Carrero, J. J., Andersson Franko, M., Obergfell, A., Gabrielsen, A. & Jernberg, T. Hs-CRP level and the risk of death or recurrent cardiovascular events in patients with myocardial infarction: a healthcare-based study. *J. Am. Heart Assoc.* **8**, e012638 (2019).
85. Jiangping, S. *et al.* Assessment of coronary artery stenosis by coronary angiography: A head-to-head comparison with pathological coronary artery anatomy. *Circ. Cardiovasc. Interv.* **6**, 262–268 (2013).
86. Lin, C.-F., Chang, Y.-H., Chien, S.-C., Lin, Y.-H. & Yeh, H.-Y. Epidemiology of Dyslipidemia in the Asia Pacific Region. *Int. J. Gerontol.* **12**, 2–6 (2018).
87. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **37 Suppl 1**, S81-90 (2014).
88. Williams, B. *et al.* 2018 ESC/ESH guidelines for the management of arterial hypertension. *Eur. Heart J.* **77**, 71–159 (2019).
89. Samadoulougou, S. *et al.* Validated methods for identifying individuals with obesity in health care administrative databases: A systematic review. *Obes. Sci. Pract.* **6**, 677–693 (2020).

90. Huang, P. L. A comprehensive definition for metabolic syndrome. *Dis. Model Mech.* **2**, 231–237 (2009).
91. La Rosa, P. S. *et al.* Hypothesis testing and power calculations for taxonomic-based human microbiome data. *PLoS One.* **7**, e52078 (2012).
92. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **37**, 852–857 (2019).
93. Segata, N. *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol.* **12**, R60 (2011).

Figures

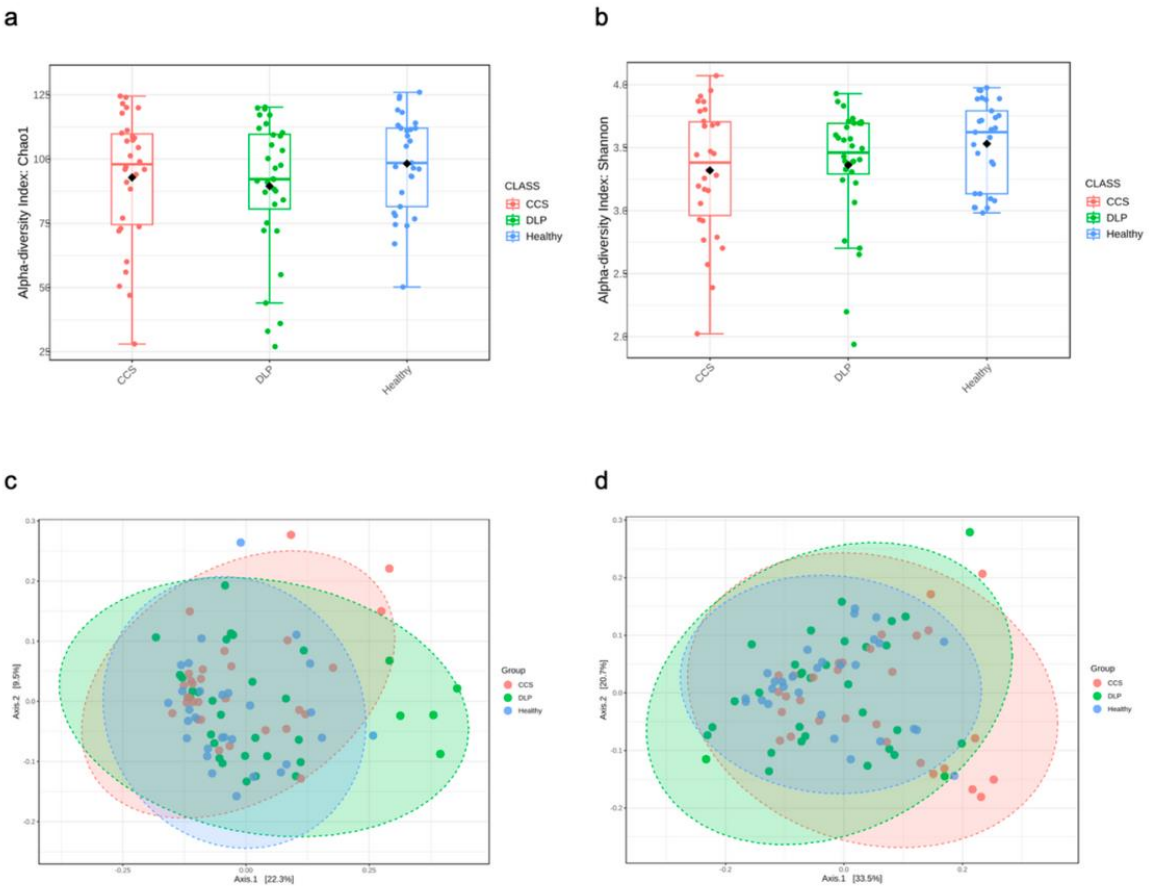

Figure 1

Analysis of alpha- and beta-diversity of microbial composition in the three patient groups. Diversity within bacterial communities was measured by The Chao1 index (a), The Shannon diversity index (b) The

principal coordinate analysis (PCoA) of beta diversity based on the unweighted UniFrac distance (c) and the weighted UniFrac distance (d). Kruskal-Wallis H test was used in the statistical test of the alpha diversity (No statistically significant difference found). Permanova (Permutational multivariate analysis of variance) test was used in the statistical test of the beta diversity. Statistically significant differences found at p<0.001. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

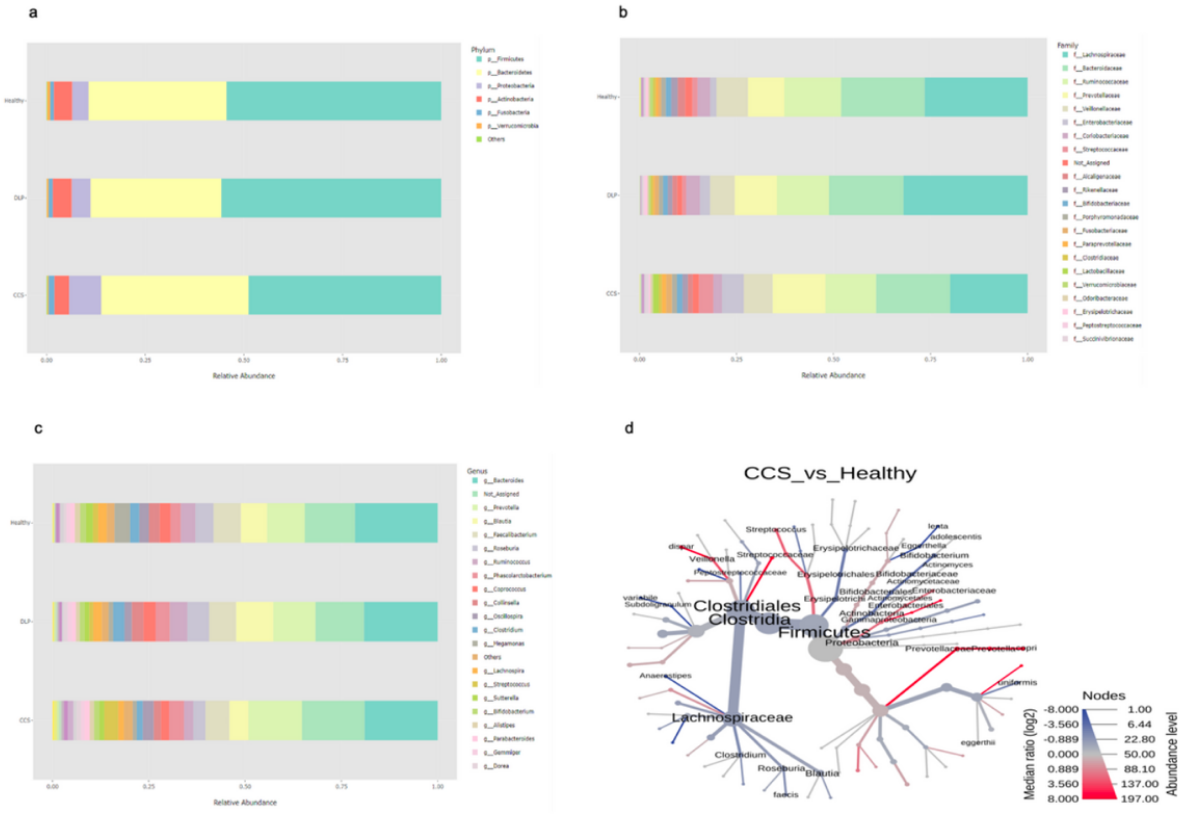

Figure 2

The relative abundance of bacterial taxonomic profile in the feces at the phylum (a) family (b) and genus (c) level. The phylogenetic heat tree (d) in comparison of bacterial microbiota between CCS and healthy groups shows the bacteria composition at the species level. The color of nodes and edges represents the mean change in operational taxonomic units (OTUs) richness at each taxonomic group, with red indicating greater richness in CCS and blue in healthy, while node size represents OTUs richness of each taxonomic group across the dataset. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

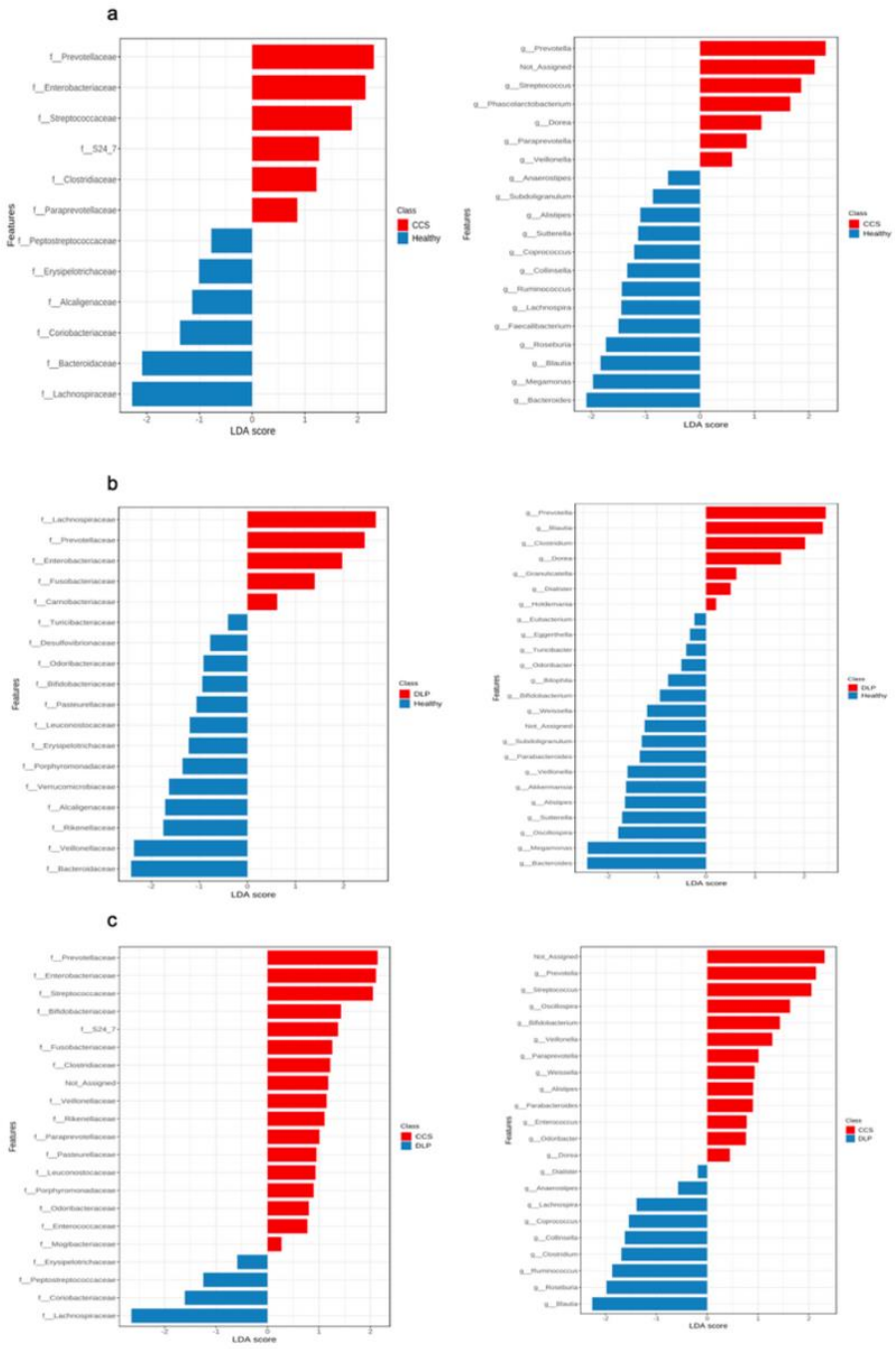

Figure 3

The linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota between CCS patients and healthy volunteers (a), dyslipidemia patients and healthy volunteers (b), and CCS patients and dyslipidemia patients (c). CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

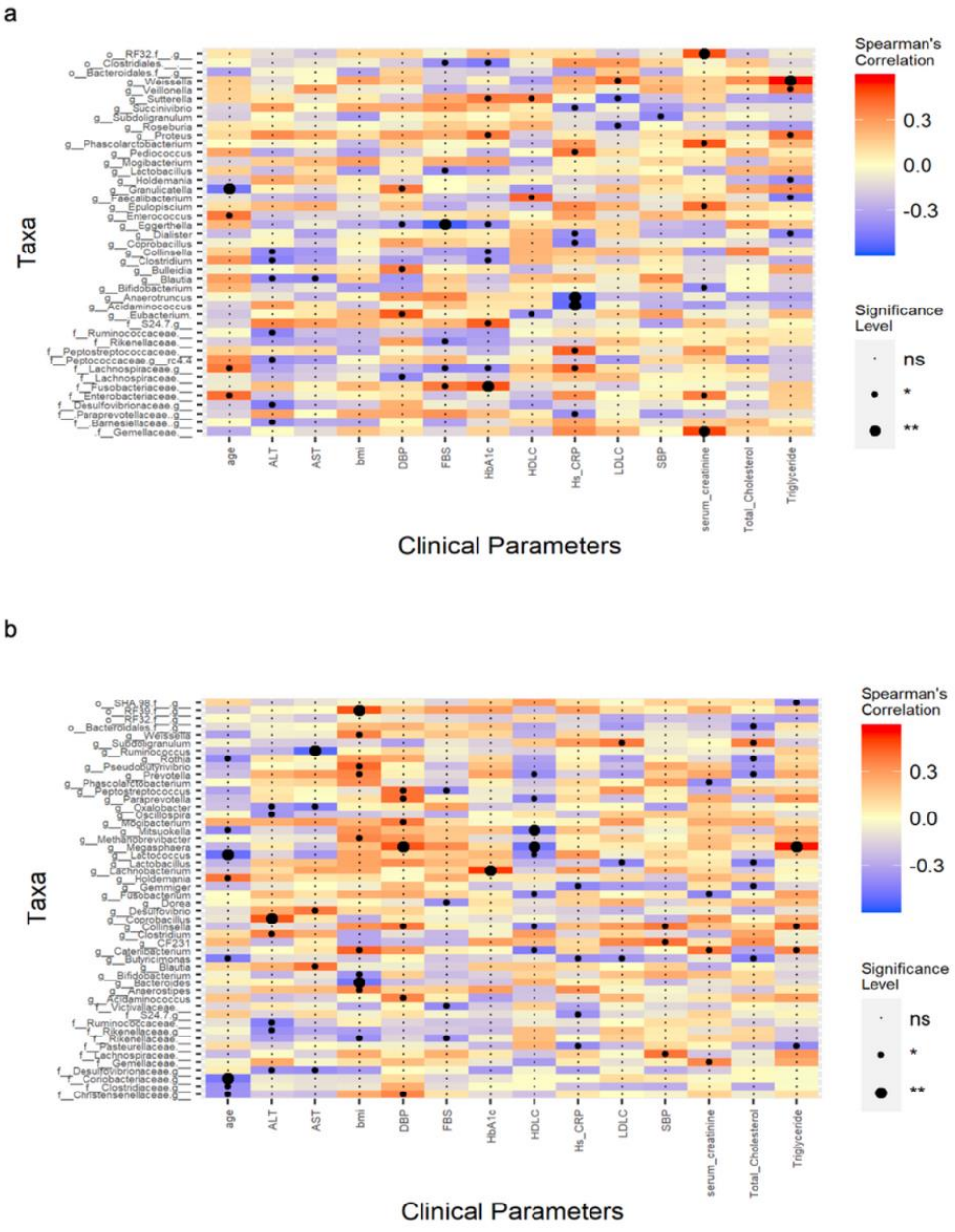

Figure 4

Spearman's correlation analysis between the clinical indexes and the microbiota. CCS patients undergoing coronary angiography group (a), dyslipidemia patients' group (b). The colour represents positive (red) or negative (blue) correlations, and *P < 0.05, **P < 0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HbA1C, hemoglobin

A1C; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoproteincholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure

REFERENCES

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