

POST-PRANDIAL SERUM CONCENTRATIONS OF
TRIMETHYLAMINE-*N*-OXIDE IN OVERWEIGHT AND OBESE
ADULTS WITH LOW AND HIGH SYSTEMIC INFLAMMATION

by

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GLOSSARY

Abbreviations

ALT – alanine aminotransferase
AST – aspartate aminotransferase
BIA – bioelectrical impedance analysis
BMI – body mass index
CHOL – cholesterol
CKD – chronic kidney disease
CVD – cardiovascular disease
DMB – 3,3-Dimethyl-1-butanol
eGFR – estimated glomerular filtration rate
ESRD – end stage renal disease
FFA – free-fatty acids
FMO – flavin-containing monooxygenase
FMO3 – flavin-containing monooxygenase 3
FMT – fecal microbiota transplantation
eGFR – estimated glomerular filtration rate
GI – gastrointestinal
GLU – glucose
GM-CSF – granulocyte macrophage colony-stimulating factor
HDL – high density lipoprotein
HFD – high-fat diet
hsCRP – high sensitivity C-reactive protein
IL – interleukin
LCMS – liquid chromatography mass spectrometry
LPS – lipopolysaccharide
MCP-1 – monocyte chemoattractant protein-1
NF- κ B – nuclear factor - kappa beta
NHANES – National Health and Nutrition Examination Survey
PBS – phosphate buffered saline
Q-TOF – quadrupole – time of flight
SCFA – short chain fatty acids
T2D – Type 2 Diabetes
TLR4 – toll-like receptor 4
TG – triglyceride
TMA - trimethylamine
TMAO – trimethylamine-*N*-oxide
TNF- α – tumor necrosis factor – alpha
UHPLC – ultra-high performance liquid chromatography

ABSTRACT

Purpose: Elevated trimethylamine-N-oxide (TMAO) levels have been implicated in the development of atherosclerosis. The impact of a high-fat meal on the post-prandial TMAO response in healthy overweight and obese adults was explored, accounting for additional variables that may affect the rate of TMAO conversion. **Methods:** Forty overweight and obese men and women participated in a high-fat meal challenge containing 50 g fat. Blood samples were collected at fasting and hourly in the 4 hours after meal ingestion and tested for concentrations of TMAO, liver enzymes, lipids, creatinine, insulin, and inflammatory markers. An initial mixed-effects model was constructed to analyze TMAO changes in participants who classified as having low or high levels of systemic inflammation accounting for factors that may influence TMAO concentrations. Backward refinement of the initial model was performed based off p-values. **Results:** Model refinement found that the log TMAO response was best explained by inclusion of estimated glomerular filtration rate (eGFR), insulin over time, a linear interaction between time and inflammation phenotype, and random effects on subject. Log TMAO after the meal was not different between inflammation phenotypes ($p = 0.33$). Estimated GFR was the most important determinant in the log TMAO response ($p = 0.03$), with lower log TMAO observed with higher eGFR values. In conclusion, serum TMAO concentrations during a high fat-meal challenge were strongly determined by eGFR, but not by fasting inflammation status.

CHAPTER ONE

INTRODUCTION

Development of the Problem

It may come as no surprise that American waistlines are growing larger. A nationally representative survey of U.S. adults in 2011-2012 found that 37.7% of Americans were obese.¹ Obesity, often defined as having a body mass index (BMI) equal or greater than 30 kg/m², is a serious public health concern as obesity raises the risk of cardiovascular disease (CVD), chronic kidney disease (CKD), hypertension, type II diabetes (T2D), and mortality.²⁻⁴ The financial burden of obesity and such related conditions is staggering with total healthcare costs to treat obesity-related conditions projected to reach 860.6 - 956.7 billion US dollars by 2030.⁵

Obesity is characterized by chronic low-grade systemic inflammation.⁶ Different from classic inflammation where there is an acute and rapid rise of inflammatory markers to injury or infection, chronic low-grade inflammation has increased systemic levels of pro-inflammatory cytokines like C-reactive protein, tumor necrosis factor – alpha (TNF- α), and interleukin (IL)-6 over an extended period of time with no resolution.⁷ Chronic inflammation underlies many obesity-related conditions like CVD, metabolic syndrome, and T2D.⁸⁻¹⁰ The initial stimulus for elevated inflammation with obesity is unclear. Macrophage accumulation and their proinflammatory activation in expanding adipose tissue is thought to be a major contributor to inflammation, but is not likely the only contributor, to inflammation. There is evidence to believe the microorganisms housed in

our gut, called the gut microbiota, may be involved in the development of obesity and chronic low-grade inflammation.^{11,12}

We live in symbiosis with the microbes in our gastrointestinal (GI) tract. In return for a relatively constant environment and supply of nutrients, gut microbes metabolize substrates that go undigested by host enzymes to help supply vitamins, produce short chain fatty acids (SCFA), and produce other compounds that can be absorbed and utilized by the host.^{13,14} One gut-derived compound is trimethylamine (TMA). Bacterial metabolism of particular compounds within red meat, eggs, and fish produces TMA.¹⁵ TMA is absorbed into host circulation from the gut and oxidized to trimethylamine-*N*-oxide (TMAO) by flavin-containing monooxygenases (FMO) in the liver, predominately FMO3.¹⁶⁻¹⁸ Oxidation of TMA by FMO3 was recently found to be modulated by the hormone insulin.¹⁶

Trimethylamine-*N*-oxide has been shown in murine models to negatively affect cholesterol and sterol metabolism in macrophages and the liver.¹⁵ Homeostatic imbalance of cholesterol transport is a key component leading to plaque growth on arterial walls. Cholesterol accumulation in macrophages, such as foam cells, can promote inflammation in atherosclerotic plaques.¹⁹ Increased serum TMAO has been linked with the development of atherosclerosis and increased risk of cardiovascular disease,^{18,20,21} conditions which feature chronic inflammation,¹⁰ suggesting TMAO may play an important role in the promotion of systemic inflammation. Several studies have reported that TMAO promotes pro-inflammatory pathways in animal models, but the relationship between post-prandial TMAO and inflammation in humans is unclear.^{22,23} As humans

spend most of their day in a post-prandial state, it is important to consider how TMAO concentrations may change after ingestion of a meal.

Triglyceride concentrations have been positively correlated with inflammation.²⁴ Free fatty acids (FAAs) are known to promote inflammation by activating pro-inflammatory pathways in immune cells like mononuclear cells and leukocytes.^{25,26} A high-fat meal rich in saturated fatty acids may be beneficial in exploring how changes in inflammation may influence the post-prandial TMAO response.

Purpose

The aim of the study is to examine whether fasting inflammation status and insulin may influence fasting and post-prandial serum TMAO concentrations. Specifically, the goal was to determine if the post-prandial TMAO response to a high-fat meal is different between overweight and obese individuals who differ in inflammation after initially accounting for additional variables, such as insulin concentrations, that have been found to influence the conversion of TMA to TMAO. Overweight and obese individuals were examined as they are more likely to have excess adipose tissue to drive chronic inflammation. If high serum TMAO is linked with conditions that have high systemic inflammation, it is speculated that higher fasting and post-prandial serum TMAO may be found in individuals who have high levels of inflammation but who are considered healthy. Additionally, if TMAO conversion is suppressed by insulin, then it is possible that individuals with low systemic inflammation, who are more likely to be insulin sensitive, would have a different TMAO response to a high-fat meal than high

inflammation individuals. A high-fat meal challenge was used to introduce TMA precursors for potential TMAO production.

Implications

This research will provide information about the relationship between TMAO and inflammation in humans. TMAO has only recently come into the spotlight as a factor for atherosclerosis,¹⁷ and our understanding of its influence on inflammation in humans is limited. It is important to incorporate microbiota-dependent metabolites like TMAO in our investigation of disease mechanisms to fill gaps of understanding in current processes. Future research opportunities following this study could lead to more targeted approaches on TMAO research, particularly regarding systemic inflammation and dietary intervention in humans.

Hypothesis

It is hypothesized that serum TMAO response to a high-fat meal challenge differs between high (HI) and low (LO) inflammation phenotypes and that insulin is a factor in the difference in the post-prandial TMAO response.

Delimitations

1. The study is restricted to males and females aged 18-55 years old.
2. The study is restricted to individuals who have not recently taken antibiotics.

Limitations

1. The results of the study cannot be generalized to individuals under the age of 18 or over the age of 55.
2. The results of the study cannot be generalized to individuals with cardiovascular, gastrointestinal, or immunological conditions.

CHAPTER TWO

LITERATURE REVIEW

Trimethylamine-*N*-oxide and Disease

TMAO is a small organic, odorless simple amine that has been previously studied for decades in marine organisms. It is thought to assist marine organisms with buoyancy, protein folding and stability, and act as an osmolyte.²⁷ In 2011, TMAO came to immediate and widespread attention in human research as a potential risk factor for CVD.¹⁷ Since the seminal paper by Wang and colleagues, higher TMAO levels also been associated with increased diabetes risk, increased risk of first stroke in hypertensive patients, major adverse cardio-and cerebrovascular events, and CKD.^{21,28-31} Direct influence of TMAO on host function has been demonstrated in mice, with TMAO supplementation promoting renal fibrosis and interstitial fibrosis in the heart.^{22,28} Through intensive research in mice and humans, it is known now that TMAO formation is first and foremost dependent on the bacteria residing the gut.

Gut Microbial Role in TMAO Production

The bacteria in our gut have diverse metabolic pathways. Pathways range from carbohydrate and lipid metabolism to vitamin metabolism. Specific taxa of anaerobic bacteria involved in the TMAO production process are capable of metabolizing choline from the diet. In addition to choline, microbial metabolism of *L*-carnitine, γ -butyrobetaine, and phosphatidylcholine can also ultimately lead to TMA production (Fig.

1).^{15,16,32} With bacterial choline metabolism, TMA lyase cleaves the bond between carbon and nitrogen to form TMA and acetaldehyde. Once produced, TMA is absorbed from the distal GI tract into portal circulation. Oxidation of TMA by hepatic FMOs forms TMAO.¹⁷ Flavin-containing monooxygenase 3 (FMO3) is reported to be the most active enzyme in TMA to TMAO conversion.^{16,18} The formation of TMAO in humans is reliant upon TMA uptake from the gut and into circulation.

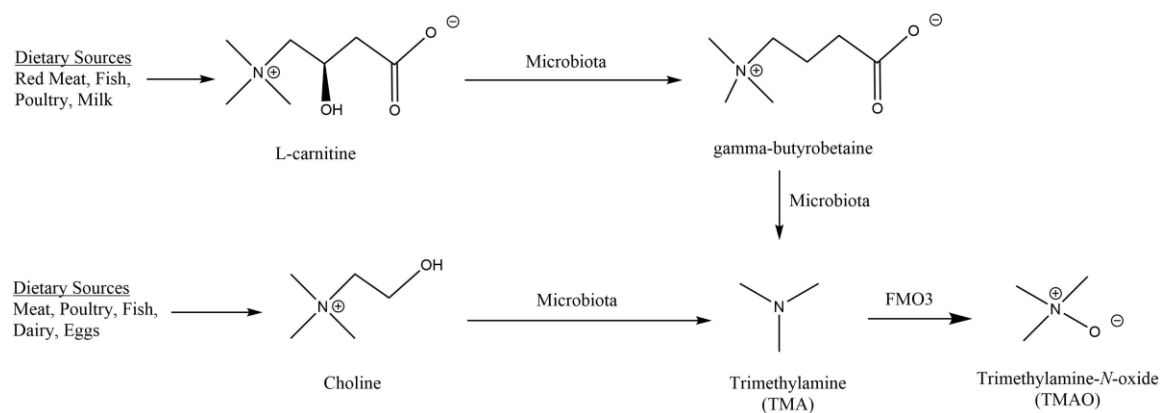


Figure 1. Dietary sources of choline and L-carnitine and their conversion into TMA and TMAO. Foods listed in dietary sources are rich in L-carnitine and choline. FMO3 is the hepatic enzyme flavin-containing monooxygenase 3. Hydrolysis of phosphatidylcholine produces choline phosphate, an intermediate that leads to choline with an additional kinase-mediated reaction.

The importance of gut bacteria in TMAO formation has been tested by targeting microbial pathways. Suppression of the mouse gut microbiota with antibiotics reduced the introduction of TMAO in plasma after oral administration of phosphatidylcholine by >100-fold.¹⁷ TMAO was detectable only with the re-establishment of the microbial community in the antibiotic-treated mice.¹⁷ In humans, serum TMAO was lower after antibiotic suppression even with a large dietary intake of L-carnitine.¹⁵ Similar to the

mice, detectable TMAO formation during the *L*-carnitine challenge had resumed after at least three weeks post-antibiotic treatment. In a different approach, the introduction of the choline analogue, 3,3-Dimethyl-1-butanol (DMB), in murine models decreased TMA lyase activity.³³ TMA lyase normally cleaves the carbon and nitrogen bond in choline using a glycine radical, but without the carbon and nitrogen bond in the analogue, lyase activity was disrupted and circulating TMAO levels decreased.³³ Together, the inhibition of TMA formation and the reduction of plasma TMAO through antibiotic suppression highlight the critical role gut microbes play in TMAO production.

Factors Affecting TMAO

TMAO concentrations can be influenced through diet, the gut microbiota, regulation of FMO3, genetics, glomerular filtration, and potentially, age (Fig. 2). These factors modulate substrate metabolism and availability, TMA to TMAO conversion, and the rate at which TMAO is removed from the body.

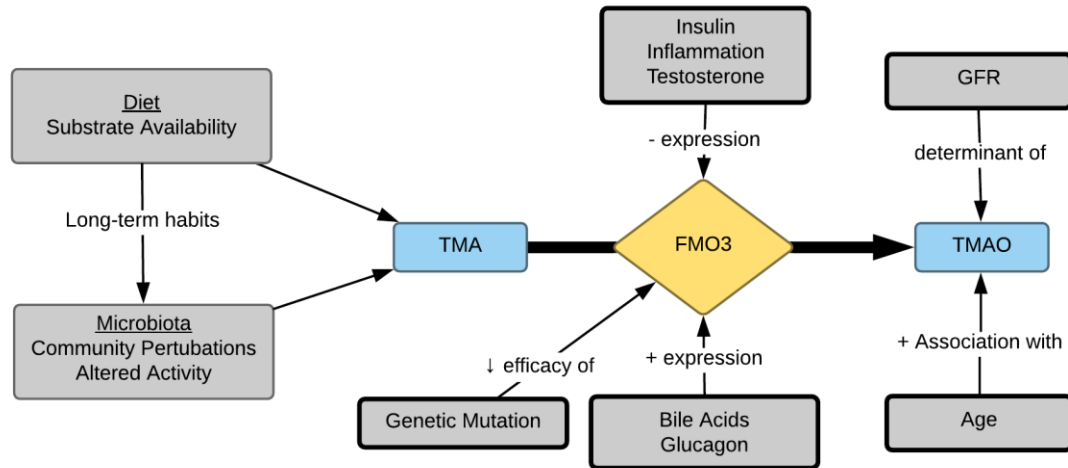


Figure 2. Simple schematic of factors that affect TMAO concentrations. Bolded boxes indicate factors that may have an influence on TMAO concentrations. Abbreviations: trimethylamine, TMA; flavin-containing monooxygenase 3, FMO3; trimethylamine-N-oxide, TMAO; glomerular filtration rate, GFR.

Diet

Changes to diet can impact TMA substrate availability and production for later hepatic oxidation. Increased TMA substrate availability from increased dietary intake of eggs, dairy, meat, poultry, and seafood can raise circulating TMAO concentrations.^{15,34} Mice on an 8-week Western diet, rich in TMA precursors such as *L*-carnitine and choline, had increased fasting plasma TMAO levels compared to mice on a normal diet.³⁵ Though the TMAO increase was reversed when DMB was introduced to mice.

Long-term dietary habits such as vegetarianism and veganism were also shown to have differences in TMAO production.¹⁵ Vegans demonstrated a negligible increase in TMAO after *L*-carnitine ingestion but increased labelled *L*-carnitine in plasma, compared to omnivores, where the fasting to peak change in labelled TMAO was approximately 14 μM .¹⁵ The stark difference in TMAO between dietary groups may, in part, be due to

altered microbial communities between vegans, vegetarians, and omnivores.^{36,37}

Negligible increases in plasma TMAO but large increases in the plasma concentrations of one TMAO precursor support a reduced capacity to metabolize of TMA precursors in vegans.

Microbial Perturbation

Altered microbial activity, such as through antibiotic treatment or through inhibited bacterial enzymes, can also affect TMAO levels even in the presence of increased substrate availability. By suppressing bacteria capable of forming TMA and blocking enzyme activity required for TMA formation, less TMA is available for absorption and subsequent oxidation by FMO3.^{15,17} Altered microbial activity can also be the result of microbial dysbiosis. Researchers have recently explored the possibility that fecal microbiota transplantation (FMT), defined as the “transfer of gut microbiota from a healthy donor to introduce or re-establish a stable microbial community in the gut,” may be able to help other health conditions besides *Clostridium difficile* infections.^{38,39} Surprisingly, a 2018 study found FMT from a vegan donor to metabolic syndrome patients did not alter the composition of intestinal microbiota, fasting plasma TMAO concentrations, or vascular inflammation.⁴⁰ The vegan-donor FMT did, however, increase urinary excretion of isotope-labelled TMAO. Thus, the influence of dysbiotic communities on TMAO production remains unclear.

FMO3 Regulation

Additionally, regulation of FMO3 can alter TMAO levels. Regulators of FMO3 include insulin, inflammation, testosterone, and bile acids.^{16,18,41} The oxidation of TMAO by FMO3 is downregulated by insulin, a key hormone in glucose uptake.¹⁶ Insulin downregulated FMO3 expression by 60% in rat hepatocytes, a process remediated by inhibiting a regulator of insulin signaling.¹⁶ Lipopolysaccharide (LPS)-stimulated inflammation was also shown to downregulate FMO3 by almost two-thirds.⁴¹ Testosterone downregulated FMO3 mRNA and decreased plasma TMAO. Upregulation of FMO3 and increased TMAO levels were observed with glucagon and dietary bile acids, important in glucose metabolism and dietary lipid absorption, respectively.¹⁸ Increased FMO3 expression was also found in morbidly obese adults compared to controls though the exact process for how the increase occurs is unknown.¹⁶ Greater FMO3 expression led to increased TMAO levels and has been recently suggested to have even broader metabolic effects such as increased hepatic glucose secretion and lipogenesis.⁴² Less FMO3 expression could lead to decreased TMAO production and potentially, the accumulation of TMA in the serum.

Genetics

Severe accumulation of TMA is also known as trimethylaminuria, a genetic disorder characterized by a strong fish odor in the breath, sweat, and urine. The condition is autosomal recessive and is caused by a mutation in the FMO3 gene.⁴³ The resulting folded enzyme has a partial or fully reduced ability to catalyze the oxidation of nitrogen

in TMA. As TMAO cannot be formed, TMA accumulates and is excreted from the body instead of TMAO, which is odorless.

Glomerular Filtration Rate

Glomerular filtration rate, or flow rate of blood through the glomerulus, is an excellent measure of overall kidney function. An estimate of GFR can be derived using levels of serum creatinine, an end product of creatine phosphate in muscle. Estimated GFR was found to be the most important determinant of TMAO from a multivariate analysis with age, gender, albumin, subjective global assessment, and diabetes mellitus status.³⁰ For every one unit increase in eGFR, TMAO would be expected to decrease by 0.414 $\mu\text{M/L}$ after accounting for the aforementioned variables.³⁰ Likewise, decreases in eGFR would increase TMAO. Estimated GFR was lowest (median 61 [45-82] ml/min/1.73 m²) in the highest TMAO tertile of a diabetic cohort.⁴⁴ Low GFR is indicative of kidney damage and precedes kidney failure, with a GFR below 60 ml/min/1.73 m² increasing the risk of CKD complications.^{45,46}

If TMAO production were to surpass ability to clear TMAO via urine, levels would expectedly rise. This is supported by a study examining TMAO accumulation in hemodialysis patients, where individuals with end stage renal disease (ESRD) had 40-fold higher plasma TMAO (2 ± 1 vs 77 ± 26 μM) and decreased TMAO urinary clearance (165 ± 72 vs 219 ± 78 ml/min) compared to healthy controls, even with dialysis.⁴⁷ Urinary clearance of TMAO was found to be higher (219 ± 78) in healthy adults with no known renal disease compared to clearance rates of creatinine (119 ± 21)

or urea (55 ± 14).⁴⁷ Previous research found that >95% of isotope labelled ingested TMA was excreted via urine as TMAO in the first 24 hours after TMA ingestion.⁴⁸

Age

Lastly, recent research has suggested that advancing age influences TMAO concentrations. A recent study reported higher TMAO concentrations in old Fischer rats compared to young rats.⁴⁹ The high TMAO concentrations in old rats were reduced to concentrations comparable to young rats with the introduction of DMB, the choline analogue. The rats also displayed impaired endothelial dysfunction that was ameliorated with the DMB treatment. Impaired arterial dilation is a characteristic of atherosclerotic arteries and endothelial dysfunction and is also greatly impacted by advancing age.⁵⁰ The decline in endothelial and arterial function with aging is related to increased expression of pro-inflammatory cytokines.⁵¹

TMAO and Inflammation

As there is evidence that inflammation downregulates FMO3, individuals with higher inflammation may have a different TMAO concentrations than those low inflammation. A study of CKD stage 3-5 patients found that TMAO concentrations in CKD patients were positively associated with IL-6 ($\rho = 0.42$, $p < 0.0001$) and high sensitivity C-Reactive Protein (hsCRP, $\rho = 0.17$, $p < 0.022$).³⁰ High levels of hsCRP, an acute phase protein secreted by the liver in response to inflammation, is often used as an indicator of systemic inflammation. Different TMAO concentrations were observed between inflamed (hsCRP ≥ 10 mg/L) and non-inflamed (hsCRP < 10 mg/L) CKD

patients with inflamed patients having higher TMAO ($p < 0.002$).³⁰ However, findings from later stage CKD patients are not generalizable to healthy overweight and obese individuals. Understanding the relationship between TMAO and inflammation in a more generalizable human population is required.

Researchers have examined the effects of direct TMAO administration in canine and murine models as well as human cell cultures. Local injection of TMAO into canine hearts increased expression of p65 nuclear factor - kappa beta (NF- κ β), IL-1β, IL-6, and TNF- α relative to controls.⁵² Administration of TMAO in mice also increased activation of major intracellular regulators, leading to increased nuclear abundance of p65 NF- κ β and numbers of cyclooxygenase 2 and IL-6.⁵³ Mice who had TMAO incorporated into a HFD had increased levels of serum monocyte chemoattractant protein-1 (MCP-1) relative to mice on control and HFD.⁵⁴ Elevated MCP-1 is of particular importance as this chemotactic cytokine regulates macrophage and monocyte infiltration into tissues.⁵⁵ Similarly, increased gene expression of pro-inflammatory compounds was also observed with TMAO-treated cultures of human endothelial and smooth muscle cells.⁵³ FMO3, not necessarily TMAO, has also been shown to influence inflammatory regulators in the liver. FMO3-knockout mice displayed evidence of hepatic inflammation, specifically, increased gene expression of Cd68, a macrophage marker.⁴² There is building evidence demonstrating TMAO promotes inflammation. More research *in vivo* would prove helpful in understanding the effect of natural TMAO production on inflammatory pathways.

Limited research has examined the influence of inflammation on systemic TMAO. One study examined effects of *Citrobacter rodentium* infection and LPS injection on FMO3 expression.⁴¹ *C. rodentium* infection and LPS were separately used to induce inflammation in mice. Both treatments decreased FMO3 expression, with the largest decrease in FMO3 expression seen with *C. rodentium*. Evidence thus far suggests that inflammation decreases TMAO levels by downregulating FMO3. Differences in inflammation models may stem from infection-induced inflammation being independent of toll-like receptor (TLR) 4 and LPS-induced inflammation being TLR4-dependent.⁴¹

Post-prandial TMAO response

The post-prandial TMAO response in healthy adult men and women has been studied by Koeth and colleagues in response to a high *L*-carnitine dietary load.¹⁵ The *L*-carnitine challenge consisted of a pill of isotope-labelled *L*-carnitine via pill (250 mg) and for a subset of the male and female participants, the pill (250 mg) plus an 8-oz. steak (180 mg). Modest changes in unlabeled *L*-carnitine and TMAO occurred after the meal, which researchers described as being due to the large total pool of *L*-carnitine and TMAO in the body relative to the amount. New TMAO formation detectably increased after concomitant *L*-carnitine ingestion but did not return to baseline concentrations after 24 hours. Additionally, the post-prandial TMAO response was markedly reduced in vegans and vegetarians versus omnivorous subjects no matter the *L*-carnitine load ingested. To what extent their reported post-prandial responses are due to challenge is not clear as

methodology does not mention if participants received additional meals within the 24-hour period.

The TMAO response to ingestion of a variety of animal sourced foods has also been studied in healthy young men.³⁴ Cho and colleagues conducted a crossover feeding trial with foods rich in TMAO and its dietary precursors and examined TMAO concentrations before and hourly for 6 hours after meal ingestion. Post-prandial plasma TMAO concentrations were 46-62 times greater with a TMAO-rich meal (fish) compared to the choline-rich (egg), carnitine-rich (beef), and control meal (fruit). They additionally found that serum TMAO increased 15 minutes after ingestion of fish suggesting that the absorption of TMAO into circulation can occur without gut microbial metabolism. These findings support the influence of meal choice on circulating TMAO.

Two studies have examined the post-prandial TMAO response after a high-fat meal.^{56,57} Boutagy and colleagues examined the 4-hour post-prandial TMAO response to a 63% fat, 850 kcal meal (two sausage, egg, and cheese breakfast biscuits) in 10 healthy, non-obese men before and after a 5-day high-fat diet (HFD).⁵⁶ The HFD did not affect fasting TMAO concentrations but did increase the post-prandial TMAO response. TMAO concentrations gradually increased from baseline with hour 4 having the highest concentration ($2.51 \pm 0.33 \mu\text{mol/L}$). Though there was a lack of evidence for a high-fat meal effect on post-prandial TMAO response before HFD, it was worth noting that TMAO peaked at hour 2 ($1.8 \pm 0.32 \mu\text{mol/L}$) then declined. Baugh and colleagues also examined the 4-hour post-prandial TMAO response to the same 63% fat meal but in 11 overweight and obese men and women before and after a 6-week standard diet (30%

fat).⁵⁷ The diet did not affect fasting or post-prandial TMAO levels. Taken together, a HFD may result in higher TMAO levels in the post-prandial period compared to a standard diet. Larger sample sizes that also include women are required to better examine the post-prandial TMAO response.

Summary

TMAO is a gut-derived compound that has recently come into the spotlight as a potential risk factor for CVD. The evidence thus far in humans suggests TMAO is positively associated with inflammatory markers. Inflammation, insulin, and testosterone has been shown to influence TMAO concentrations by regulating FMO3, the predominant liver enzyme that converts TMA to TMAO. Research in the post-prandial TMAO response, particularly to a high-fat meal, has not accounted for changes in insulin, potential sex differences, age, or how the response may differ between individuals with different levels of inflammation. These factors should be considered in examining the post-prandial response as they have been implicated in the regulation of TMAO production.

CHAPTER THREE

METHODS

Subject Characteristics

A convenience sampling observational cohort was studied, with 40 healthy overweight and obese men and women recruited for study. Inclusion criteria include: 18-55 years old, BMI 27-35 kg/m². Exclusion criteria included antibiotic treatment within the past 90 days, regular use of anti-inflammatory medications, use of estrogen-only contraceptive, presence of wheat or dairy allergies, presence of heart disease, pregnancy, and the presence of any musculoskeletal, cardiovascular, GI, or immunological condition that could interfere with the study.

Experimental Design

The study was a comparative research design within a single Montana community and was conducted from March 2016 to June 2018. The Montana State University Institutional Review Board approved this study (#MM021116-FC). The study consisted of two visits to the MSU Nutrition Research Laboratory (NRL) for all participants. Scheduling the first visit occurred upon confirmation of eligibility. The first visit involved a verbal review of the informed consent, completion of three one-page self-report questionnaires, and analysis of body composition and cardiorespiratory fitness. The second visit occurred within two weeks after the first visit involved blood collection

before and after a meal, outlined in Figure 3. The total time commitment for each participant was approximately seven hours.

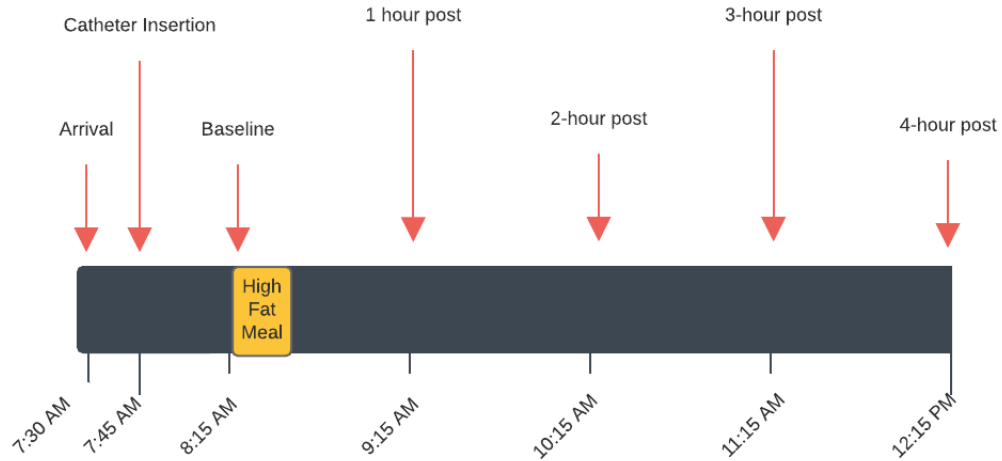


Figure 3. Approximate timeline of blood collection times for fasted participants during the high-fat meal challenge. Collection occurred immediately before the high-fat meal challenge and on the hour, every hour for four hours after they started their high-fat meal.

Recruitment

Subjects were recruited through word of mouth, e-mail, and flyers (Appendix A) posted on the MSU campus and within predetermined locations in the community. Interested participants contacted researchers at the MSU Nutrition Research Laboratory. A telephone screening was conducted by the researchers to determine eligibility. All interested participants were verbally asked about each inclusion and exclusion criteria. Participants were also asked about supplement use (i.e., vitamins, herbs, probiotics) to which their responses were recorded in detail. The purpose of the research, its risks, and

benefits were verbally described to all participants before they gave their written informed consent.

Body Composition

Body composition data were collected from the first visit and was determined through bioelectrical impedance analysis (BIA). The SECA mBCA 515 (Hamburg, Germany) was used to perform the BIA and was previously proven as a valid tool to analyze body composition.⁵⁸ Specifically, data retrieved from the BIA included participant fat mass (%) and estimated volume of visceral adipose tissue (l).

Cardiorespiratory Fitness

All participants were also asked to complete a modified Bruce protocol on a treadmill for determination of a calculated absolute VO₂ max at their age-predicted heart rate max in their first visit. The speed and grade of the treadmill (Woodway GmbH D-79576, Weil am Rhein, Denmark) were manually changed with each progressive three-minute stage until the participant reached 85% of their age-predicted maximal heart rate. More information on the modified Bruce protocol used for this study can be found in Appendix B. Expired gases were collected for analysis via a metabolic cart (ParvoMedics TrueMax 2400 Metabolic System, Sandy, Utah, USA). Heart rate data was also collected during the test using the Polar Wearlink heart rate monitor (Kempele, Finland) and sent wirelessly to the metabolic cart. Heart rate (bpm) and VO₂ (ml/kg/min) data from each participant were input into a simple linear regression model to predict the absolute VO₂ at

the age-predicted maximal heart rate based on the equation presented by Tanaka, Monahan, and Seals.⁵⁹

High-Fat Meal Challenge

The second visit involved baseline blood collection in the morning after an overnight fast, and subsequent blood sampling after the ingestion of a high-fat meal. The high-fat meal contained salted butter (58.3 g; Tillamook) over three pieces of whole wheat toast (127.5 g; Wheat Montana). The total energy content of the meal was 714 kcal, with approximately 43.1% from fat. This corresponds to 50 g fat, 54 g carbohydrate, 12 g protein. Water was also provided with the meal; caffeinated black tea (Bigelow) was provided instead for participants who habitually consume coffee.

Blood samples were obtained from all participants at the following time points during their second visit to the lab: immediately before ingestion of a high-fat meal and 1, 2, 3, 4-hr after the time they began their meal. During the second visit, subjects expended little energy between blood draws such as reading a book or working on a computer.

Venous blood was collected from a cannula inserted into the antecubital vein by a physician or nurse on the research team. Blood was collected into 8.5 mL endotoxin-free serum separating and heparinized vacutainer tubes (BD Vacutainer, Franklin Lakes, New Jersey, USA). The serum tube was allowed to clot for 15 minutes at room temperature before centrifugation (3000 rpm, 15 min). The resulting serum was aliquoted and frozen at -80°C until analysis for creatinine, CRP, insulin, inflammatory cytokines, and TMAO.

Lipid, Glucose, and Liver Enzyme Analysis

Whole blood collected in heparinized blood collection tubes (BD Vacutainer) was immediately analyzed using Picollo Lipid Panel Plus discs (Abaxis, Union City, California, USA) on the Picollo Xpress Chemistry Analyzer (Abaxis) for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (CHOL), high density lipoprotein (HDL), triglyceride (TG), and glucose (GLU), all with units micrograms per deciliter. Presence of enzymes ALT and AST in serum can be indicative of hepatocellular damage; the ratio of AST to ALT is often used to determine alcoholic liver disease from nonalcoholic fatty liver disease (NAFLD).⁶⁰

Creatinine and eGFR

Serum was run on Picollo MetLyte Plus CRP discs to determine fasting creatinine levels (mg/dL). Serum creatinine, age, gender, and race were input into the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation to estimate glomerular filtration rate (eGFR), an indicator of renal function.⁶¹ This study utilized the CKD-EPI equation as it provides better accuracy of eGFR values compared to other eGFR equations, such as the Modification of Diet in Renal Disease (MDRD) Study equation, especially at higher values.⁶¹ Considering that the median eGFR for this cohort of overweight and obese adults was 8.3 ml/min/1.73 m² more than the National Health and Nutrition Examination Survey (NHANES) median (94.5 ml/min/1.73 m²), the CKD-EPI

equation was appropriate for GFR estimation. The CKD-EPI equation is as follows:

$$GFR = 141 \times \min\left(\frac{Scr}{\kappa}, 1\right)^\alpha \times \max\left(\frac{Scr}{\kappa}, 1\right)^{-1.209} \times 0.993^{Age} \times 1.018[\text{if female}] \\ \times 1.159[\text{if black}]$$

where Scr is serum creatinine level, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, and min and max respectively represent the minimum or maximum of Scr/ κ or 1.⁶¹ The eGFR is on the natural scale with units mL/min/1.73 m².

High sensitivity CRP

Fasting serum concentrations of hsCRP (mg/mL) were determined using the high sensitivity CRP ELISA Kit (MP Biomedicals, Solon, OH). Procedures for CRP were performed according to manufacturer instructions. All samples were tested in duplicate wells within the same run of the assay, with the mean used for statistical analysis. The mean intra-assay coefficient of variation was 8.1%.

Insulin

Insulin levels (μ IU/mL) in blood serum at each time point were determined using the Insulin ELISA Kit (MP Biomedicals, Solon, OH). Insulin ELISA procedures were performed according to manufacturer instructions. All samples were tested in duplicate wells, with the mean used for statistical analysis. Mean intra-assay coefficient of variation was 15.0%.

Inflammation Phenotyping

Blood serum was analyzed for concentrations of inflammatory cytokines (Bio-Rad Bio-Plex 200 HTS) using high-sensitivity Luminex multiplexing as prepared by Millipore. Selected inflammatory cytokines to be detected include IL-1 β , IL-6, TNF- α , granulocyte macrophage colony-stimulating factor (GM-CSF), IL-17A, and IL-23, all with units picograms per deciliter. All serum samples were run in duplicate with the mean used for analysis. Mean intra-assay coefficients of variation for Luminex multiplexing were 9.8, 13.1, 11.5, 13.7, 10.9, and 10.9% for IL-1 β , IL-6, TNF- α , GM-CSF, IL-17A, and IL-23, respectively.

The multi-cytokine approach was used instead of hsCRP to determine systemic inflammation. Participants were deemed high (HI) inflammation if they were above the group median at baseline in four or more inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-17A, IL-23, and GM-CSF), and low (LO) inflammation if above the group median at baseline in two or less cytokines. Individuals high in specifically three cytokines are designated neither LO nor HI and were removed for analysis between inflammation phenotypes. Values above the group median were considered high.

TMAO

Frozen serum samples were delivered to the Bothner Lab on MSU campus for determination of TMAO concentrations. When ready for analysis, metabolite fractions were extracted via a protein precipitation using methanol followed by metabolite fraction concentration. Samples were then analyzed by liquid chromatography mass spectrometry

(LCMS). Analysis was completed on an Agilent 6538 quadrupole-time of flight (Q-TOF) mass spectrometer in positive mode in conjunction with an Agilent 1290 ultra-high performance liquid chromatography (UHPLC) system using a BEH-HILIC normal phase column. Acetonitrile with 0.1% formic acid was used as mobile phase B while water with 0.1% formic acid was used as mobile phase A in a 6-minute method with a linear gradient from 60% to 90% B.

TMAO and TMA standards were also obtained and were used to create standard solutions with concentrations of 1 nM, 10 nM, 100 nM, 1 μ M, 5 μ M, 10 μ M, 50 μ M and 100 μ M. Standard solutions were analyzed by LCMS and standard curves were created. LCMS analysis occurred within 24 hours of extraction and all samples and standards were analyzed concurrently. Post-experimental quantification of TMA and TMAO was completed using MassHunter to determine peak area. Peak area was then extrapolated using the standard curves to determine concentration (μ Mol).

Statistical Procedures

Statistical analyses were performed in RStudio (R version 3.4.3). Descriptive statistics of the participants' physical characteristics, metabolic profile, and inflammation markers by inflammation phenotype were performed with the Welch two sample t-test when variance was not equal or the two sample t-test when variance was equal. The insulin response over time by phenotype and accounting for subject was examined using a general linear model and analyzed using a Type II F-test.

Beanplots of the distribution of serum TMAO were plotted by inflammation phenotype at each time point.⁶² Serum TMAO concentrations over time were grouped by participant and faceted by inflammation phenotype using ggplot.^{63,64} Non-normality and low sample size warranted further investigation of baseline TMAO in participants. The log of TMAO was additionally evaluated with a permutation test for independent samples. Differences in fasting TMAO concentrations were also examined between inflamed and non-inflamed classifications based off of hsCRP values using the Welch two-sample t-test.³⁰

As diets were not controlled leading up to blood collection, a non-linear mixed-effects model was used to examine differences in the TMAO response after a high-fat meal challenge between inflammation phenotypes and accounting for age, AST/ALT ratio, sex, eGFR, insulin over time, and subject-to-subject variation in serum TMAO.⁶⁵ The initial model to examine fasting and post-prandial TMAO concentrations by inflammation phenotype was created with TMAO as the response (outcome variable), random effects on subject as a factor, fixed effects on age, AST/ALT ratio, sex, eGFR, a two-way interaction between inflammation phenotype (LO, HI) and a quartic polynomial on time, and a two-way interaction between insulin and a quartic polynomial on time. Fixed and random effects were predictor variables. The quartic polynomial was determined to be the highest estimable polynomial order. The TMAO response was log transformed to improve the variance and normality of standardized residuals in the initial model. The five time points from 35 overweight and obese adults who classified as LO or HI inflammation were included in analysis. A total of three insulin concentrations were

missing due to high coefficients of variation, one timepoint each from three participants, bringing the total number of observations to 172.

Model refinement was performed by stepping down the polynomial degree one step at a time from the initial model. Main effects were dropped if there was weak evidence to support inclusion, but the interaction between time and phenotype was maintained during refinement as it relates to the research question. Model reduction was determined off the strength of evidence against the null hypothesis using Type III F-tests, in which every test is conditional on every variable in the model.⁶⁶ Model refinement was stopped when the phenotype by time interaction reached the smallest p-value. Estimates of R-squared for the refined model were generated using the `r.squaredGLMM` function in the MuMin package.⁶⁷

CHAPTER FOUR

RESULTS

Subject Characteristics

Forty subjects, 24 women and 16 men, successfully completed the full protocol and were included in data analysis. Participants were 21 to 55 years of age with a mean (SD) age of 36.4 (10.1) years and BMI of 30.5 (2.0) kg/m².

Inflammation

After phenotype classification, 19 participants were designated as LO inflammation and 16 as HI inflammation. Five participants did not classify as LO or HI and were not included in phenotype comparisons or the mixed-effects modelling. Participant characteristics by LO and HI inflammation phenotypes are reported in Table 1. There was little to no evidence to suggest a difference in age, BMI, body composition, cardiorespiratory fitness, and fasting serum metabolic markers between LO and HI inflammation phenotypes. However, there was very strong evidence supporting a difference between LO and HI inflammation phenotypes in five cytokines: IL-1 β , IL-6, IL-17A, IL-23, and TNF- α with the HI inflammation phenotype having higher cytokine concentrations (Table 2). Distribution of all six fasting cytokines by inflammation phenotype can be seen in Figure 4.

Table 1. Physical characteristics and fasting metabolic profile by inflammation phenotype.

	LO (n=19)	HI (n=16)	p-value
Number of women	12	9	NA
Age (years)	36.4 ± 10.5	35.3 ± 10.3	0.75
BMI (kg/m ²)	30.3 ± 1.9	30.6 ± 2.0	0.66
FM (%)	35.0 ± 5.9	36.7 ± 7.8	0.47
VAT (l)	2.1 ± 1.3	2.2 ± 1.1	0.79
VO ₂ max (ml/kg/min) ¹	42.8 ± 8.7	41.7 ± 10.5	0.74
Glucose (mg/dl)	96.2 ± 6.2	98.6 ± 7.5	0.31
Serum triglycerides (mg/dl)	130.5 ± 87.2	151.1 ± 84.1	0.49
HDL cholesterol (mg/dl)	53.7 ± 14.5	49.2 ± 19.1	0.46
Total cholesterol (mg/dl)	182.9 ± 31.0	177.8 ± 29.1	0.61

Data represent mean and standard deviation. P-values were determined by a two sample t-test. Abbreviations: low inflammation phenotype, LO; high inflammation phenotype, HI; body mass index, BMI; fat mass, fat mass; visceral adipose tissue, VAT; maximal oxygen consumption, VO₂ max; high density lipoprotein, HDL.

¹ indicates participant values were calculated. The VO₂ max data is calculated based off a submaximal treadmill test. See methods for more details.

Table 2. Concentrations of six pro-inflammatory inflammation markers by inflammation phenotype.

	LO	HI	p-value
GM-CSF	36.2 (26.2 – 118.1)	120.0 (51.0 – 265.1)	0.049
IL-1β	1.5 (1.2 – 2.2)	3.4 (2.7 – 4.2)	< 0.001
IL-6	2.3 (1.3 – 2.9)	5.7 (4.7 – 7.7)	<0.001
IL-17A	11.0 (9.4 – 14.8)	22.7 (21.3 – 32.7)	<0.001
IL-23	347.4 (211.4 – 434.7)	1160.0 (679.3 – 1682.3)	< 0.001
TNF-α	6.0 (4.2 – 7.9)	10.1 (8.7 – 11.5)	< 0.001

Data represent median and 25th and 75th interquartile range (low – high). All values are reported in picograms per milliliter. P-values were determined by a two sample t-test. Abbreviations: low inflammation phenotype, LO; high inflammation phenotype, HI; granulocyte macrophage colony-stimulating factor, GM-CSF; Interleukin, IL; tumor necrosis factor, TNF.

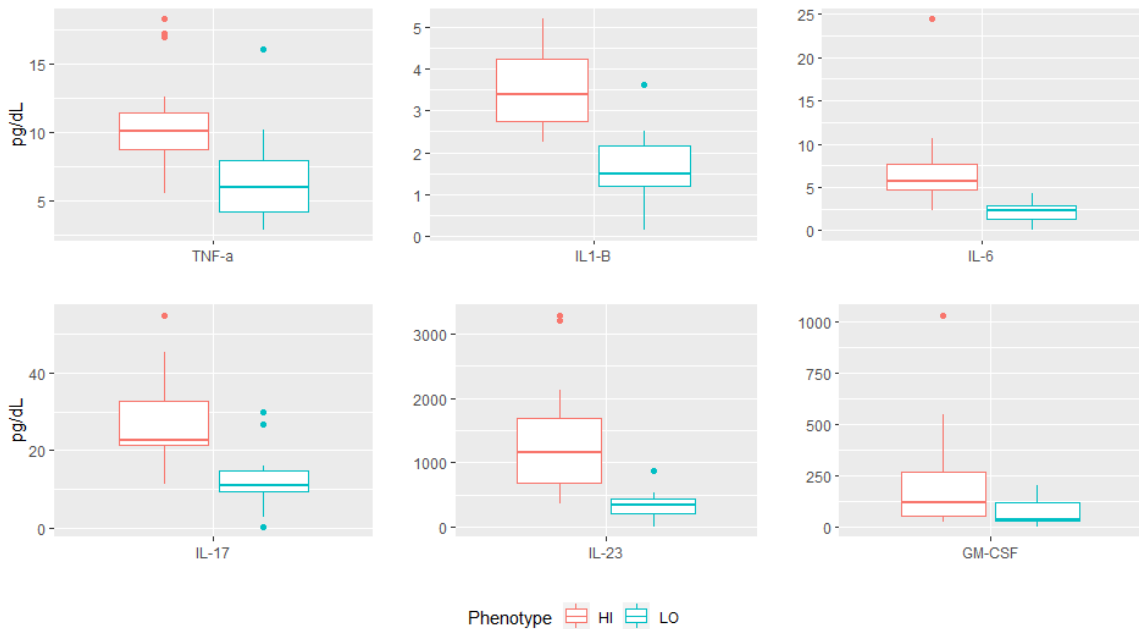


Figure 4. Boxplots of fasting serum inflammation markers by inflammation phenotype. Sixteen individuals classified as HI inflammation phenotype and nineteen classified as LO inflammation phenotype. Note scaling differences in the y-axis for each cytokine. Abbreviations: granulocyte macrophage colony-stimulating factor, GM-CSF; Interleukin, IL; tumor necrosis factor, TNF.

Insulin

Insulin responses before and after the high-fat meal grouped by inflammation phenotype are represented in Figure 5. Serum insulin peaked one-hour after meal ingestion in most participants. Insulin concentrations did not differ by phenotype by time after accounting for subject ($F(4,129) = 0.3301$, $p = 0.857$).

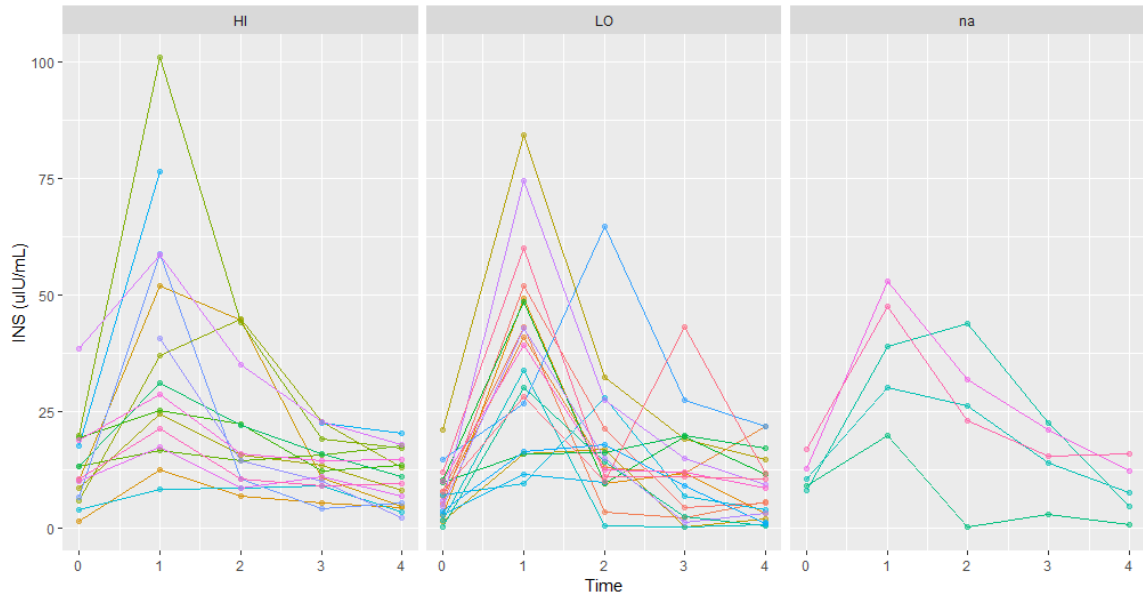


Figure 5. Serum insulin concentrations before and after ingestion of the high-fat meal challenge, grouped by inflammation phenotype. Two HI and one LO individual each had one missing insulin value. Abbreviations: high inflammation phenotype, HI; low inflammation phenotype, LO; na, did not classify.

Creatinine and eGFR

Serum creatinine concentrations were used in conjunction with age, gender, and race to estimate GFR. Creatinine was not different between inflammation phenotypes ($t_{0.435} = 29.3$, $p = 0.667$). Additionally, there was little to no evidence to suggest that eGFR was different between phenotypes ($t_{0.718} = 28.3$, $p = 0.477$). Creatinine concentrations and eGFR by phenotype are represented in Figure 6.

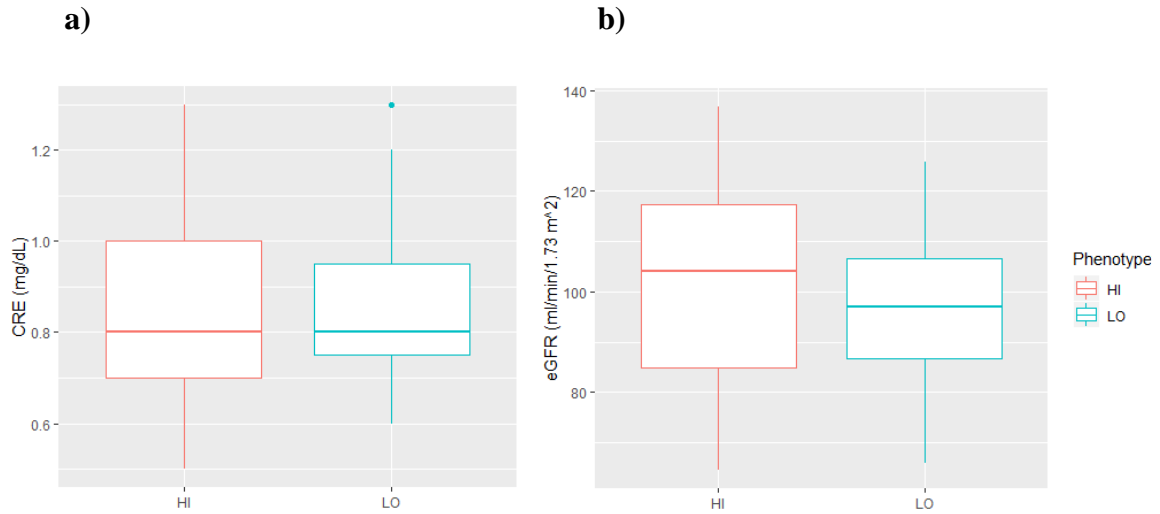


Figure 6. Fasting serum a) creatinine (CRE) and b) estimated glomerular filtration rate (eGFR) by inflammation phenotype. Abbreviations: high inflammation phenotype, HI; low inflammation phenotype, LO.

hsCRP and Fasting TMAO

Serum concentrations of hsCRP were assessed to examine inflammation in participants. Serum hsCRP was not different between LO (2.82 ± 2.95 mg/L) and HI (3.93 ± 3.34 mg/L) inflammation phenotypes ($t_{-1.031} = 30.3$, $p = 0.311$).

Levels of hsCRP were grouped into inflamed ($\text{hsCRP} \geq 10$ mg/L) and non-inflamed ($\text{hsCRP} < 10$ mg/L) groups based off criteria originally presented by Missailidis and colleagues.³⁰ Only two participants had a large enough hsCRP value to group them as inflamed. There was little to no evidence to suggest a difference in fasting TMAO concentrations between inflamed and non-inflamed groups ($t_{-0.280} = 1141$, $p = 0.822$).

Post-prandial TMAO

The majority of fasting and post-prandial TMAO concentrations were below 10 μMol (Fig. 7). Several participants had relatively high ($>10 \mu\text{Mol}$) fasting and post-prandial serum TMAO. A permutational test found fasting log TMAO was not different between inflammation phenotypes ($Z = -0.055$, $p = 0.96$). Mean serum TMAO was generally lower in LO versus HI participants at each time point (Fig. 8a), but there was little to no statistical evidence supporting a difference between phenotypes (Table 3). The right-hand skewed distributions of TMAO did indicate that TMAO was a good candidate for the log transformation for the mixed-effects model (Fig. 8b).

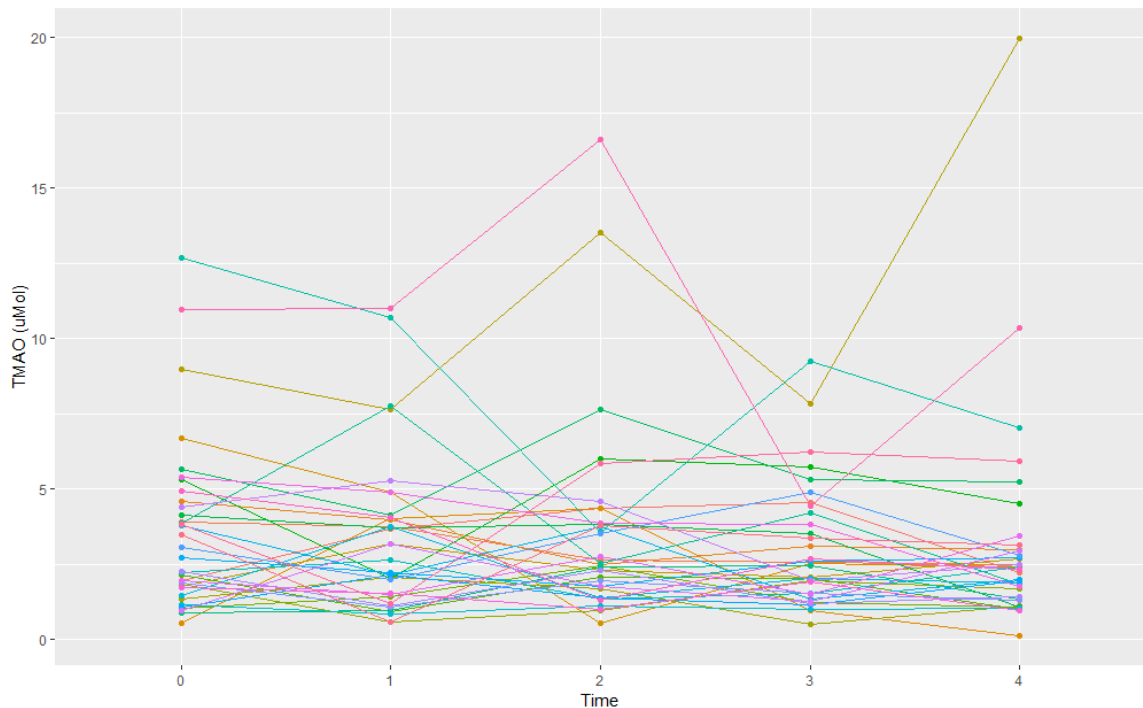


Figure 7. Serum TMAO concentrations by participant before and after ingestion of the high-fat meal challenge. Each colored line represents data from one participant. Time is in hours, with zero representing the fasting, or pre-meal, time point.

Table 3. Fasting and post-prandial TMAO concentrations by inflammation phenotype.

	LO	HI	<i>p</i>-value
0 (Fasting)	3.7 ± 3.0	3.4 ± 2.6	0.80
1-hr post	3.6 ± 2.4	3.1 ± 2.9	0.58
2-hr post	3.8 ± 3.5	3.3 ± 3.8	0.66
3-hr post	3.3 ± 2.4	2.6 ± 1.4	0.27
4-hr post	3.7 ± 4.3	2.5 ± 2.3	0.30

Data represent mean and standard deviation. All values are reported in μMol . P-values were determined by a two sample t-test. Abbreviations: low inflammation phenotype, LO; high inflammation phenotype, HI.

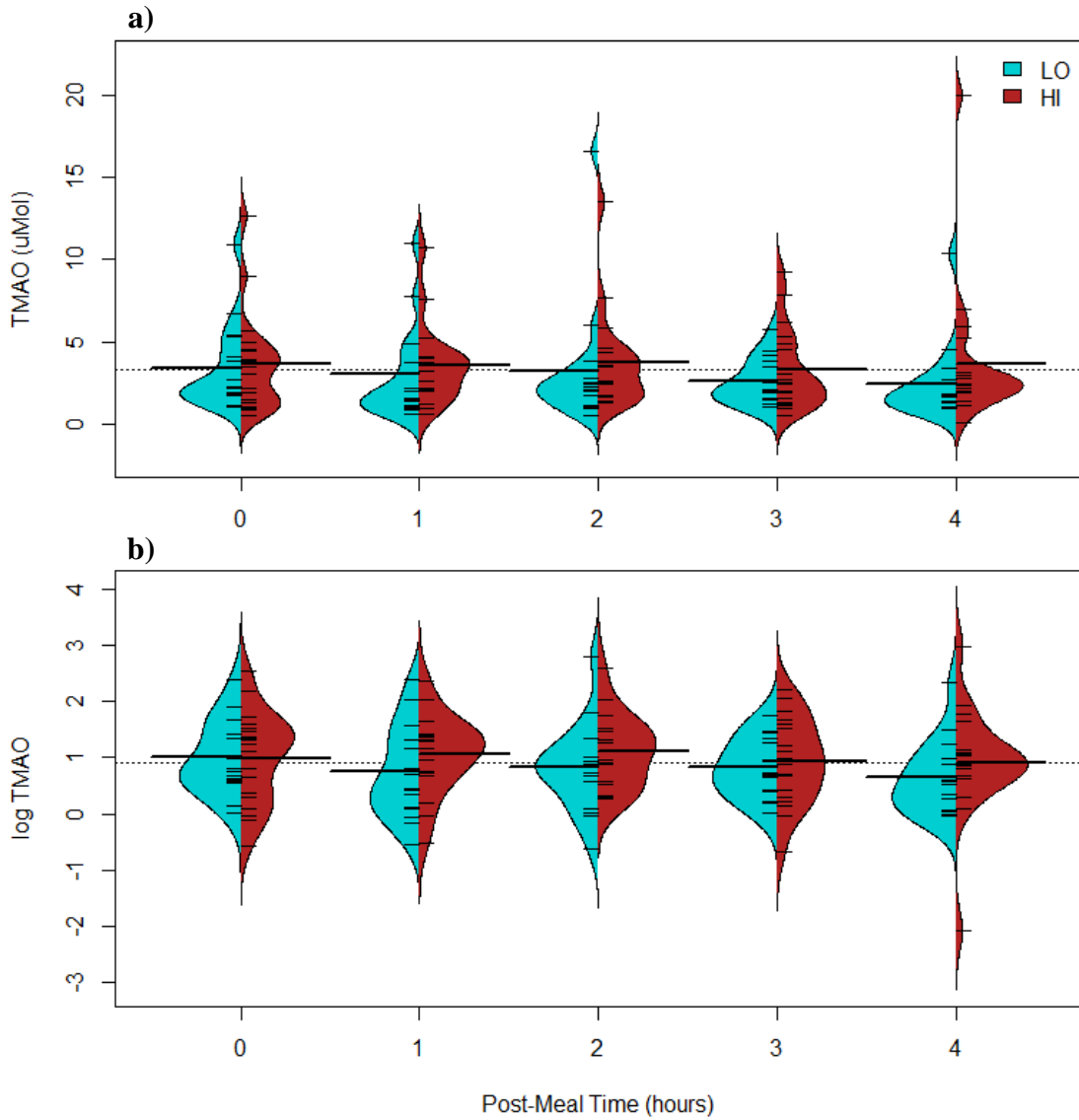


Figure 8. Beanplot depicting distributions of a) unlogged and b) logged serum TMAO concentrations before and after the high-fat meal challenge. The dashed horizontal line across all timepoints indicates the overall mean TMAO concentration. The solid horizontal line is the mean TMAO concentration for each time by phenotype combination. Abbreviations: high inflammation phenotype, HI; low inflammation phenotype, LO.

The initial mixed-effects model was designed to examine the log TMAO response during the high-fat meal challenge, accounting for interindividual variability and other variables that may influence serum TMAO. Age, gender, the quartic polynomial, the cubic polynomial, AST/ALT ratio, and quadratic polynomial were sequentially removed one at a time from the initial model based off the strength of evidence. The final theoretical model is as follows:

$$\log(TMAO) = \beta_0 + \beta_1 \text{Time}_{ij} + \beta_2 I_{\text{Phenotype=HI},i} + \beta_3 \text{INS}_{ij} + \beta_4 \text{eGFR}_i + \beta_5 I_{\text{Phenotype=HI},i} \text{Time}_{ij} + \beta_6 \text{INS}_{ij} \text{Time}_{ij} + \text{Subject}_i + \varepsilon_{ij}$$

where i is subject, j is the j th observation on i th subject, $I_{\text{Phenotype=HI}}=1$ for HI inflammation phenotype and 0 for LO inflammation phenotype, INS is insulin, subject $\sim N(0, \sigma^2_{\text{sub}})$, and $\varepsilon_{ij} \sim N(0, \sigma^2_{\varepsilon})$. Table 4 provides a summary of the refined final model.

Table 4. Summary of the final mixed-effects model for the logged TMAO response.

	Estimate	SE	DF	t-value	p-value
(Intercept)	2.056	0.512	133	4.018	<0.001
Time	-1.341	0.900	133	-1.491	0.138
Phenotype, HI	-0.150	0.191	32	-0.785	0.438
INS	0.005	0.004	133	1.272	0.205
eGFR	-0.011	0.005	32	-2.225	0.033
Time*Phenotype, HI	-1.028	1.053	133	-0.977	0.331
Time*INS	0.096	0.061	133	1.570	0.119

Abbreviations: standard error, SE; degrees of freedom, df; high inflammation, HI; insulin, INS; estimated glomerular filtration rate, eGFR.

Model refinement resulted with an interaction between inflammation phenotype and time as linear. In Figure 9a, log TMAO increased during the post-prandial period from baseline in LO participants, while HI participants had decreased log TMAO over time. However, there was little to no evidence of a difference in log serum TMAO

concentration during the high-fat meal challenge between LO and HI inflammation phenotypes, controlling for other aspects of the model ($F(1,133) = 0.953$, $p = 0.331$).

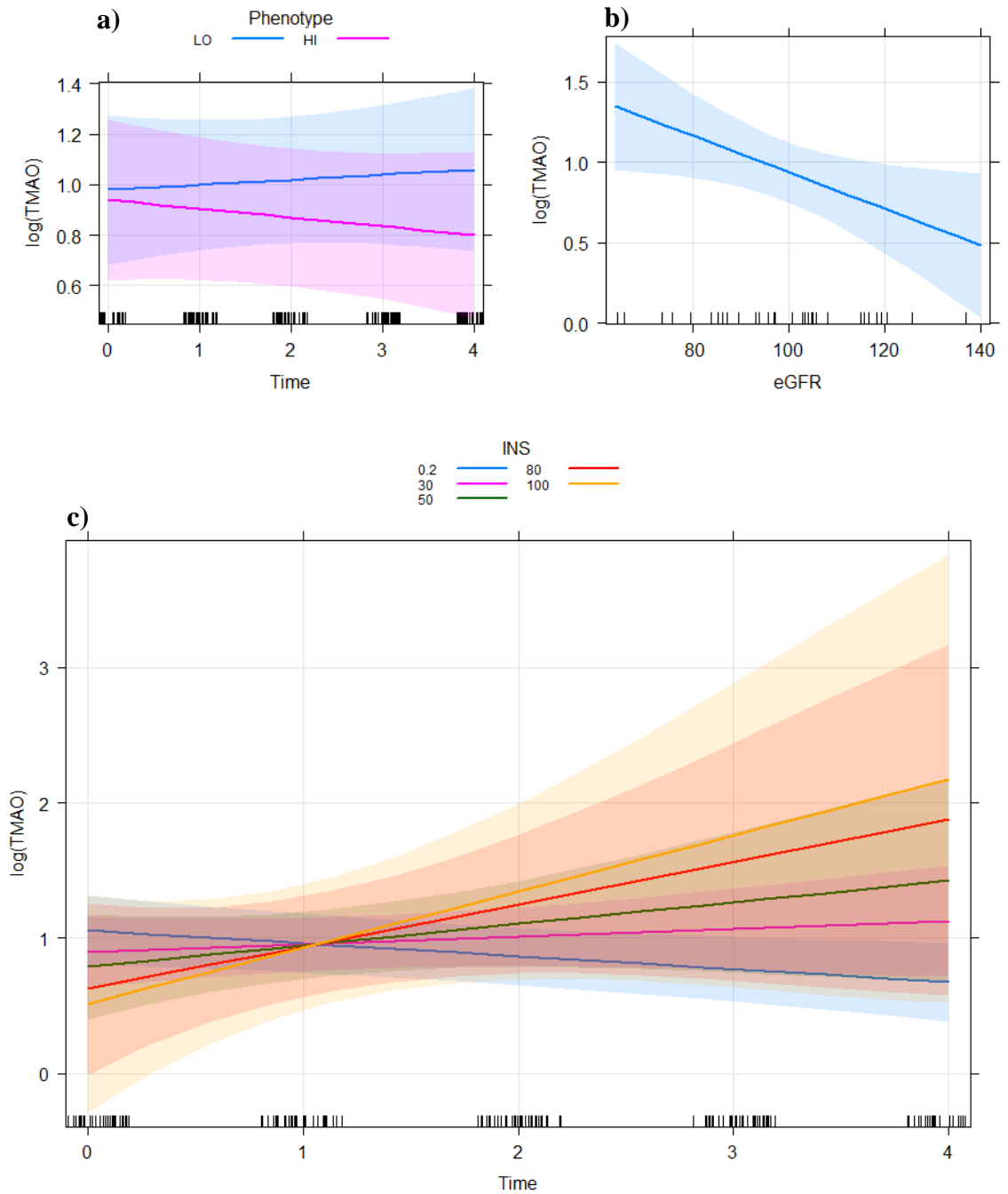


Figure 9. Effects plots of a) eGFR b) Phenotype and time interaction and c) Insulin (INS) and time interaction. Abbreviations: high inflammation phenotype, HI; low inflammation phenotype, LO; trimethylamine-*N*-oxide, TMAO

Model refinement supported inclusion of eGFR in the model. There is strong evidence supporting the contribution of eGFR to log TMAO, after accounting for interindividual variability, phenotype by time interaction, and insulin by time interaction ($F(1,32) = 4.949, p = 0.033$). The decrease in log TMAO with the rise of eGFR can be seen in Figure 9b. Log TMAO is estimated to be, on average, 0.011 units less, for every 1 ml/min/1.73 m² rise in eGFR (95% CI: -0.022 to -0.001, $t_{32} = -2.225, p = 0.033$).

The interaction between time and insulin was included in the final model. There is some evidence to suggest insulin concentrations over time contribute to the log TMAO response, after accounting for interindividual variability of the response and other fixed effects in the model ($F(1,133) = 2.466, p = 0.119$). Participants who had higher post-prandial insulin concentrations had increased log TMAO over time than participants who had insulin concentrations below 30 $\mu\text{IU/mL}$ (Fig. 9b). Log TMAO actually decreased in participants with lowest grouping of insulin concentrations.

The estimated correlation of two log TMAO concentrations on the same subject after accounting for subject-to-subject variation, eGFR, insulin over time, phenotype by time, and all underlying main effects, is 0.49. The two R-squared estimates, one showing variance explained by fixed effects (marginal) and then fixed and random effects (conditional), are 0.109 and 0.548, respectively.

Diagnostic plots of the final model were generated and used to examine model assumptions. Random effects distribution has a right-hand skew from the normal distribution in the quantile-quantile plot of random effects (Fig. 10a). Left-hand skew in the normal plot for residuals indicates there are extreme values outside of a normal

distribution (Fig. 10b). Constant variance is fair given the even spread of standardized residuals in the residuals versus fitted plot (Fig. 10c). Additionally, the linearity assumption appears to be met (Fig. 10c). As blood draws are not prone to shared dependency, the independence assumption is not violated.

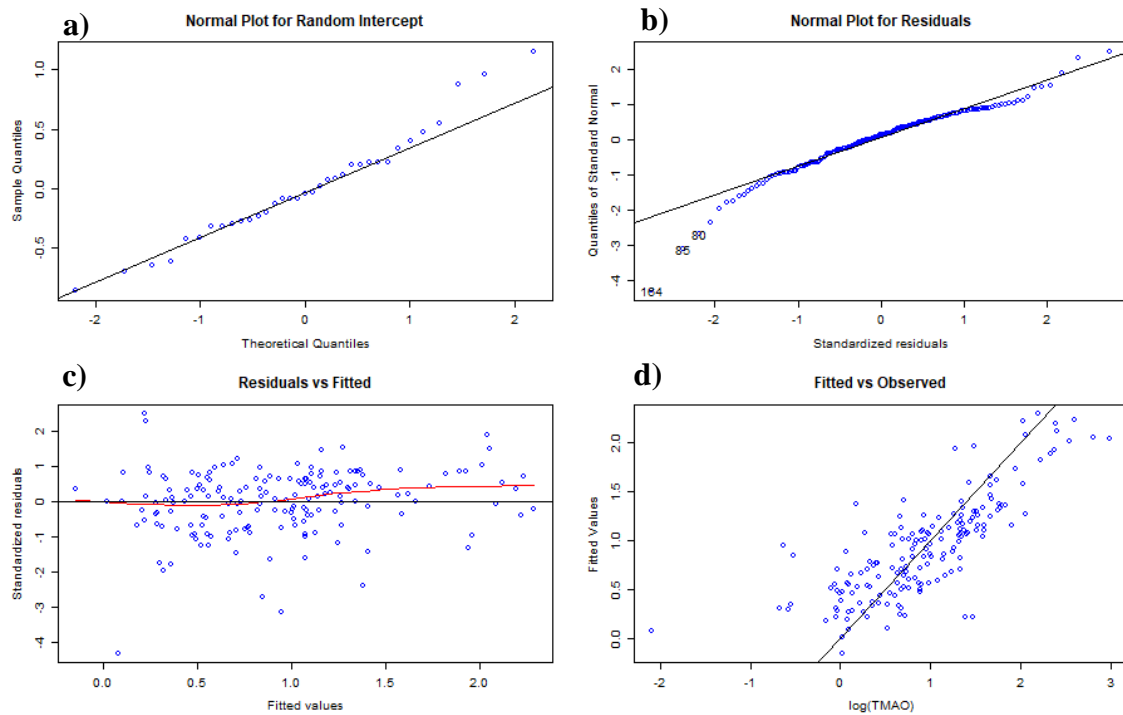


Figure 10. Series of diagnostic plots for the final mixed-effects model on the logged TMAO response.

CHAPTER FIVE

DISCUSSION

Introduction

The purpose of this study was to investigate how post-prandial serum TMAO concentrations may be influenced by host inflammation status and changes in insulin concentrations after a high-fat meal in overweight and obese adults. It was hypothesized that the serum TMAO response to a high-fat meal would differ between LO and HI inflammation phenotypes and that insulin was a key factor in this difference in response. An initial mixed effects model to examine the logged TMAO response was built to accommodate factors that may influence TMAO and refined based off the strength of evidence. Having higher baseline levels of inflammation was not shown to be an important determinant of the logged TMAO in the post-prandial period, accounting for subject-to-subject variation in TMAO, insulin over time, and the phenotype by time interaction. However, there was moderate evidence to suggest that insulin concentrations were an important determinant in post-prandial log TMAO. Notably, estimated GFR was found to be the most important determinant in the logged TMAO response.

Subject Characteristics and Inflammation

Participants who completed the study met all eligibility criteria. Nineteen participants classified as the LO inflammation phenotype and 16 classified as HI at baseline. Participants who were LO were similar to their HI counterparts in physical

characteristics including BMI, FM, VAT, and calculated $VO_{2\text{ max}}$. Glucose, triglycerides, and cholesterol levels are indicators of metabolic health and were found to be in normal ranges but not different between LO and HI.⁶⁸ Inflammation was one factor that distinguished LO and HI participants. The strong difference between phenotypes in all six pro-inflammatory cytokines supports the use of the LO and HI phenotyping criteria in this study.

Insulin

The high-fat meal in this study contained 54 g of carbohydrates, enough of a carbohydrate load to induce changes in blood glucose, but not enough to reach hyperglycemia in most healthy individuals. In response to elevated blood glucose, insulin is secreted by β -cells in the pancreas. Insulin is essential during the post-prandial period to remove and store glucose from the blood to adipose, liver, and muscle tissue, an inability of which results in hyperglycemia. Having elevated levels of glucose and insulin over time is positively associated with inflammation and chronic disease development.²⁴

A general linear model was used to examine insulin changes over time between inflammation phenotypes and accounting for subject. An overall time effect for insulin was observed for all participants with most participants reaching peak concentrations one hour after the meal, consistent with the large body of literature on post-prandial insulin. Insulin concentrations were not found to differ between inflammation phenotypes after accounting for subject. Lack of a difference in insulin concentrations between phenotypes may be partially due to the healthy status of participants. While diabetes was not

explicitly an exclusion criterion, none of the participants who enrolled and completed the study had Type I or Type II diabetes.

Creatinine and eGFR

A degradation product of creatine, creatinine is often used as indicator of muscle mass and to estimate GFR.⁶¹ Creatinine levels were previously not found to be different between obese adults who differ in metabolic health status, particularly relevant as metabolically healthy individuals often have lower systemic inflammation than those who are not metabolically healthy.^{69,70} Higher systemic inflammation has also associated with decreased eGFR.⁷¹ In this study, creatinine concentrations and eGFR were not different between LO and HI inflammation phenotypes.

hsCRP and Fasting TMAO

C-reactive protein is commonly used an indicator of systemic inflammation. Higher levels of CRP have been associated with obesity and are found to be an independent risk factor for CVD.^{9,72,73} LO and HI inflammation phenotypes did not differ in hsCRP concentrations, an interesting finding considering the difference between all six pro-inflammatory cytokines. The sole use of CRP can make it difficult to determine the level of systemic inflammation as the production of CRP can be initiated from infection, inflammatory conditions, or even use of drugs and oral contraceptives.^{74,75}

A prior study with CKD patients examined differences in fasting TMAO between inflamed and non-inflamed patients, with inflamed status defined as having a fasting

hsCRP concentration above or equal to 10 mg/L.³⁰ The same hsCRP grouping criteria was used in this cohort of healthy overweight and obese adults to examine inflammation status and TMAO. Only two individuals had an hsCRP above 10 mg/dL, and the results did not support a difference in fasting serum TMAO between inflamed and non-inflamed status. A hsCRP concentration of 10 mg/L is higher than the normal laboratory range, typically indicating acute inflammation.^{73,76} A moderate hsCRP, 3 mg/L, is a more appropriate comparison for chronic, low-grade inflammation. Fasting TMAO concentrations were not different even after revising the inflamed and non-inflamed grouping criteria with this moderate hsCRP cutoff, though it did generate more evenly sized groups than the higher hsCRP cutoff.

Post-prandial TMAO

The refined mixed effects model included fixed effects for a time by phenotype interaction, insulin over time, eGFR, and random effects for subject. The high conditional to marginal coefficient of determination indicates variability in the logged serum TMAO concentrations was most explained by subject to subject random effect and supports the use of a mixed-effects model, over a model with strictly fixed effects, to examine the logged TMAO response in this cohort. High variability in log TMAO may, in part, be a result of residual TMAO produced, or ingested via fish, from recent meals and/or a result of natural variation between individuals.

Inflammation status was forced into the model as there is literature to suggest that inflammation may influence TMAO concentrations.^{30,31,41,44} To the authors knowledge,

inflammatory markers have not been tested nor evaluated in studies thus far examining the post-prandial TMAO response in humans, though it has been previously been suggested as a future evaluation marker.^{15,34,56,77} This study utilized fasting levels of six inflammatory markers to designate HI or LO inflammation phenotypes to evaluate the log TMAO response and found the response was not different between phenotypes. It is possible that low levels of systemic inflammation are not a large enough stimulus to downregulate FMO3 expression, in contrast with the downregulation observed with a bacteria or LPS-induced infection.⁴¹ It is also possible that lack of dietary control affected fasting and post-prandial TMAO concentrations, potentially obscuring detection of a difference between phenotypes.

Based on experiments in rat models,¹⁶ it was hypothesized that higher serum insulin concentrations in the post-prandial period would be concomitant with decreased serum TMAO concentrations. Insulin treatment was previously demonstrated to partially suppressed FMO3 expression.¹⁶ Theoretically, the downstream effects of less expression of FMO3 could be less efficient conversion of TMA to TMAO. Results from the refined mixed effects model suggested that insulin concentrations during the post-prandial period may partially explain log TMAO concentrations. Contrary to what was expected, log TMAO increased over the course of four hours with higher post-prandial insulin concentrations after the high-fat meal challenge. Smaller post-prandial insulin concentrations had a lesser gain of log TMAO, with the lowest insulin concentrations decreasing in log TMAO. While there is some evidence to suggest that post-prandial

changes in insulin may influence post-prandial TMAO, other factors are likely at play that would promote elevated TMAO.

Previous research has found that TMAO is inversely associated with eGFR, a finding also reflected in this study.^{30,78} In fact, eGFR was the most important determinant of the log TMAO response. Lower eGFR can result in the accumulation of TMAO, a finding seen with impaired kidney function.⁴⁷ Low GFR also results in the decreased secretion of urea into urine. It is thought from mammalian and deep-sea fish studies that TMAO may actually increase to counteract the protein destabilizing effects of increased urea in the blood by favoring protein folding and even restoring enzyme function.^{27,79,80} Inclusion of BUN in future examination of TMAO responses in humans may be informative. In addition, the impact of TMAO on human enzymes is poorly understood and worth further evaluation.

Limitations

The main limitation was that diet was not controlled for leading up to blood collection and the high-fat meal challenge. Lack of dietary control before the high-fat meal challenge may potentially explain the interindividual variability in serum TMAO concentrations, as ingestion of foods rich in TMA precursors in the 24 hours prior to blood collection has been shown to give serum TMAO concentrations well above fasting.¹⁵ The high-fat meal of toast and butter provided to participants in this study is not a rich source of TMAO but did contain unquantified amounts of choline and betaine, both TMA precursors. It is unknown how much new TMAO was generated in response to the

high-fat meal challenge. Future studies may consider using isotope-labeled dietary precursors to better track formation of new TMAO.¹⁵

While variables that may influence TMAO levels such as insulin, inflammation, age, sex, and indicators of liver and renal function were accounted for in the initial model, there is the possibility of unmeasured confounding in modelling the log TMAO response. For example, the gut microbiota and FMO3 expression were not examined in this study but are known to influence TMAO concentrations in humans. Further examination of FMO3 expression may also be helpful in examining TMAO in obese adults as high BMI ($>33 \text{ kg/m}^2$) has previously been linked with increased relative levels of FMO3 mRNA.¹⁶

Conclusion

The goal of this study was to better understand how inflammation status impacts the post-prandial TMAO response to a high fat meal. This is especially important for several reasons. The first being that chronic, low-grade inflammation is a feature of metabolically abnormal obese adults and is related to chronic conditions such as CVD. Fat has increasingly become a major component of the Western Diet, and humans spend most of their day in a post-prandial state. Circulating TMAO has been implicated in the atherosclerosis with mounting evidence suggesting TMAO acts on macrophages, promotes fibrosis of tissues, and alters cholesterol metabolism. Understanding the post-prandial TMAO response can help elucidate early mechanisms for the development of CVD and potentially lead to improved recommendations and early treatments.

It was found that inflammation status did not impact post-prandial log TMAO in healthy overweight and obese adults even after accounting for factors that may influence the rate of TMA to TMAO conversion. Estimated GFR was the most important determinant of log TMAO, with some evidence suggesting post-prandial changes in insulin also influence TMAO concentrations. These results demonstrate that baseline systemic inflammation may not play as large of a role as eGFR on the post-prandial TMAO response to a high-fat meal.

The current study also helped inform statistical considerations for evaluating the post-prandial TMAO response. A mixed effects model may be more appropriate than a strictly fixed effects model to examine TMAO response as it accounts for subject to subject variation in TMAO.

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APPENDICES

APPENDIX A

RECRUITMENT FLYER



**You are invited to participate
in a Research Study**

Inflammation and the gut microbiome study

You may eligible if you are:

- 18-55 years old
- have a BMI 28-35
- have not taken antibiotics in the last 90 days
- do not have heart disease
- have no wheat or dairy allergies
- not pregnant

More questions? Interested?
 (406) 994-5001
 msunutritionlab@gmail.com

Researchers at MSU are conducting a research study on how factors related to health are influenced by microorganisms in the gut.

What to Expect: Two visits to MSU's Nutrition Lab. The 1st visit involves collecting body measurements and performing a sub-maximal fitness test. The 2nd visit involves ingesting a high-fat meal and blood collection.

Benefits: information on your body composition, blood pressure, and fasting glucose and blood lipid panel.

Compensation: You will be compensated up to \$100.

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APPENDIX B

MODIFIED BRUCE PROTOCOL

Participant #: _____	Visit One Submaximal Testing	Tech. Initials _____
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Age: _____

Age-Predicted HRmax = 220 - _____ = _____

85% of APhRmax = 0.85 x _____ = _____

Modified Bruce Protocol						
Stage	Time	Grade	Speed	HR	RPE	Notes
	Minutes	%	MPH	bpm		
1	0:00-2:59	0	1.7			
2	3:00-5:59	5	1.7			
3	6:00-8:59	10	1.7			
4	9:00-11:59	12	2.7			
5	12:00-14:59	14	4.0			
6	15:00-17:59	16	5.4			