Intestinal Permeability and Microbial Dysbiosis in Acute Coronary Syndrome (MIACS study)

Tarik Alhmoud
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Henry Lin, MD, Member

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Intestinal Permeability and Microbial Dysbiosis in Acute Coronary Syndrome (MIACS study)

BY

Tarik Z. Alhmoud

DOCTOR OF MEDICINE, MD

THESIS

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biomedical Science
(Clinical Research Concentration)

The University of New Mexico

Albuquerque, New Mexico

May 2018
DEDICATION

إهداء إلى والدي العزيز:

الأستاذ الدكتور زهير محمد الحمود

أسال الله عز و جل أن يبارك و يطيل في عمره.
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Intestinal Permeability and Microbial Dysbiosis in Acute Coronary Syndrome (MIACS study)

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MD, University of Jordan, 2007

MS, University of New Mexico, 2017

ABSTRACT

Background: Acute Coronary Syndrome (ACS) is a leading cause of morbidity and mortality. Metabolic syndrome and obesity are major risk factors for atherosclerosis and ACS. Dysbiosis plays an important role in metabolic syndrome and obesity. Studies show a markedly increased risk of heart attacks in patients with high levels of the pro-atherogenic metabolite trimethylamine-N-Oxide (TMAO). TMAO is produced by the intestinal microbial flora through metabolism of dietary phospholipids; Gram-negative bacteria (Phylum Proteobacteria) is the major source of TMAO metabolism. Patients with obesity and metabolic syndrome have a defective intestinal tight-junctional (TJ) barrier, which allows paracellular permeation of luminal antigens such as lipopolysaccharides (LPS; endotoxins); leading to endotoxemia. Endotoxins are also associated with increased risk of atherosclerosis and cardiovascular disease. We aim to study the hypothesis that patients with ACS have dysbiosis, including higher proportion of Gram-negative bacterial species capable of...
producing the pro-atherogenic metabolite TMAO, and test if dysbiosis in ACS patients is associated with increased intestinal permeability and endotoxemia.

**Methods:** This is a prospective case-control study conducted at a single university hospital. We enrolled ACS patients (within 72 hours of acute cardiac events) and healthy controls. Relevant clinical and demographic data were collected. Stool microbiome composition was examined using the 16S ribosomal RNA (Illumina) method to identify microbiota taxonomic genera. LPS serum level was measured by an ELISA-based method. The intestinal TJ barrier function was evaluated using lactulose-to-mannitol urinary excretion ratio (L/M ratio). Serum TMAO was measured using a mass spectrometry.

**Results:** We enrolled 19 patients and 19 controls. ACS patients had increased relative abundance of Gammaproteobacteria and Proteobacteria compared to healthy controls, with mean proportions of 1.8 ± 3.0 vs 0.2 ± 0.4 % (P = .04) and 4.1 ± 3.8 vs 2.1 ± 1.7% (P=.056), respectively. L/M-ratio was three times higher in ACS patients compared to healthy controls (.06 ± .07 vs .023 ± .02, P = .014).

There was no difference in the mean serum LPS or TMAO levels.

Conclusion: ACS patients have dysbiosis and increased intestinal permeability. Further studies are required to find the role of dysbiosis in acute cardiac events.

**Keywords:** dysbiosis, Intestinal tight Junctional barrier, Intestinal permeability, Proteobacteria, Gammaproteobacteria, TMAO, Endotoxemia, LPS, Endotoxins, Acute Coronary Syndrome and myocardial infarction.
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Chapter One

Introduction

Acute Coronary Syndrome (ACS; heart attacks) is a leading cause of morbidity and mortality in developed countries, and is growing in prevalence in developing countries; according to the World Health Organization (WHO) fact sheets (1). Metabolic syndrome and obesity—both major risk factors for atherosclerosis and ACS—are emerging as a worldwide pandemic (2). Metabolic syndrome is defined [by the National Cholesterol Education Program (NCEP), Adult Treatment Panel III (ATP III)] as a cluster of: central obesity, hyperglycemia, hypertriglyceridemia, low high-density lipoprotein (HDL) levels and hypertension--three out of five criteria are required to confirm diagnosis (3). WHO requires evidence of Insulin Resistance (such as impaired glucose tolerance or Diabetes Mellitus type II (DMII)) to fulfill the definition of metabolic syndrome (4). Despite the increasing prevalence of ACS, obesity and metabolic syndrome, our understanding of the underlying pathophysiology and what links these conditions is still evolving.

The Intestinal Microbiome

In the twenty-first century, technology is advancing gene sequencing and it’s bioinformatic applications, this has transformed microbiome genomics and molecular biology sciences. Next Generation Sequencing (NGS) platform allows detailed study of biological samples (such as human feces) in less than 24
hours. Investigators can run tens of samples simultaneously with immediate bacterial DNA/RNA sequencing and analysis of large amounts of produced data. Prior to the availability of gene sequencing, microbiologists and scientist used bacterial cultures to identify bacterial species in a biological specimen; however, most organisms could not be cultured, and the process was costly and time-consuming (5).

The wide use of NGS and metagenomics, allowed rapid advancement of our understanding of the intestinal microbiome composition. Metabolomics allow us to study the function of the microbiome. The number of publications about the microbiome has exponentially increased over the last decade (6). The intestinal microbiome has been linked to many diseases including atherosclerosis, airways diseases, brain disease and cancer among others (7, 8), and multiple studies in this area has shed light on the important role of intestinal microbial flora in metabolic syndrome and obesity (4, 5). The term intestinal microbiota refers to micro-organisms living in the human intestine, this include bacteria, archaea, viruses and fungi. The term microbiome is used to describe the community of microorganisms living in a certain habitat and the surrounding environment as well. We will use the term intestinal microbiota to refer to the intestinal bacteria in this study.

The intestinal microbiome contains hundreds of bacterial species (in older studies) (9); the number increased to thousands with the new bacterial gene sequencing methods. Over the last decade, the intestinal microbiome is increasingly recognized as an organ that serves different functions, most
importantly influencing our metabolic function and immune homeostasis (10, 11). The human genome contains about 20,000 genes (12) and the intestinal microbiome has ~150 times more genes than the human genome (13). It is not surprising that alterations in the intestinal microbiome (dysbiosis) are associated with multiple diseases such as metabolic syndrome and obesity (14, 15).

Intestinal microbiota are composed of more than one thousand species of bacteria. Several major bacterial Phyla make up the entire intestinal microbiota in humans: Bacteroidetes, Firmicutes and minorities of Actinomyces; Proteobacteria and Verrucomicrobia. The intestinal microbiota varies widely between individuals and in the same individual at different times (16, 17). However, the function of intestinal microbiota is very similar in different people, based on the shot-gun metagenomic studies, predicting the enzymatic and metabolic pathways of different organisms (13).

**Terms and Definitions**

Figure 1. below include definitions for many technical terms used in the microbiome field (6, 18, 19).
Definitions

**Microbiota**
Organisms living in a certain environment such as a body organ. Microbiota include bacteria, archaea, viruses and fungi

**Microbiome**
Community of organisms (microbiota) and the surrounding environment

**Metagenome**
Sum of genomes and genes of all organisms

**Metabolome**
Metabolites produced by microbiota and host cells in a certain environment, this include proteins, lipids, saccharides etc.

**Metatranscriptome**
Indicates the active genes that are transcribed by microbiota in a certain environment; a collective profiling of the mRNA from all organisms

**Prebiotic**
Nutrients that promote growth of healthy microbiota

**Probiotic**
Living microorganism or consortium of microorganisms that confers health benefits to the host, as a supplement

**Next-generation sequencing (NGS)**
Also known as High Throughput Sequencing; is a method for fast and cost-effective DNA and/or RNA sequencing. NGS is used to study Eukaryotic and Prokaryotic cells
Figure 1. (cont.)

**Shot-gun sequencing**

Is a special case of High Throughput Sequencing, where long strands of DNA are sequenced with the goal for whole genome analysis.

**16S rRNA gene**

Is part of the Prokaryotic 30S ribosome subunit. The 16S rRNA gene is well preserved and is used to identify specific bacterial species or genera. Some bacterial strains have multiple 16S rRNA genes.

**Operational Taxonomic Unit (OTU)**

A classification system based on the similarity of a taxonomic reference gene (e.g. 16S rRNA). Organisms with greater than 97% similarity are classified as a unique OTU.

**Alpha Diversity**

Alpha diversity or local diversity depend on the number of microbial species in each sample, and the evenness of species.

**Beta Diversity**

A measure of diversity between different samples (how many species are shared between different samples).

**Chao1 index**

Is a measure of Alpha diversity, with focus on richness of microorganisms in a sample.

**Principal Component Analysis PCA**

A complex statistical method used in data analysis. It is useful in visualizing
distance and relatedness between different populations and is commonly used in genetics. The first component (first axis) account for the highest variability in data, in comparison to other components (second and third axes).

**Intestinal Tight Junctional (TJ) Barrier**

Small and large intestines are key organs for absorption of nutrients, water and minerals. With a length of 15 feet (on average) and a large surface area [more than 40 square meters and up to 300 square meters in older studies] (20). Most of the human microbiota are present in the intestines, the number of bacterial cells exceeds the number of human cells [1-10 times higher] (21). Inside human intestines live trillions of bacteria; where the body is exposed to numerous bacterial and dietary antigens, and potentially harmful substances. We are separated from the outside world by an intestinal epithelial monolayer (compared to the keratinized, thick skin epidermis). Intestinal epithelial cells are held together by tight junctions; which form a barrier between cells, often referred to as the intestinal tight junctional (TJ) barrier. TJs act as a physical and functional barrier against penetration of harmful substances and bacterial antigens in the intestinal lumen.

**Defective Intestinal Tight Junctional Barrier**

Patients with diseases affecting the small and/or large intestines often have a defective intestinal tight junctional barrier, which leads to an increased intestinal permeability (leaky gut). Examples of such conditions include celiac disease,
ulcerative colitis and Crohn’s disease (22). These individuals are at a higher risk for cardiovascular complications (23). Moreover, defective intestinal tight junctional (TJ) barrier with increased intestinal permeability, also occurs in many systemic diseases, such as alcoholic and non-alcoholic steatohepatitis, obesity, DMII and metabolic syndrome. Paracellular permeation of luminal antigens from the intestinal lumen to the blood stream, appears to be an important pathogenic factor in these conditions(24-26).

**Lipopolysaccharides (LPS; Endotoxins)**

Lipopolysaccharides (LPSs; endotoxins) are a major component of Gram-negative bacteria outer-membrane structure. In the presence of a defective intestinal TJ barrier, harmful bacterial antigens such as endotoxins translocate to the blood stream resulting in endotoxemia (27). Studies from our lab have shown that LPS causes an increase in intestinal tight junction permeability in vitro and in vivo (28).

Endotoxins are associated with increased risk of atherosclerosis and cardiovascular events; subjects with high levels of endotoxins face a threelfold increased risk of developing atherosclerosis (29). The mechanism may include direct damage to endothelial cells, which stimulate macrophage transformation into foam cells; promoting atherosclerosis (30). The Endotoxin molecules bind Toll-Like Receptor-4 (TLR-4), which is present in intestinal, immune and endothelial cells, and form a receptor cluster with the Cluster of Differentiation-14 (CD14). TLR4 polymorphism confers reduced risk of atherosclerosis (31). CD14
promoter polymorphism is a risk factor for ACS and atheromatous plaque vulnerability (32). Investigators detected Proteobacteria (pathogenic Gram-negative bacteria) in atherosclerotic plaques (from endarterectomies) (33), and blood levels of Proteobacteria DNA was associated with increased risk of cardiac events (Odds ratio 1.56, 95%CI 1.12–2.15) (34).

**Trimethylamine-N-Oxide (TMAO)**

Intestinal microbiota are not inert organisms or bystanders; they play an important role in metabolism. Metabolites produced by the intestinal microbiota affect human health and diseases, of which TMAO is an important example. Dietary phospholipids such as phosphatidylcholine and L-carnitine are metabolized by intestinal bacteria to trimethylamine (TMA), subsequently TMA is absorbed and converted into TMAO in the liver (by hepatic flavin monooxygenases 3 (FMO3)) (35, 36). Recent studies revealed an association between trimethylamine-N-Oxide (TMAO) and risk for cardiovascular events (36, 37). In a large study (n = 4,007) elevated baseline TMAO plasma level was associated with increased risk of major cardiovascular event (hazard ratio of 2.54) after three years of follow-up (37). In animal studies, TMAO’s proatherogenic effect was visible by inducing atherosclerosis (38). In addition, high TMAO blood levels were associated with higher odds of newly diagnosed DMII (39). Phylum Proteobacteria is the major source of TMAO metabolism in humans (40).
Aims of the Study

It is known that TMAO, and endotoxemia (with underlying defective intestinal TJ barrier) are risk factors for future ACS events. Intestinal Microbiota--Proteobacteria in particular--is the primary source of TMAO and endotoxins. The intestinal microbiota composition, during or shortly after an acute cardiac event (ACS) remains unclear. We aim to study the hypothesis that patients with ACS have dysbiosis, including higher proportion of Gram-negative bacterial species (Proteobacteria), which contribute to the production of the pro-atherogenic metabolite TMAO. We aim to assess the intestinal TJ barrier function and serum endotoxin levels shortly after acute cardiac events.
Chapter Two

Material and Methods

This is a prospective case-control study performed in a single tertiary center; University of New Mexico Hospital. The study was approved by a UNM Institutional Review Board (IRB) committee with a protocol number 13-625. The study was conducted between March 11th, 2014 and November 30th, 2016.

Inclusion Criteria

Inclusion Criteria for Cases

Patients admitted to UNM Hospital cardiology service with Acute Coronary Syndrome. We used the definition of ACS as described by the American Heart Association:

A-STEMI: diagnosis is made by clinical symptoms (chest pain at rest) with ST elevation >1mm in two contiguous EKG leads, or new LBBB (Left bundle branch block) ± troponin-I > 0.12 ng/ml.

B-NSTEMI: This is defined by one of the following criteria (with elevated troponin level): 1. Resting chest pain (angina) >20minutes in duration 2. New onset angina <2 months, 3. Increasing angina in frequency/intensity or duration and elevated cardiac markers; troponin > 0.12 ng/ml.
Unstable Angina: 1. Resting angina >20 minutes in duration 2. New onset angina <2 months, 3. Increasing angina in frequency/intensity or duration. Cardiac biomarkers are normal in these patients. All patients were recruited in the study within 72 hours of admission, and after Coronary Angiography and Percutaneous Coronary Intervention (PCI) were completed.

**Inclusion Criteria for Healthy Controls**

Subjects in this group had normal body mass index (18.5 < BMI < 24.9), no history of hypertension, diabetes or dyslipidemia, no prior heart attacks or strokes, no tobacco use and no antibiotic use within three months prior to enrollment.

**Exclusion Criteria**

- Age less than 19 years.
- Pregnant women (pregnancy test verification for women in child-bearing age).
- Prisoners.
- Adults unable to consent.
- Sick patients including:
  
  I. Glomerular filtration rate (GFR) less than 60 (abnormal kidney function).
  II. Cardiac Ejection fraction (EF) < 45% (for the intestinal permeability test).
III. Patients requiring pressors including but not limited to dopamine or norepinephrine.

IV. Patients who received thrombolytics like tissue plasminogen activator.

V. Patients requiring care in the Intensive Care Unit (ICU).

VI. Patients requiring an intra-aortic balloon pumps (IABP).

VII. Patient requiring a Coronary Artery Bypass Surgery (CABG).

VIII. Patients requiring a trans-venous pacemaker.

**Recruitment**

All patients were screened for eligibility by a physician investigator. Healthy controls (matched to cases by age and sex) were enrolled from a UNM Clinical and Translational Science Center (CTSC) volunteer list. All subjects were offered the opportunity to participate in the study by one of the study team members. Subjects were enrolled after obtaining an informed consent. Each subject provided blood and stool samples. Urine samples were collected for some of the patients after a delayed approval by UNM institutional IRB. All samples were frozen at -80° C until usage. Relevant clinical and demographic data were documented.

**Study Endpoints**

The primary end point of the study is the relative abundance of intestinal Proteobacteria in ACS patients compared to healthy controls. Secondary end
points include serum endotoxin and TMAO levels and assessment of the intestinal TJ barrier function using the lactulose/mannitol urine excretion test.

**Statistics**

Sample size was calculated to achieve a statistical power >80%, and a level of significance; \( \alpha = .05 \) for different end points of the study.

**Intestinal Microbiota Data Statistics**

16S rRNA data was analyzed to detect alteration of the relative abundance of the four-major intestinal microbial Phyla; including the phylum Proteobacteria (which includes pathogenic Gram-negative bacteria). Our hypothesis is that one out of the four major bacterial phyla (Proteobacteria) will be more abundant in ACS patients compared to healthy controls. A non-parametric t-test (Satterthwaite test) was used to compare the proportion of Proteobacteria in ACS patients versus controls (due to unequal variance in data).

Physiologically, Proteobacteria makes up ~ 5-8% of the human microbiome; to detect a 30% increase in this phylum in the ACS patient group, and to achieve a power of > .8 and \( \alpha \) of 0.05, eighteen (18) subjects are required in each group. We anticipated that about 20-25% of subjects would not provide all the required samples; therefore, we planned to recruit 25 subjects per group.
How results were interpreted

The intestinal microbiota composition was displayed as relative abundance of different intestinal bacteria at the Phylum, Class, Order, Family and Genus levels. At each level, results were displayed using Bar-Charts to identify the microbiota taxonomic phyla and genera. A bioinformatics team (Los Alamos National Labs) helped analyze 16S rRNA data by providing various Alpha Diversity, Beta Diversity and Principal Component Analyses. It should be noted that our sample size might not be adequate to detect all differences at the Genus level.

Endotoxin and Intestinal Permeability Data Statistics

Sample size was calculated to achieve statistical power (>80%) and a level of significance $\alpha = .05$. Standard unpaired t-test used to compare the endotoxin levels between ACS patients and controls, we predicted the mean serum LPS to be at least 100% higher in ACS patients compared to controls, 18 subjects per group are required to detect this effect size; based on preliminary results from our alcoholic hepatitis (AH) study, the serum LPS level in AH patients was $420 \pm 50.3$ vs $18.9 \pm 8.8$ in healthy controls.

For the intestinal permeability test, we used the Mann-Whitney-Wilcoxon nonparametric test as the data was not normally distributed. To detect a 100% increase in intestinal permeability in the ACS group compared to the control group; 17 patients per groups are required, based on our prior alcoholic hepatitis study the lactulose to mannitol ratio in patients compared to controls was $.23 \pm$
.08 vs .02 ± .003; we didn’t expect the same degree of increased intestinal permeability in ACS patients.

**How Results Were Interpreted**

These studies are intended to determine the intestinal barrier function in patients with ACS and healthy controls. The studies are expected to show that ACS patients have an abnormal intestinal TJ barrier function with an increased intestinal permeability to the paracellular marker lactulose. Lactulose to mannitol ratio (L/M ratio) is a well-validated marker for intestinal permeability; L/M ratio is expected to be higher in ACS patients compared to health controls. The results will show the mean increase in L/M ratio and the proportion of ACS subjects who have increased intestinal permeability. Similar approach will be used for serum endotoxin levels, the results are intended to show the difference in endotoxin levels between the two groups and what proportion of ACS patients have increased endotoxin levels.

**Intestinal Microbiota Sequencing and Analysis**

**DNA Extraction**

DNA extraction from stool samples was performed using the ZR Fungal/Bacterial Miniprep Kit (Zymo D6005). Approximately 150mg stool was homogenized in a bead bashing tube at 6 m/s for 60 seconds on the MP Bio FastPrep 24 homogenizer. Homogenized stool samples were then processed per the
miniprep kit protocol. Extracted DNA was filtered using Spin-X Centrifuge Tube filters (Costar 8160) prior to quantification.

DNA Sequencing

MetaVx™ Mammalian Library Preparation and Illumina MiSeq Sequencing Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, LLC. (South Plainfield, NJ, USA. DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and DNA quality was checked on a 0.6% agarose gel. Sequencing library was constructed using a MetaVx™ Mammalian Library Preparation kit (GENEWIZ, LLC., South Plainfield, NJ, USA). Briefly, 50 ng DNA was used to generate amplicons that cover V3 and V4 hypervariable regions of bacteria and Archaea16S rDNA. Indexed adapters were added to the ends of the 16S rDNA amplicons by limited cycle PCR. Sequencing libraries were validated using an Agilent 4200 TapeStation system (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer’s instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2x250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument.
16S rRNA Analysis

Sequence analysis by QIIME pipeline. The 38 stool samples were processed through QIIME analysis pipeline (v1.9.1, Caporaso et al 2010a). Raw paired-end Illumina® fastq files were merged, de-multiplexed, quality filtered (-q 19 –p 0.5 –n 1) and resulted in 4,821,145 filtered sequencing reads. OTUs (operational taxonomic units) were assigned using an open-reference OTU picking protocol, where UCLUST (Edgar 2010) was applied to search sequences against a subset of the Greengenes database (DeSantis et al 2006) filtered at 97% identity. Reads were assigned to OTUs based on their best hit to this database at greater than or equal to 94% sequence identity. Reads that did not match a reference sequence were clustered by UCLUST to assign novel OTUs. The OTUs were further filtered at minimum OTU abundance 2 (in number of sequences) to retain the OTU. The cluster centroid for each OTU was chosen as the OTU representative sequence. OTU representative sequences were then classified taxonomically using the Greengenes taxonomy string by the best matching Greengenes 16S rRNA sequence. In addition, OTU representative sequences were aligned using PyNAST (Caporaso et al 2010b) against a template alignment of the Greengenes database and phylogenetic trees were constructed using FastTree (Price et al 2010). The alpha-diversity estimates, beta-diversity estimates, taxonomy summary table, group comparison were performed within QIIME using “core_diversity_analyses.py” script with even subsampling at minimum 1,000 sequences per sample (41-45).
Measurement of serum Endotoxin, Intestinal Permeability and TMAO Levels

A 20-ml sample of peripheral blood was collected from each participant by venipuncture. Serum level of endotoxin (LPS) was measured using a Chromogenic Limulus Amoebocyte Lysate Quantification kit (Thermoscientific-88282, IL, USA).

Assessment of Intestinal Permeability: After an overnight fast (8 hours), all participants drank a 50-ml aqueous solution containing 2 gm mannitol and 5 gm lactulose. Following ingestion, urine was collected for 5 h. Participants were not allowed to consume food but were allowed to drink water during the 5 h urine collection period. At the end of the collection period, the total urine volume was recorded, and 20 ml of the urine was used for processing. The concentration of mannitol and lactulose present in the urine was determined by an enzyme-based assay (Megazyme, Ireland) and quantified by spectrophotometry. Results were calculated as a ratio of lactulose to mannitol excreted in urine (46, 47). Serum TMAO levels was measured using Liquid Chromatography Mass Spectrometry (Cleveland Heart labs, Ohio, USA).
Chapter Three

Results

Subject Characteristics

Nineteen ACS patients and similar number of healthy controls were recruited. ACS patients were slightly older than healthy controls (mean age difference of five years). ACS patients were overweight (mean BMI of 28.3) with a 4-point higher Body Mass Index (BMI) than healthy controls. Patient characteristics at baseline are shown in Table 1.

Table 1. Baseline Characteristics of patients and healthy controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ACS group (n=19)</th>
<th>Control group (n=19)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>54.4 ± 2.2</td>
<td>49 ± 1.6</td>
<td>.07</td>
</tr>
<tr>
<td>Gender M (n, %)</td>
<td>14 (74)</td>
<td>13 (68)</td>
<td>0.7</td>
</tr>
<tr>
<td>BMI (kg/m²) (mean ± SD)</td>
<td>28.31 ± 1.9</td>
<td>24.3 ± .39</td>
<td>.004</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whites</td>
<td>13(68)</td>
<td>14(74)</td>
<td>0.72</td>
</tr>
<tr>
<td>Others</td>
<td>6(32)</td>
<td>5(26)</td>
<td></td>
</tr>
</tbody>
</table>
Most patients had STEMI (89%), the mean troponin level was 30.07 ng/mL and most patients (63%) had a single vessel disease. Full clinical characteristics of patients are shown in Table 2.

Table 2. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol use</td>
<td>7(37)</td>
</tr>
<tr>
<td>Tobacco use</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>4(21)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>4(21)</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>11(58)</td>
</tr>
<tr>
<td>Past Medical History</td>
<td></td>
</tr>
<tr>
<td>Family history of CAD$^1$</td>
<td>13(68)</td>
</tr>
<tr>
<td>Diabetes Mellitus Type II</td>
<td>4(21)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6(32)</td>
</tr>
<tr>
<td>Prior ACS$^2$</td>
<td>2(10)</td>
</tr>
<tr>
<td>Admission diagnoses</td>
<td></td>
</tr>
<tr>
<td>STEMI$^3$</td>
<td>17(89)</td>
</tr>
<tr>
<td>NSTEMI$^4$</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Intervention</td>
<td></td>
</tr>
<tr>
<td>Coronary angiography with PCI$^5$</td>
<td>19(100)</td>
</tr>
<tr>
<td>Stent placement</td>
<td>18(95)</td>
</tr>
<tr>
<td>Number of affected vessels</td>
<td></td>
</tr>
<tr>
<td>One-vessel disease</td>
<td>12(63)</td>
</tr>
<tr>
<td>Two-vessel disease</td>
<td>6 (32)</td>
</tr>
<tr>
<td>Three-vessel disease</td>
<td>1 (5)</td>
</tr>
</tbody>
</table>
Table 2. (cont.)

<table>
<thead>
<tr>
<th>Mean Troponin level</th>
<th>30.07 ng/mL</th>
</tr>
</thead>
</table>


**Intestinal Microbiome**

Deep sequencing (minimum sequence count of 37971) of fecal samples revealed more than one thousand bacterial species. Bacteria with at least 97% similarity in 16S rRNA gene were assigned a unique Operational Taxonomic Unit (OTU).

**Alpha Diversity**

**Number of OTUs**

We sequenced all samples to detect all available OTUs as shown in the observed-OTUs rarefaction plot (figure 2A, B). Rarefaction plot is a marker of sample richness, in figure 2A, all sample curves reach a plateau, which indicates that no more OTUs can be found with further sequencing. The OTUs rarefaction plot are displayed per group in figure 2B.
Figure 2. Observed OTUs. (A) rarefaction plot of number of OTUs per sample. (B) rarefaction plot showing number of OTUs per group.
In both groups, the number of observed OTUs (mean +/- SD) was more than 1000 (1928.9 ± 1165.1 vs 1336.2 ± 611.7, P-value = 0.19), more variability is noted in the ACS patient group (figure 3).

![Figure 3. Number of observed OTUs per group.](image)

**Phylogetic Diversity**

Phylogenetic diversity (PD) is an indicator of biodiversity, PD is measured by adding the branch lengths of a phylogenetic tree to quantify diversity of a sample (48). PD was measured for all samples (figure 4A). Our results showed no significant difference in PD between the two groups: mean PD +/- SD in ACS patients vs healthy controls was 111.63 ± 45.65 vs 86.55 ± 25.69, P-value = 0.16. (figure 4B).
Chao1 Index

The Chao1 index; a measure of sample richness was performed for all subjects (figure 5A). The Chao1 index was similar in ACS patients and healthy controls: mean Chao1 index was $3303.3 \pm 1747.6$ vs $2415.8 \pm 977.7$, P-value = 0.2, (figure 5B).
Beta diversity

This is a measure of diversity between different samples.

Unique Fraction Distance (Unifrac)

The unique fraction (unifrac) distance (weighted for abundance) was uniform; a mean distance of ~ 0.4 was noted between samples within the same group and between samples within different groups. A Unifrac distance between group one and group two is defined as the fraction of branches of the phylogenetic tree that lead to members of either group, but not group one and group two (49) [figure 6].
Figure 6. Weighted unique fraction group distances.
Principal Component Analysis

Principal Component analysis (PCoA) of Unifrac distance showed similar distribution of ACS and healthy subjects along the three PC axes; no distinct group clustering was noted (figure 7).

Figure 7. Principal component analysis of weighted unique fraction distance.
Taxonomic Summaries

Bacterial Phyla

16S rRNA analysis revealed the relative abundance of intestinal major bacterial phyla in individual subjects, we compared the levels between ACS patients and healthy controls. Both groups had Bacteroidetes and Firmicutes as the two major phyla, and a small percentage of Proteobacteria and Actinobacteria, as expected from prior studies (Figure 8 A, B).
The mean relative abundance of the Phylum, Proteobacteria; Class, Gammaproteobacteria and Family, Enterobacteriaceae was higher in ACS patients compared to controls. This was statistically significant only at the Gammaproteobacteria level (figure 9A-D).
Figure 9. Levels of Proteobacteria Gammaproteobacteria and Enterobacteriaceae. (A) mean percentage of fecal Proteobacteria in ACS patients compared to healthy controls. (B) mean percentage of fecal Gammaproteobacteria in ACS patients compared to healthy controls. (C) mean percentage of fecal Enterobacteriaceae in ACS patients compared to healthy controls. (D) summary of Proteobacteria, Gammaproteobacteria and Enterobacteriaceae levels.
The other major bacterial phyla were similar between the two groups as shown in figure 10A-D.
Figure 10. Bacteroidetes, Firmicutes and Actinobacteria (A) mean percentage of fecal Bacteroidetes in ACS patients compared to healthy controls. (B) mean percentage of fecal Firmicutes in ACS patients compared to healthy controls. (C) mean percentage of fecal Actinobacteria in ACS patients compared to healthy controls. (D) summary of Bacteroidetes, Firmicutes and Actinobacteria.
Bacterial Genera

Relative abundance of different bacterial OTUs was calculated in ACS patients and healthy controls, and in individual subjects. A specific OTU infers specific bacterial genus (not adequate to identify a specific species or strain) (figure 11A, B), for legend see supplementary figure1.
More than twenty bacterial genera had more than 2-fold difference between the two groups, a summary is shown in table 3.
Table 3. Bacterial genera with more than 2-fold difference between the two groups. Red color indicates higher abundance

<table>
<thead>
<tr>
<th>Bacterial genera</th>
<th>Healthy controls</th>
<th>ACS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobrevibacter</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Coriobacteriaceae</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Bacteroidales</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Rikenellaceae;</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Odoribacter</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Paraprevotellaceae</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Paraprevotella</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Prevotella</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Christensenellaceae</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lachnobacterium</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Roseburia</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Acidaminococcus</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Dialister</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Megamonas</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Megasphaera</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Veillonella</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Erysipelotrichaceae</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>[Eubacterium]</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Bilophila</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Succinivibrio</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Intestinal Permeability Test**

The lactulose-to-mannitol ratio (L/M ratio) was calculated by dividing the fractional (percentage) of urinary excretion of lactulose over the fractional excretion of mannitol. Fifty percent of patients (n=8) and most controls (n=16) completed the L/M permeability test. The L/M ratio was three times higher in ACS patients (figure 12A, B).
Figure 12. Lactulose-to-mannitol ratio (L/M ratio). (A) L/M ratio in ACS patients and healthy controls. (B) mean L/M ratio in ACS patients versus controls.
Lipopolysaccharides; Endotoxin Levels

The mean serum LPS level was less than 1 ng/ml in both groups (figure 13).

![P-value = 0.9](image)

Figure 13. Mean serum LPS level in ACS patients compared to healthy controls

Trimethylamine-N-Oxide (TMAO)

TMAO was detected in blood samples from all subjects. No difference in the TMAO level was detected between the two groups (figure 14).
Results Summary

All major results for both groups are summarized in table 4.

Figure 14. Mean serum TMAO level in ACS patients and healthy controls.
Table 4. Major test results for patients and controls

<table>
<thead>
<tr>
<th>Test result</th>
<th>ACS patients (n=18)</th>
<th>Healthy controls (n=18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria (% ± SD)</td>
<td>4.1 ± 3.8</td>
<td>2.1 ± 1.7</td>
<td>.056</td>
</tr>
<tr>
<td>Gammaproteobacteria (% ± SD)</td>
<td>1.8 ± 3.0</td>
<td>0.2 ± 0.4</td>
<td>.04</td>
</tr>
<tr>
<td>Enterobacteriaceae (% ± SD)</td>
<td>1.5 ± 2.9</td>
<td>0.2 ± 0.4</td>
<td>.07</td>
</tr>
<tr>
<td>Bacteroidetes (%± SD)</td>
<td>36 ± 19</td>
<td>32 ± 17</td>
<td>0.6</td>
</tr>
<tr>
<td>Fermicutes (%± SD)</td>
<td>57 ± 19</td>
<td>60 ± 17</td>
<td>0.5</td>
</tr>
<tr>
<td>Actinobacteria (%± SD)</td>
<td>1.4 ± 1.5</td>
<td>1.8 ± 1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>LPS¹ (ng/mL ± SD)</td>
<td>1.01 ± 0.5</td>
<td>1.12 ± 0.35</td>
<td>0.9</td>
</tr>
<tr>
<td>L/M² ratio (± SD)³</td>
<td>.06 ± .07</td>
<td>.023 +/- .02</td>
<td>.014</td>
</tr>
<tr>
<td>TMAO⁴ (uM ± SD)</td>
<td>5.8 ± 4.8</td>
<td>5 ± 2.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

¹Lipopolysaccharide (endotoxin). ²Lactulose-to-mannitol ratio. ³Only 8 patients completed this test along with 16 healthy controls. ⁴Trimethylamine-N-Oxide.
Chapter Four
Discussion
Summary and Discussion of the Results

We found that patients with acute cardiac events (ACS) have a nine-fold increase in intestinal Gammaproteobacteria (this Class includes opportunistic pathogenic Gram-negative bacteria); compared to healthy controls. Proteobacteria level was 2-folds higher in ACS patients, but didn’t reach statistical significance (P=0.056). The Proteobacteria (facultative anaerobes) level detected in stool samples (luminal fraction) is expected to be much lower than what is actually present on the mucosal surface of distal intestine (mucosa-associated fraction) (50). ACS patients have clear dysbiosis compared to healthy controls; up to 72 hours after an acute cardiac event. After delayed IRB approval, we performed the intestinal permeability test--lactulose-to-mannitol fractional excretion ratio (L/M ratio) -- in 50% of ACS patients (n=8), and in most healthy controls (n=16). The L/M ratio was three times higher in patients compared to controls; L/M ratio was 0.06 ± .07 vs 0.02 ± .02, P=0.014. The levels of serum endotoxins (lipopolysaccharides/LPS) and Trimethylamine-N-Oxide (TMAO) were similar in both groups, this was unexpected given the significant dysbiosis and the higher relative abundance of Gammaproteobacteria in the ACS group.

Proteobacteria contributes the most in the variation of the human intestinal microbiome genome function; a study combined human stool metagenomic data (from healthy participants) from North America, China and Europe; the mean Proteobacteria abundance was 1%--much lower than Bacteroidetes and
Firmicutes—however, it explained the most variation in gene families of the intestinal microbiome (51). Intestinal Proteobacteria is the most unstable of the four-major intestinal bacterial phyla [Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria] (52). It is not surprising that high levels of Proteobacteria is a key feature of dysbiosis in many inflammatory and metabolic conditions (53). For examples, Proteobacteria is more abundant in Inflammatory bowel disease (IBD) patients. Furthermore, dysbiosis with alteration in Proteobacteria; Enterobacteriaceae and E-coli differentiates Non-Alcoholic Steatohepatitis (NASH) from obesity-associated intestinal microbiome in the absence of NASH (54). Some studies showed negative correlation between Proteobacteria and Diabetes Miletus type II (DMII) (55), however, other studies showed that DMII patients have higher abundance of the Class Betaproteobacteria; not Gammaproteobacteria (56, 57). A recent study on atherosclerotic cardiovascular disease (218 patients) showed increased Enterobacteriaceae (a major Family within the Class Gammaproteobacteria) compared to controls (58).

Our study explored the intestinal microbiome in the setting of acute coronary syndrome; we studied dysbiosis and related intestinal barrier function, endotoxin and TMAO levels shortly after subjects had an acute cardiac event, with a goal to find associations between the study end points and acute cardiac events; and to understand what makes an atherosclerotic plaque vulnerable. There is a key role for inflammation and activation of immune cells inside the atherosclerotic plaques and rupture of the fibrous cap (59), with subsequent clot formation; leading to
myocardial ischemia/infarction during acute cardiac events. Endotoxins bind to Toll-like receptor 4 (TLR4) which is present in many cell types including endothelial cells and macrophages (foam cells inside atherosclerotic plaques) (31), studying the role of endotoxins in plaque vulnerability is intuitive. Previous studies confirmed clear correlations between dysbiosis, higher levels of endotoxins/TMAO and developing a future cardiovascular event, but such data are scarce for patients during acute cardiac events. We showed an increase in Gammaproteobacteria in ACS patients but that was not associated with increased serum endotoxin levels. Gram-negative bacteria produce different endotoxins—with major variation in the immunogenic Lipid A portion—that bind to TLR4. Endotoxins (with different lipid A) activate innate immune system and trigger a largely variable immunogenic response (60, 61). The determination of different types of endotoxins present in the ACS patients was beyond the scope of this study. We found no difference in TMAO levels between the two groups, this result is consistent with a recent study in patients with acute stroke and Transient Ischemic Attacks [TIAs] (62); patients had lower levels of TMAO compared to controls. This doesn’t contradict the previous large studies showing increased risk of acute cardiac events in subjects with high levels of TMAO; it is possible that TMAO is associated with a long-term risk of cardiovascular
events—and levels at the time of the event can be normal or low. In addition, TMAO levels are known to fluctuate widely overtime; over the course of one-year TMAO levels correlated only weakly ($R^2=0.29$, $P=.003$) in the same subjects (63).

**Strengths and Limitations**

**Strengths**
This study has multiple strengths; we enrolled patients with a clear-cut diagnosis of acute coronary syndrome. All patients had an acute event and underwent percutaneous coronary intervention (PCI) for STEMI ($n=17$) or NSTEMI($n=2$), the mean troponin I level of 30.07 ng/ml. The majority of patients (89%) had their first episode of ACS and had no prior stroke or cardiac event. Our study examined the microbiome composition and dysbiosis in ACS patients, along with studying the intestinal barrier function and a microbiome originated antigen (LPS) and metabolite (TMAO).

**Limitations**
Our study is not short of limitations, ACS patients had higher BMI compared to healthy controls, we performed a subgroup analysis and our results were not different when measured in patients with normal BMI (data not shown). We enrolled patients and obtained samples within 72 hours of the ACS events. Microbiome composition changes rapidly and changes can be noticed within 24-48 hours after hospitalization. Patient enrollment within few hours after going
through a major health event is difficult; especially when they go through a PCI procedure. A prior study enrolled stroke patient within 48 hours of the event. Subgroup analysis of patients enrolled with in the first 24 hours was not different in microbiome composition from patients enrolled after 24-48 hours (62). Our patients were mainly recruited, and samples obtained within 48 hours of the event (89% of cases).

Only 50% of our patients completed the intestinal permeability test due to delayed IRB approval. The results should be interpreted with caution due to the small sample size. Compared to a prior alcoholic hepatitis study (performed in our Digestive Disease Center); L/M ratio in healthy controls was similar (.02 ± .003). However, the L/M ratio in alcoholic hepatitis was greater than ACS patients (0.2 ± .08). We think that ACS patients have true increase in L/M ratio, however, it’s unclear if this is due to the cardiac event and norepinephrine surge or was present at baseline and contributed to the event.

**Future Directions**

A study at a larger scale is required to examine the role of the intestinal permeability in ACS. In microbiome studies, it is important to understand dysbiosis and the microbiome composition in different disease conditions “who’s there?”. However, it is more important to understand what is the function of the bacteria, which enzymatic pathways are activated and what metabolites are produced “What they’re doing?”. Metagenomic microbiome studies such as LPS biosynthetic pathway will be helpful, as there is a great variation in the genes
responsible for LPS production. Metatranscriptomic studies will put greater insight on the potential function of the microbiome and interactions with the host.

Internal sampling of the ruptured plaque is challenging, but it is possible to examine the PCI instruments to have a closer look at the bacterial antigens in-situ. Future study looking at causality of the microbiome in ACS are prudent. This will open the door for interventional studies targeting the microbiome function. Targets for microbiome therapy includes probiotics and prebiotics to alter enzymatic pathways affecting metabolite production, tightening of the intestinal TJ barrier, modifying the microbiome with short chain fatty acids, bile salts, and bacterial antigens and enzymes.

**Conclusion**

Dysbiosis affects patients with ACS, with an increase in intestinal permeability. Further studies are required to find the role of dysbiosis in plaque vulnerability (biomarkers for plaque vulnerability) and to examine if the dysbiosis and leaky gut are mere associations, or key contributing factors in acute cardiac events.
REFERENCES


