Investigating the Deleterious Effects of the Gut Metabolite Trimethylamine N-Oxide on Bioenergetics and Functional Capacities.

Kevork Hratchia Atamian

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By: Kevork Hratchia Atamian

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Signed by the final Examining Committee:

_____ Chair

Robert Panenic, MA
_____ Examiner

Dr. Robert Kilgour
_____ Examiner

Dr. Peter John Darlington
_____ Supervisor

Dr. Andreas Bergdahl

Approved by _____
Dr. Nancy St-Onge, Chair of Department

_____2026

_____
Dr. Pascale Sicotte, Dean of Faculty of Arts and Science

Abstract

Investigating the Deleterious Effects of the Gut Metabolite Trimethylamine N-Oxide on Bioenergetics and Functional Capacities.

Kevork Hratchia Atamian

Cardiovascular disease (CVD) is the leading cause of death worldwide (Vaduganathan *et al.*, 2022). In 2011, the gut microbiome was linked to CVD through the metabolite trimethylamine N-oxide (TMAO), which is causally associated with atherosclerosis and reduced cardiac function (Wang *et al.*, 2011). TMAO is formed when gut bacteria metabolize dietary quaternary amines into trimethylamine (TMA), which is then oxidized in the liver. Mitochondrial dysfunction is a hallmark of many forms of CVD, particularly heart failure, where impaired ATP production contributes to exercise intolerance and decreased functional capacity (Zhou & Tian, 2018).

Despite the known associations between TMAO and CVD, its effects on mitochondrial respiration remain poorly defined, and its role in physical performance has not been studied (Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021). This thesis investigated the effects of chronic TMAO supplementation on mitochondrial function in cardiac, hepatic, vascular, and skeletal muscle tissues, as well as on physical performance in a murine model. We also examined whether high-intensity interval training (HIIT) could mitigate TMAO accumulation.

Key findings were: (1) HIIT did not reduce plasma TMAO concentrations; (2) TMAO generally did not impair cardiac mitochondrial respiration; and (3) unexpectedly, TMAO improved balance and maximal exercise capacity when combined with exercise.

These results challenge the current understanding of TMAO as solely deleterious and highlight the need for further research. The findings also emphasize the importance of non-pharmacological strategies for managing TMAO levels and improving cardiovascular and functional outcomes.

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I have been a student who has faced many challenges and roadblocks to success due to academic difficulties throughout my youth. That young struggling student would never have dreamed of going through graduate school in the future. I am proud of the distance I have come but, it would not have been possible without the support of the following people.

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1.1 Theoretical Context

Cardiovascular disease (CVD) is the number one cause of death worldwide and is responsible for the reduction in quality of life for millions of people every year (Vaduganathan *et al.*, 2022). There has been a significant focus on tackling this issue in academia. Research groups from many fields are aiming to find solutions from different angles. Treatment for CVD involves the use of pharmacological interventions, such as beta-blockers, ACE inhibitors and statins, or lifestyle modifications including exercise training and dietary adjustments. Although these pharmacological and behavioral interventions target cardiac function directly, there still remains variability in CVD improvement. As such there is a growing need to approach CVD treatment from a different angle. Recent studies have begun to explore the influence of the gut microbiome, a collection of bacteria colonizing the intestinal tract, on the development of CVD. My project investigates a metabolite called trimethylamine N-oxide (TMAO) produced by the microbiome and liver, which is linked with cardiovascular disease. CVDs limit the flow of the body's energy resources, reduce physical capacity, and decrease the ability to perform physical activities. Elevated levels of TMAO may be negatively related to the energy production during physical activities. Before discussing TMAO and its effects, some fundamental background theory will be reviewed to help understand the reasoning behind the design of this research project. This chapter will outline the underlying physiological pathways responsible for heart function and how their failure can impact functional capacity measurements.

1.2 Cardiovascular Disease (CVD)

Cardiovascular disease (CVD) is a broad category of illnesses affecting the heart and blood vessels, including conditions such as ischemic heart disease, hypertension, and cardiomyopathies. Many

forms of CVD are characterized by impaired cardiac performance due to structural or functional changes in the myocardium. These changes often involve reduced oxygen delivery, fibrosis, or altered energy metabolism, compromising the heart's ability to maintain adequate blood flow to meet the body's demands (Zhang *et al.*, 2021). One common pathological mechanism across several CVDs is a disruption in myocardial bioenergetics. This includes impairments in ATP production, which can lead to weakened or irregular cardiac contractions and ultimately reduced cardiac output (Zhou & Tian, 2018). By compromising energy availability in cardiomyocytes, bioenergetic dysfunction may contribute to symptoms such as fatigue and exercise intolerance frequently observed in individuals with cardiac dysfunction.

1.2.1 Causes of Mitochondrial Dysregulation in the Heart

In CVDs characterized by impaired oxygen delivery and/or increased workload (e.g., ischemia, hypertrophy), myocardial mitochondria are often observed to transition from lipid to glucose metabolism as their primary energy source. Although fatty acids provide more energy, they require more oxygen to complete the process of oxidative phosphorylation. Therefore, when the heart is starved of oxygen, it resorts to using carbohydrates which conserves myocardial oxygen consumption but produces less adenosine triphosphate (ATP) per molecule of fuel used when compared to fat metabolism. The shift in energy production is correlated with cardiac hypertrophy and reduced ejection fraction (Neglia et al., 2007; Byrne et al., 2016). Studies conducted in mice with different phenotypes, report on different bioenergetic profiles in the cardiac mitochondria. The dyslipidemic ApoE$^{-/-}$ mice display a subtly different cardiac mitochondria bioenergetic profile than C57BL/6 (reduced NADH-linked flux at rest but preserved maximal capacity with tighter coupling), a strain-specific physiology to keep in view when interpreting results (Rocha *et al.*, 2013).

1.3 Mitochondrial Respiration

The mitochondria are double-membraned organelles that produce ATP from adenosine diphosphate (ADP) using an electrochemical energy gradient called oxidative phosphorylation. The electrochemical gradient is built up in the intermembrane space by pumping hydrogen ions (H^+) – protons – from shuttle molecules sourced by the Krebs cycle. Complexes I, III and IV move H^+ ions from the mitochondrial matrix to the intermembrane space (Figure 1-1). The electrons are taken from the redox reaction and shuttled to Complex IV. When a sufficient electrochemical gradient is produced, Complex V (otherwise known as ATP synthase) will pass hydrogen back into the mitochondrial matrix, which will drive the phosphorylation of a molecule of ADP to make ATP. Oxygen molecules are the terminal acceptors of the electrons shuttled to Complex IV, where they react with two H^+ ions to form H_2O. For reference, Oxygraph-2k data in permeabilized cardiac fibres show that young ApoE$^{-/-}$ (dyslipidemic) hearts have markedly lower Complex I–linked basal (ADP-restricted) oxygen flux than C57BL/6 (~11 vs ~21 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$), while maximal ADP-stimulated respiration is similar; coupling indices are higher in ApoE$^{-/-}$ (ACR ≈12 vs 5; RCR 3.0 vs 2.3), indicating tighter OXPHOS coupling despite lower basal flux(Rocha *et al.*, 2013). Together, these features provide concrete flux benchmarks and establish ApoE$^{-/-}$ as a physiologically relevant dyslipidemic model. Hence it is a valid platform to test our thesis hypotheses on TMAO-related changes in cardiac bioenergetics and functional capacity against the C57BL/6 standard. Because ATP generation by OXPHOS ultimately powers cardiomyocyte contraction and skeletal muscle work, even subtle shifts in substrate use or coupling efficiency can scale up to measurable differences in balance, strength, and maximal exercise performance; we therefore turn next to the functional and exercise capacity outcomes used in this thesis.

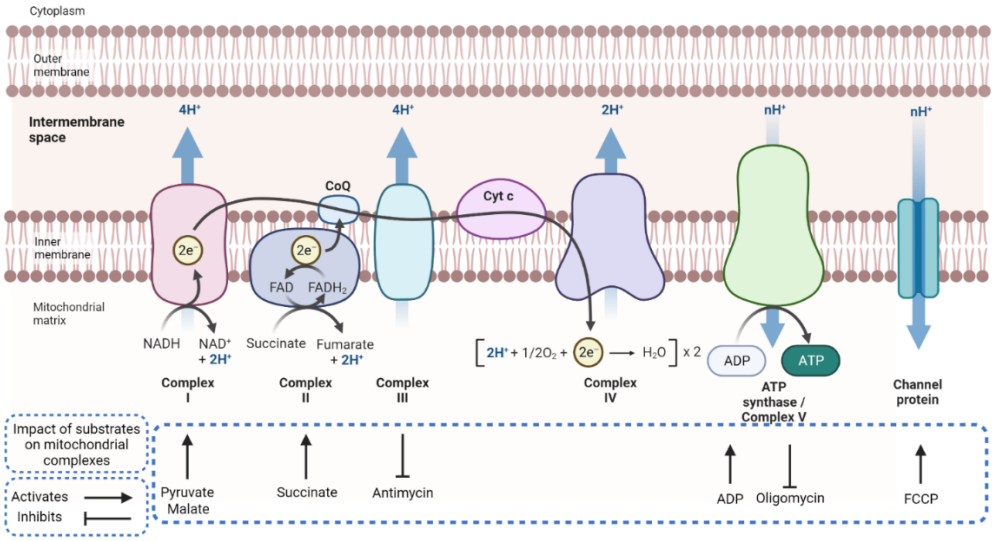

Figure 1-1 - Electron transport chain and the impact substrates have on its complexes. (Biorender.com)

1.4 Functional and Exercise Capacities

Building on the mitochondrial framework above, functional capacity, the whole-animal expression of cellular energy supply, will be discussed below. They demonstrate how well integrated organ systems translate ATP availability into movement needed for activities of daily living. Functional capacity measures an individual's ability to move in a manner that allows them to perform their activities of daily living (ADLs). Balance and static muscular strength are common metrics of functional capacity. With increased age, individuals tend to have decreased functional capacities, which can lead to falls and injuries, which in turn lead to complications, reduced quality of life and even death (Del Buono et al., 2019). Exercise capacity refers to an individual's ability to perform physical activity. Aging reduces skeletal muscle mitochondrial density and function, reducing exercise capacity (Short *et al.*, 2005). Reduced exercise capacity is also linked to decreased quality of life and is a symptom observed in individuals with decreased cardiac function. In order to have both good functional and exercise capacities, the cardiovascular, pulmonary and musculoskeletal systems must be properly functioning (Arena et al., 2007).

1.4.1 Balance – Functional Capacity

Balance is the ability to return to a state of stability from a point of instability. This functional capacity measurement is crucial to prevent falls and injuries while performing activities of daily living (Pollock et al., 2000). To have a stable stance, the body's center of gravity must be above the base of support – the surface area outlined by the points of contact with the floor on which they stand. To do this, the individual must have adequate neuromuscular control to make the fine motor adjustments according to the changes sensed by the vestibular system in the ear. People with reduced cardiac output would generally have decreased oxygen perfusion to their muscles, making small and rapid muscular adjustments difficult.

In animal-based studies, a rota-rod is used to measure balance. Maintaining stance on the accelerating rotarod requires continuous, high-frequency activation of postural muscles and reliable neuromuscular transmission—processes that are acutely dependent on mitochondrial ATP production and Ca^{2+} buffering in skeletal muscle and at the neuromuscular junction. Thus, decrements in mitochondrial oxidative capacity manifest as early fatigability and impaired micro-corrections (de Haas *et al.*, 2017).

1.4.2 Static Muscular Strength – Functional Capacity & Anaerobic Exercise Capacity

Strength, particularly grip strength, is necessary for many daily tasks such as holding objects or grasping onto surfaces to assist in balance. Strength can be evaluated by multiple parameters, each emphasizing different energy systems. Static muscular strength is assessed under isometric conditions: when quantified as time to failure while supporting a posture, as in the hanging wire test performed in the experiments below, it measures isometric endurance (fatigue resistance). Patients with reduced oxygen delivery to skeletal muscles will have increased fatiguability (Aartsma-Rus & Putten, 2014; Hoffman & Winder, 2016; Keller-Ross *et al.*, 2019). This is partly

due to reduced mitochondrial density and a shift from oxidative phosphorylation to a greater preponderance of anaerobic energy production.

In animal-based studies, the four-limb hanging test measures static muscular strength. The four-limb hanging test assesses isometric endurance rather than peak force, as such, performance (often summarized as holding impulse = body mass × hang time) depends strongly on mitochondrial function: enhancing biogenesis improves hang time, whereas impaired mitochondrial dynamics reduces it (Gill *et al.*, 2018).

1.4.3 Maximal Exercise Capacity

Exercise capacity is an individuals' maximal tolerance to physical activity. Chronic aerobic exercise can elevate mitochondrial density and function in both animal and humans, improve skeletal muscle oxidative capacity which ultimately increases exercise capacity (Porter *et al.*, 2015; Feng *et al.*, 2025).

Individuals with CVDs often exhibit reduced tolerance to physical activity. (Del Buono et al., 2019). As such, changes in exercise tolerance, as measured by the VO_2 max test, can be used to assess the heart's health. Importantly, VO_2 max reflects both central delivery and peripheral utilization; thus, the mass and quality of locomotor skeletal muscle, particularly the legs, contribute materially to overall exercise tolerance, and peripheral skeletal muscle abnormalities can independently depress VO_2peak in heart failure. The root cause of the decrease in exercise tolerance can be multifactorial and will depend on the cause of each individual's reduction in cardiac function. Although there can be severe reductions in exercise tolerance and functional movement abilities, exercise can be used as an efficient tool to help recover those losses.

1.5 High-Intensity Interval Training (HIIT)

There is substantial evidence that exercise is an effective intervention for improving quality of life, with benefits across various of physiological systems (Atakan *et al.*, 2021). Exercise prescriptions can be customized in frequency, intensity, duration, and type to elicit specific physical or physiological adaptations. As such, structured exercise is increasingly recognized for general health and as a therapeutic tool for managing chronic diseases. Unlike pharmaceutical treatments, lifestyle interventions like exercise typically have minimal adverse effects. However, adherence can be challenging due to social barriers and the perceived effort required (Weston et al., 2014). Among the various modalities, high-intensity interval training (HIIT) has gained substantial attention for being time-efficient while producing comparable or superior adaptations to longer-duration moderate-intensity training (Nicolò & Girardi, 2016). HIIT is generally well-received, with positive enjoyment scores from participants, making it a promising option for both clinical and non-clinical populations (Oliveira *et al.*, 2018; Sabag *et al.*, 2022). It involves cycles of high-intensity effort followed by active recovery, with customizable ratios and intensities based on the individual's needs and capacity.

With appropriate supervision and training, and contrary to outdated beliefs, high-intensity interval training (HIIT) is not only feasible but has been repeatedly demonstrated to be safe and effective in patients with certain levels of heart failure and even in heart transplant recipients (Franklin *et al.*, 2020; Yu *et al.*, 2022). A systematic review by Yu et al. (2022) found that HIIT significantly improved peak oxygen uptake (VO_2peak), exercise tolerance, and quality of life in heart failure patients as well as heart transplant recipients (Yu *et al.*, 2022). Furthermore, a recent meta-analysis by Costa et al. (2023) concluded that exercise-based cardiac rehabilitation programs – including HIIT – lead to marked improvements in cardiorespiratory fitness, muscle strength, and functional status in heart transplant recipients (Costa *et al.*, 2023). These findings highlight the critical role

of structured exercise, even of higher intensities, in previously thought to be exercise-intolerant populations. However, in patients with more advanced heart failure (NYHA Class III and IV), HIIT might not be appropriate. As such, the inclusion of HIIT in experimental models is justified and evidence-based, especially when investigating functional capacity and cardiometabolic adaptations.

In murine models, HIIT is well established and consistently outperforms moderate-intensity continuous training for metabolic, cardiovascular, and skeletal-muscle adaptations. In diet-induced obesity and related models, HIIT reduces body weight and fat mass, improves insulin sensitivity and glucose tolerance (Wang et al., 2017; Martinez-Huenchullan et al., 2019; Dos-Santos et al., 2023; Lu et al., 2025). In skeletal muscle, HIIT increases fatigue resistance, mitochondrial respiratory chain complex expression and muscle mass/fiber size; it also improves physical performance and frailty indices in aged mice (e.g., better treadmill endurance and gait speed) (Seldeen et al., 2018a; Yamada et al., 2021). Across these studies, whole-body cardiorespiratory fitness and performance improve, reported as higher VO_2-linked outcomes or validated surrogates such as greater maximal running speed/distance and time to exhaustion—supporting the inclusion of a murine HIIT paradigm when interrogating functional capacity and cardio-metabolic mechanisms.

1.1.1 Physiology of HIIT

Many physiological adaptations, categorized under central and peripheral adaptations, occur due to HIIT. Central adaptations include increased maximal stroke volume, maximal cardiac output and blood volume (Atakan et al., 2021). The increase in cardiac output as an adaptation to aerobic exercises has been associated with cardiomyocyte hypertrophy and, more specifically, left ventricular hypertrophy. The reasoning is that this hypertrophy is an adaptation which allows the

heart to keep up with the chronic increase in demand during HIIT (Carvalho et al., 2021). This hypertrophy is reported in many studies with HIIT regardless of the different intensities and durations of their stages. On the peripheral side, research done on humans show that HIIT produces clear adaptations in the working limb muscles: oxygen delivery and use improve, fatigue resistance increases, and modest gains in muscle size and force endurance are often observed. Practically, these changes show up as longer time-to-exhaustion and better performance on limb-focused tasks (e.g., treadmill running, hanging/rotarod tests). Together with central adaptations, these peripheral changes help explain the HIIT-related improvements in VO_2max and functional capacity (MacInnis & Gibala, 2017; Atakan *et al.*, 2021). In mice, HIIT (treadmill/sprint) produces parallel peripheral adaptations: improved endurance/peak running speed, enhanced limb fatigue resistance, and small or variable changes in muscle mass. Central cardiac remodeling is less consistently reported; performance improvements primarily reflect metabolic and neuromuscular efficiency. These murine effects align with our hindlimb-focused functional tests (e.g., rotarod/hanging/treadmill)(Seldeen *et al.*, 2018*b*).

1.5.1 HIIT and CVD

In disturbed cardiac bioenergetics, interval-based high-intensity exercise are important in reversing left ventricular remodelling and improving endothelial function, quality of life and life expectancy (Wisløff et al., 2007). Multiple studies using HIIT as a treatment modality have shown it improves left ventricular ejection fraction (Sabag et al., 2022). Another cardiovascular-related metric which is a predictor of all-cause mortality is arterial stiffness – the inability of arteries to comfortably expand and return to their initial shape to accommodate the natural variations in blood volume and pressure (Vlachopoulos et al., 2010). Aerobic exercises, such as HIIT, which increase heart rate, help reduce arteries' stiffness and conductance (Atakan et al., 2021; Sabag et al., 2022). Individuals with cardiovascular disease often experience chronic kidney disease (CKD) as a comorbidity due

to the increased strain on the kidneys. Glomerular filtration rate (GFR) is a measure that quantifies the rate at which the kidney can filter the necessary particles from the blood. It is often used as a marker of kidney health. Many studies have investigated the impact of physical activity on patients living with CKD. Although they have found HIIT to be safe and effective at improving VO2max and other cardiovascular measures, GFR remained unchanged between control and exercising groups (Hamada et al., 2016; Watson et al., 2018; Arazi et al., 2022).

1.5.2 HIIT and Functional Capacities

An individual's VO2max is a predictor of all-cause mortality in both sexes (Blair et al., 1996; Lee et al., 2011; Kaminsky et al., 2013). HIIT increases peak aerobic VO2max (an individual's maximum ability to extract oxygen from inspired air), which improves the ability to effectively perform activities of daily living (Weston et al., 2014). Mitochondrial content determines the primary substrate for ATP production in skeletal muscles. At a given submaximal workload, greater mitochondrial content shifts fuel use toward higher fat oxidation and is associated with lower lactate accumulation. HIIT increases mitochondrial biogenesis and content in skeletal muscle in humans (MacInnis & Gibala, 2017) and in skeletal and cardiac muscle in murine models (Carvalho *et al.*, 2021). The increased presence of mitochondria will decrease lactate production, the byproduct of anaerobic production of ATP. As a result, individuals would have an increased lactate threshold, meaning increased exercise tolerance and reduced fatigue. A mitochondria's ability to use oxygen is called the mitochondrial oxidative capacity – higher oxidative capacity indicates improved utilization of oxidative phosphorylation. In addition to increased mitochondrial content, HIIT increases the expression of mitochondrial proteins, increasing their enzyme capacity.

The presence of free radicals – oxygen species that can cause damage in the cell and mitochondria in particular – reduces the efficiency of the mitochondria in producing ATP. Antioxidants alleviate

oxidative stress by preventing these radicals from damaging the environment. Interval-based training has been shown to increase the total antioxidant content in the plasma (Wisløff et al., 2007). Free radicals can oxidize low-density lipoproteins (LDLs), which play an important role in the forward cholesterol transport and prevent atherosclerosis. Oxidized LDLs have the opposite role in that they can perform a pro-atherogenic role in the endothelium (Poznyak et al., 2021), and due to the increased antioxidant count in interval trained people, there is a reduction in OxLDLs (Wisløff et al., 2007). Beyond lipoprotein oxidation, TMAO itself appears to modulate cellular redox tone through the glutathione system and inflammatory signaling. In endothelial and vascular models, TMAO elevates reactive oxygen species (ROS) via NADPH oxidase and NLRP3–NF-κB activation, shifts the glutathione redox couple toward oxidation (lowered GSH and GSH/GSSG), and increases lipid peroxidation, collectively favoring a pro-atherogenic milieu (Brunt *et al.*, 2020; Lei *et al.*, 2023; Florea *et al.*, 2024). Conversely, under exercise stress, TMAO has also been reported to activate the Nrf2 antioxidant pathway, which upregulates glutathione synthesis and turnover enzymes (e.g., GCL, GR, GPx), alleviates ROS burden, and improves performance (Zou *et al.*, 2024). Because HIIT augments endogenous antioxidant defenses these adaptations may intersect with TMAO's context-dependent actions on redox balance (Elokda & Nielsen, 2007; Delwing-de Lima *et al.*, 2018). Taken together, current evidence supports a nuanced view in which TMAO can be pro-oxidant and pro-inflammatory in vascular disease settings, yet may engage Nrf2–glutathione signaling during exercise to reduce oxidative stress; this duality is relevant to interpreting HIIT-related changes in OxLDL and functional capacity.

1.6 The Gut Microbiome

1.6.1 What is the Gut Microbiome?

The microbiome is the name given to the colony of commensal bacteria that inhabit the latter portion of the digestive tract, the large intestines. Containing tens of thousands of different species

and between 10^{13} and 10^{14} individual bacterial organisms, this diverse population interacts with all ingested material (Cheng & Ning, 2019). Some bacteria stay in the colon (resident), and others pass through with the feces (transient). Some bacteria are helpful to the host (beneficial symbionts), and those that are detrimental to the host (pathogens) (Hammer et al., 2019). Many, but not all, macroscopic organisms host microbiomes, many of which are co-dependent with this colony and live in a symbiotic relationship (Hammer et al., 2019). Each individual within an animal species will have a unique microbiome composition. The composition of an individual's microbiome is shaped by numerous internal (e.g., genetics, immune status) and external (e.g., diet, environment, medications) factors, resulting in significant interindividual variability that must be accounted for in microbiome research(Cheng & Ning, 2019).

1.6.2 CVDs and the Microbiome

With the recent surge in research investigating the link between the microbiome and other organs, the hyphenated term "gut-organ axis" has emerged and is being used with most organs. Studies have begun investigating the heart-gut-axis, and many associative links have been drawn between the two organs. For example, studies with patients in heart failure for extended periods of time have shown an alteration in gut microbiome composition and a trend in reduced microbial diversity (Kummen et al., 2018). The primary metabolite currently identified to be linked with the heart-gut axis is trimethylamine N-oxide, which is linked with atherosclerosis and reduced cardiac function(Wang *et al.*, 2011).

1.7 Trimethylamine N-Oxide (TMAO)

The North American diet has progressively increased in foods containing large amounts of red meat, eggs, fish, and dairy products. These foods are significant sources of quaternary amines such as choline, phosphatidylcholine, betaine, L-carnitine, and trimethylamine-N-oxide (Zhang et al., 2021). Certain intestinal bacteria that compose the microbiome take up these molecules and cleave

a trimethylamine (TMA) group during digestion. TMA comprises a central nitrogen atom bonded to three methyl groups and has a chemical formula of $(CH_3)_3N$. The intestinal epithelial cells absorb this tertiary amine and release it to the mesenteric venous system, where the liver takes it up. Here, flavin-containing monooxygenase (FMOs) enzyme groups, mainly FMO3, oxidize TMA to trimethylamine N-Oxide (TMAO) by adding an OH group to the central nitrogen, $(CH_3)_3NOH$ or $(CH_3)_3NO$- as illustrated in Figure 3 (Wang et al., 2011; Spence, 2018; Iglesias-Carres et al., 2021). TMAO and a small amount of TMA are then released into the systemic circulation. The kidney naturally filters TMA and TMAO and excretes them through the urine (Al-waiz et al., 1987; Tomlinson & Wheeler, 2017; Zhang et al., 2021). They are also readily excreted through sweat and breathing. Of the TMA produced by the microbiome, around 5% is excreted as TMA, and the remaining is in its oxidized form (Mitchell & Smith, 2016). TMA was first discovered by a German chemist in the 1800s who purified it from urine with a strong fish odour (Hofmann, 1852). Since then, it has been identified as a molecule used in the osmoregulation of fish cells. It was only in 2011 that a research group based in Cleveland and Los Angeles linked the production of TMAO by the microbiome to be directly involved in CVD (Wang et al., 2011). The publication of that group's paper started a cascade of research efforts to improve the understanding of TMAO's effects on the body. Since then, they have found it to be involved in many disease pathways, including renal, hepatic, and even some cancers (Yang et al., 2019). The largest body of evidence points towards TMAO's dose-dependent increase of risk for the development of cardiovascular disease (Schiattarella et al., 2017). Beyond its association with lipids, circulating TMAO can raise cellular ROS via NADPH-oxidase/mitochondrial pathways and ROS-TXNIP-NLRP3 inflammasome signaling in vascular endothelium, a mechanism linked to oxidative stress and endothelial dysfunction. These redox effects provide biological plausibility for TMAO's ties to atherogenesis

and help frame our OxLDL findings (Sun *et al.*, 2016). In generally healthy adults (fasting), plasma TMAO typically lies in the low micromolar range (median ~1–3 μM; upper reference ~5–6 μM), whereas cardiometabolic cohorts often show higher medians (~4–8 μM) with risk increasing in the upper quartiles (≈≥6 μM) (Tang *et al.*, 2013; Brunt *et al.*, 2021*a*; Jing *et al.*, 2022; Wang *et al.*, 2022). Levels vary with renal function and recent diet—chronic kidney disease can elevate values to ~20 μM or more, and fish-rich meals can transiently push next-day levels above ~6 μM. In rodents, chow-fed wild-type mice generally exhibit low-single-digit micromolar plasma TMAO (≈2–7 μM), with strain/sex differences partly driven by hepatic FMO3 expression (adult males typically lower than females) (Maksymiuk *et al.*, 2024).

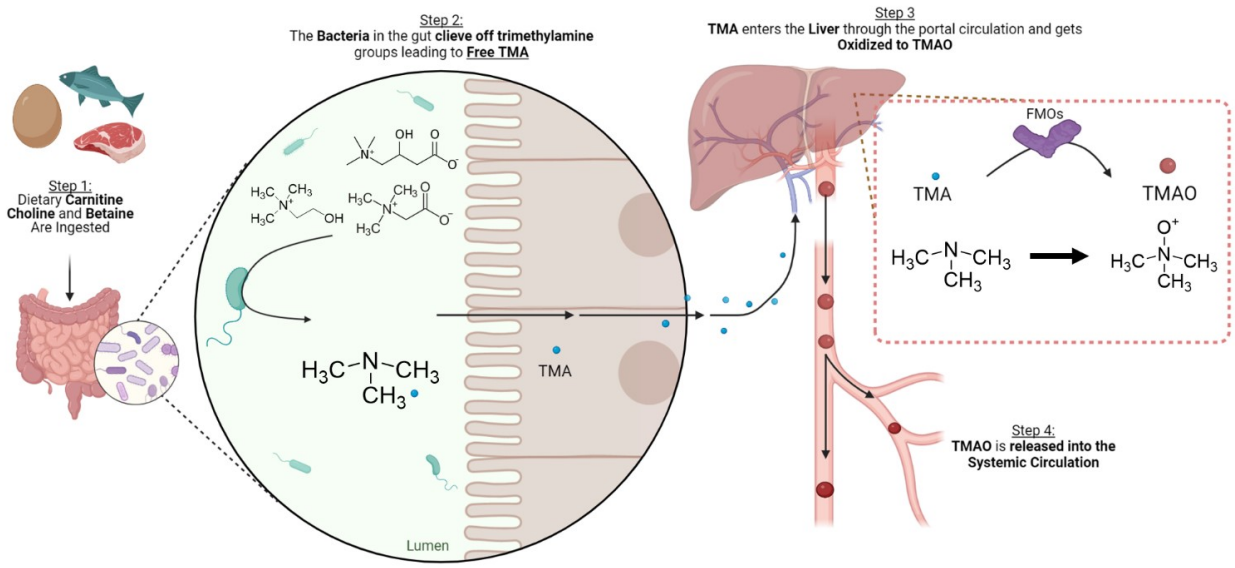

Figure 1-2 - Production of TMAO. Step 1: Ingestion of dietary quaternary amines. Step 2: Bacterial enzymes cleave trimethylamine, which is absorbed by the intestinal lining and transported to the liver. Step 3: TMA is oxidized by FMO3 in the liver to form TMAO. Step 4: TMAO is released to the systemic circulation.

1.7.1 Sources of TMAO

Trimethylamine N-oxide (TMAO) is formed in the liver through trimethylamine (TMA) oxidation, produced by gut bacteria metabolizing dietary quaternary amines. Plasma and urinary TMAO levels are influenced by four primary factors: dietary intake of precursor foods, the abundance of

TMA-producing bacteria in the microbiome, liver FMO3 enzyme activity, and excretion rates. While genetics influence microbiome composition, they play a minimal role in TMAO levels (Hartiala *et al.*, 2014; Zhang *et al.*, 2021). Among the most important dietary precursors are L-carnitine and choline. L-carnitine is a conditionally essential nutrient synthesized in the kidney, liver, and testis but primarily acquired from red meat. Despite its role in lipid metabolism and mitochondrial β-oxidation, L-carnitine supplementation is controversial; it elevates plasma TMAO and lacks strong evidence for efficacy in mitochondrial disorders, even though it is prescribed for such cases (Flanagan *et al.*, 2010; Vallance *et al.*, 2018; Simó & García-Cañas, 2020). The enzyme carnitine oxygenase/reductase cleaves TMA from L-carnitine, making it a direct TMAO precursor. Choline is also conditionally essential for membrane phospholipid synthesis (e.g., phosphatidylcholine), neurotransmitter synthesis (acetylcholine), and methyl donation in biochemical pathways. Found in red meat, eggs, and soy products, it is produced endogenously but must also be ingested (Zeisel *et al.*, 2003; Simó & García-Cañas, 2020). Additionally, direct supplementation with TMAO-rich foods like fish (especially deep-sea species) causes spikes in plasma TMAO due to its osmoregulatory role in marine animals(Yancey *et al.*, 2014; Wang *et al.*, 2022). Studies have demonstrated that TMAO can be directly absorbed into the bloodstream and that supplementation in animal models – via drinking water or intraperitoneal injection – raises plasma TMAO concentrations in a dose-dependent manner (Hu *et al.*, 2015; Li *et al.*, 2019; Videja *et al.*, 2021, 2023). Such supplementation avoids microbiome-based variability in TMA production, allowing researchers to reliably model effects. In rats, plasma TMAO half-life increases linearly with dosage; for example, a 120 mg/kg dose mixed in the drinking water results in a half-life of approximately 3.7 hours (Nnane & Damani, 2001). These mechanisms and routes

of TMAO formation and supplementation are critical to understanding its systemic effects and developing strategies for mitigating its health impacts.

1.7.2 The FMO3 Liver Enzyme

The last step before releasing TMAO into the bloodstream is to synthesize it from TMA in the liver using the flavin monooxygenase 3 enzyme (FMO3). However, consuming large volumes of TMA precursors would not be associated with large blood TMAO levels if FMO3 were inactive. This is the case with individuals with genetic mutations preventing FMO3's activity, leading to trimethylaminuria (Mackay et al., 2011). In an experiment on a mouse model with choline supplementation, female mice were found to have higher levels of plasma TMAO. This was most likely because they had a higher total hepatic FMO3 activity compared to males (Wang et al., 2011; Romano et al., 2015; Randrianarisoa et al., 2016). This difference in protein expression by sex is in line with the study by Xu and colleagues on humans, which found that FMO3 expression also depends on age, as it is higher in older individuals (Xu et al., 2017).

1.7.3 Health Impacts of TMAO

Trimethylamine-N-oxide (TMAO), a metabolite derived from dietary nutrients through gut microbial metabolism, has emerged as a significant biomarker and potential mediator of adverse health outcomes, particularly in cardiovascular disease (CVD). An expanding body of epidemiological and clinical research has demonstrated that elevated circulating TMAO levels are associated with an increased risk of developing a range of pathologies, most notably atherosclerosis, coronary artery disease, and heart failure. In addition, associations have been observed with chronic kidney disease, various malignancies, and neurocognitive decline (Roncal *et al.*, 2019; Zhang *et al.*, 2021, 2023; Coutinho-Wolino *et al.*, 2021; Jalandra *et al.*, 2021; Wang *et al.*, 2023). Importantly, elevated plasma TMAO concentrations have been consistently identified as an independent predictor of all-cause mortality, even after adjusting for traditional risk factors

and comorbid conditions (Roncal *et al.*, 2019; Chen *et al.*, 2022; Fretts *et al.*, 2022; Wang *et al.*, 2023). Concerning cardiovascular disease specifically, several studies have reported a dose-dependent relationship between TMAO levels and disease severity, including worsening New York Heart Association (NYHA) functional class, elevated B-type natriuretic peptide (BNP), and greater atherosclerotic plaque burden (Tang *et al.*, 2014; Trøseid *et al.*, 2015). These findings underscore the potential role of TMAO as a biomarker and contributor to CVD pathogenesis. As such, elucidating the underlying molecular and physiological mechanisms by which TMAO influences cardiovascular function is essential for understanding its contribution to disease progression and evaluating its utility as a therapeutic target.

1.7.4 TMAO and the Cardiovascular System

As stated above, TMAO has been shown to impact the renal, nervous and gastrointestinal systems negatively. A ground-breaking study in 2011 was the first to identify TMAO as a predictor for CVD causally, and as a promoter of atherosclerosis (Wang et al., 2011). The study by Wang and colleagues showed a dose-dependent relationship with circulating TMAO concentrations and cardiovascular disease (Wang et al., 2011). The study showed, for the first time, a link between the microbiome and the development of plaque in the aorta (Wang et al., 2011). In 2013, Koeth and colleagues performed a similar study, reinforcing the direct link between TMAO's impact on plaque buildup in the aorta and the microbiome (Koeth *et al.*, 2013). Both studies were performed in similar manners. Apolipoprotein (Apo E-/-) mice were split into four groups and fed different diets: a standard chow diet, a diet high in carnitine/choline, a standard chow diet with antibiotics to suppress the microbiome and a diet high in carnitine/choline with the same antibiotics. Apolipoprotein E knockout ($ApoE^{-/-}$) mice are genetically modified mice that lack the ApoE gene, which is essential for normal lipid metabolism. These mice spontaneously develop hypercholesterolemia and atherosclerotic plaques, making them a widely used model for studying

cardiovascular disease, particularly atherosclerosis. In both studies, the levels of plasma TMAO were also measured and were found to be highest in the groups consuming TMAO precursors without antibiotics.

1.7.5 Increase Excretion of TMAO

Renal excretion is the dominant elimination route for TMAO (approximately 95% of an oral dose within around 24 h), with fecal losses standing around 4% and breath/sweat less than 1% (Taesuwan *et al.*, 2017). Accordingly, strategies that preserve or enhance kidney function and, critically, that reduce microbial TMA production (dietary modulation and microbiome-targeted approaches) will have the largest impact on circulating TMAO. Although exercise confers broad cardiometabolic benefits, acute bouts do not increase non-renal TMAO elimination; when TMAO decreases during lifestyle programs, the effect is attributed to diet (Erickson *et al.*, 2019).

1.8 Related Research in the Literature

The present thesis aims to reduce the circulating plasma TMAO levels and reduce its negative impact on cardiac mitochondria. It will also investigate the impact of TMAO consumption on functional capacities in a murine model. Very few published papers investigate the specific information we seek to explore. Below, the relevant research papers that investigate TMAO's relationship with mitochondrial energy metabolism, exercise, and functional capacities are discussed.

The effects of TMAO on mitochondrial energy metabolism have been inconsistent across studies. In 2017, Makrecka-Kuka and colleagues showed that acute and chronic exposure to TMAO impaired mitochondrial function in rodent cardiomyocytes. When mice were acutely injected with TMAO, their respiration rates with pyruvate and malate dropped by 47% in State 1 and 34% in State 2, indicating a disruption in pyruvate oxidation. Similarly, respiration linked to fatty acid

oxidation (with palmitoyl-CoA) was reduced by 24%. However, the electron transfer system (ETS) function and Complex II-linked respiration were unaffected. They also found reduced pyruvate dehydrogenase activity, while carnitine palmitoyltransferase I remained unchanged. This suggests that TMAO limits energy production by lowering pyruvate flux and β-oxidation, without increasing reactive oxygen species (ROS). Chronic TMAO supplementation over eight weeks led to similar impairments in State 1 respiration with both carbohydrate and lipid substrates, though no changes were seen in body weight or water intake. Overall, these results suggest that TMAO interferes with substrate metabolism upstream of the ETS but does not impair the ETS directly.

In contrast, a 2021 study by Videja et al. found that long-term TMAO supplementation in a rat model of right ventricular failure helped reduce pressure overload's adverse effects. At the same time, fatty acid oxidation was still reduced by 69% in State 2 respiration, and TMAO attenuated cardiac remodelling and hypertrophy triggered by monocrotaline. This protective effect was accompanied by a metabolic shift from fatty acid use to glucose metabolism, which is less efficient but may be beneficial under stress. Similarly, a 2023 study by Naghipour and colleagues showed that acute exposure to TMAO increased mitochondrial respiration through Complex I and II, even though it impaired contractile function. These conflicting results highlight the complexity of TMAO's role in mitochondrial metabolism and suggest that outcomes depend on the experimental model, timing, and conditions.

Because of this, researchers have started looking into whether exercise could be a way to lower TMAO levels or buffer its effects. In a retrospective study with 483 participants, Argyridou et al. (2020) found that each additional 30 minutes of moderate-to-vigorous physical activity (MVPA) per day was linked to a significant drop in plasma TMAO levels. Light activity and sedentary time had no effect, and the results were independent of other factors like diet, age, and BMI. Another

study by Erickson et al. (2019) examined obese adults undergoing a 12-week exercise program. Those who combined exercise with a hypocaloric diet had a drop in TMAO. In contrast, those on an eucaloric diet saw a slight increase, suggesting that calorie intake influences the effectiveness of exercise in lowering TMAO. More recently, Zhang et al. (2023) showed that voluntary wheel running in mice improved cognitive outcomes, significantly reduced circulating TMAO, and improved gut barrier integrity and microbiota composition.

Altogether, the literature suggests that TMAO affects mitochondrial energy metabolism in a way that depends on tissue type and physiological context. In some cases, it suppresses fatty acid and pyruvate oxidation; in others, it enhances mitochondrial activity or has protective effects. Conversely, exercise is a promising way to lower systemic TMAO levels and offset its impact. However, most of the research has focused on disease models or aging populations, and little is known about how structured exercise like high-intensity interval training (HIIT) might influence TMAO metabolism in a dyslipidemic model. In addition, the impact of TMAO on physical performance outcomes – such as balance, strength, and exercise capacity – has not been well studied. This study aims to fill those gaps by testing whether HIIT can reduce plasma TMAO levels, preserve cardiac mitochondrial function, and improve functional performance in mice chronically exposed to TMAO. We hypothesize that physical activity will have a protective effect on both mitochondrial physiology and physical capacity in this context.

2 Chapter 2 – First Manuscript

Investigating the Effects of High Intensity Interval Training on the Deleterious Impacts of the Gut Metabolite Trimethylamine N-Oxide.

Kevork H. Atamian, Andreas Bergdahl

Contribution of authors

Kevork H. Atamian: animal handling, surgeries, respiration measurements, statistical analysis, preparation of manuscript.

Andreas Bergdahl: Editing of manuscript.

2.1 Abstract

Trimethylamine (TMA) produced by the gut microbiota from dietary quaternary amines (choline, carnitine, betaine) is oxidized in the liver to trimethylamine N-oxide (TMAO). It is associated with cardiovascular diseases (CVD). The dysfunction of mitochondria in cardiomyocytes results in CVD. Dietary changes reduce the presence of TMAO in blood; however, the effect of exercise remains unstudied. The objectives of this study are to examine the impact of aerobic exercise on (1) plasma TMAO levels, (2) cardiac mitochondrial function, and (3) functional capacity in dyslipidemic mice supplemented with TMAO. Four groups were used: control (sedentary), TMAO control (sedentary, TMAO supplemented), exercise (running), and TMAO exercise (running, TMAO supplemented). For eight weeks, the mice performed a five-day/week high-intensity interval training (HIIT) program during which balance, static muscular strength, and maximal exercise capacity were measured at five time points. Then, the mice were euthanized, and blood plasma and heart samples were extracted. Plasma TMAO concentration was measured using UPLC MS/MS. Oroboros O2K respirometers were used to measure mitochondrial physiology. The results showed that the HIIT exercise protocol did not reduce plasma TMAO concentrations. Mitochondrial respiration showed no differences with pyruvate, malate, ADP, or succinate. However, oligomycin-stimulated and FCCP-uncoupled flux were significantly higher in the TMAO and TMAO exercise groups than the exercise group. Functional capacities improved with TMAO supplementation. In conclusion, HIIT was ineffective at reducing plasma TMAO, and chronic TMAO supplementation did not impair basal mitochondrial function, rather, it increased leak/uncoupled capacity and improved functional capacities.

2.2 Introduction

Cardiovascular diseases (CVDs) are the leading causes of death worldwide. With the recently established link between the gut microbiome and the heart, known as the heart-gut axis, many mechanisms are left to be identified (Bui et al., 2023). The dietary quaternary amide derivative produced in the gut, trimethylamine N-oxide (TMAO), has been linked to reduced cardiac function (Wang et al., 2011; Zhang et al., 2021). TMAO was identified as a marker of mortality and is a significant factor in the determination of quality of life (Roncal et al., 2019; Chen et al., 2022; Fretts et al., 2022; Wang et al., 2023).

Efforts in the literature are aimed at reducing circulating plasma TMAO levels. However, most studies are focused on pharmaceutical interventions with negative side effects. Studies suggest that exercise and diet are a valid tool to reduce circulating plasma TMAO levels (Leal-Witt et al., 2018; Zhang et al., 2023). Exercise interventions are known to have low adherence; however, high-intensity interval training (HIIT) is more enjoyable, less time-consuming, and provides the same benefits as traditional protocols (Oliveira et al., 2018). HIIT exercise protocols are composed of repeating cycles of high intensity followed by a low intensity active recovery period. The ratio of time spent in each phase, as well as the duration of time spent performing each portion, can be customized based on the individual's needs and comfort levels and can vary from 10 seconds to multiple minutes in each phase.

Since the heart-gut axis was established, many studies have investigated the underlying mechanisms involved with TMAO leading to CVD (Bui et al., 2023). Mitochondria are central to cardiac function, and elevated TMAO has been associated with reduced cardiac performance. Therefore, we aimed to delineate the bioenergetic mechanisms by which TMAO influences cardiomyocytes. The two research papers published on the mitochondrial involvement of TMAO

are contradictory and have not established sufficient proof (Makrecka-Kuka et al., 2017; Videja et al., 2021).

One of the important symptoms of reduced heart function is a decreased ability to tolerate physical activity due to the heart's poor performance. Functional capacity measurements quantify an individual's ability to perform different physical activities. There has been no research investigating the link between functional capacities and TMAO.

Despite some model-specific variability, evidence that TMAO disrupts cardiac mitochondrial substrate oxidation and impairs contractile performance supports the expectation of lower exercise tolerance with exposure; therefore, we hypothesize that TMAO will significantly impair functional capacity (Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021)

The goals of this study are: 1. To identify if a HIIT exercise protocol could reduce the presence of TMAO in the blood. 2. To identify the impact of TMAO supplementation on mitochondria in cardiomyocytes. 3. To identify the impact of TMAO supplementation on functional capacity measurements linked to reduced heart function. The three hypotheses are: 1) HIIT protocol will reduce plasma TMAO levels; 2) TMAO will alter cardiac mitochondrial energy metabolism and, 3) TMAO will significantly impair functional capacity.

2.3 Methods

2.3.1 Animals

This randomized control trial took place in the Concordia University Animal Care Facilities. Sixty age-matched mice between six to eight weeks old were selected from the Concordia University breeding colony. Since the effect of physical activity on TMAO plasma levels was equivalent in both sexes in previous studies, the groups were composed of males and females of equal proportions (Argyridou et al., 2020). Dyslipidemic mice (Apolipoprotein E; Apo E −/−) were used

(Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021; Brunt *et al.*, 2021*b*). The mice were kept in a temperature-controlled room at 22ºC with a 12-hour light cycle. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.3.2 Groups

Mice were assigned to their groups randomly and individually housed in clear acrylic cages. Each control and exercise set contained a group that consumed regular water and one whose water was supplemented with TMAO. The four experimental groups were: Control (C), TMAO Control (T), Exercise (E), and TMAO Exercise (TE). The C group did not participate in the exercise intervention, consumed a standard chow diet, and had access to water ad libitum. The E group followed the HIIT exercise protocol defined in the "High-intensity Interval Training Protocol" section, consumed a regular chow diet, and had access to water ad libitum. The T group did not participate in the exercise intervention, consumed a regular chow diet, and had access to a TMAO-supplemented water ad libitum. The TE group followed the HIIT exercise protocol, consumed a regular chow diet, and had access to TMAO-supplemented water ad libitum. Previous research where TMAO was supplemented showed no difference in water consumption compared to control groups; therefore, we did not expect a difference (Videja *et al.*, 2021).

2.3.3 Timeline

This experiment took place over 10 weeks. In week 0, the mice were taken out of the breeding colony and underwent the acclimation week. At week 1, the mice in the exercising groups (E and TE) began the five-day-per-week HIIT protocol. In odd-numbered weeks, all groups performed functional tests (balance, strength, exercise capacity). On the last week, the day following their functional test, they were euthanized using CO_2 asphyxiation. Left atrium myocardium was used for mitochondrial respiration analysis.

2.3.4 TMAO Supplementation

TMAO was purchased from Cayman Chemical Company, Michigan, USA, as a pure crystal as aligned with previous articles. Based on the previous studies, 120 mg of TMAO per body weight was consumed daily (Makrecka-Kuka et al., 2017; Zhao et al., 2019; Videja et al., 2021, 2023). Three stock solutions were prepared based on the three most common mouse body weights (20g, 25g, and 30g). In order to keep track of appropriate nutrition and hydration, food, water and body weight were tracked three days per week (Mondays, Tuesdays, and Fridays).

2.3.5 Screening and Acclimation Period

The screening and acclimation were done according to the protocol outlined by Caru and colleagues during a five-consecutive-day period (from Monday to Friday) (Caru et al., 2019). The first day (day-1) of the acclimation protocol functioned as a screening test to identify individuals unwilling to run on the rodent treadmill. For the screening test, the mice walked for one minute at a speed of five m/min, then ran for one minute at ten m/min, followed by 15 minutes at 15 m/min. After this, they cooled down for five minutes at five m/min. Mice that refused to run for the duration of the test were removed from the study. After the first day, the acclimation protocol calls for a HIIT simulation where the mice ran at increasing repetitions of the high and low speed cycle. On day 2 the mice will begin with a warmup procedure of one minute at a speed of five m/min, another minute at ten m/min, followed by ten minutes at a speed of 15m/min. Following the warmup, the mice performed five cycles of HIIT, one minute at 17m/min, followed by two minutes at 15m/min. Once the HIIT cycles were done, the mice cooled down for ten minutes at 12m/min. On the following days, the same warmup and cool-down protocols were used, and the number of HIIT cycles increased: day-3 = six cycles, day-4 = eight cycles, day-5 = ten cycles.

2.3.6 Functional Capacity Measurements

Balance, strength and exercise capacity were measured at 2-week intervals over five time points (weeks 1, 3, 5, 7, and 9) after acclimation. The functional tests were performed sequentially in the same order: rota-rod, hanging test, and maximal exercise test. The % improvements were calculated for week 3, 5, 7, 9 compared to week 1 and were named M1, M2, M3, and M4 respectively.

2.3.6.1 Rota-Rod – Balance and Coordination

The Rota-Rod machine was used to measure neuromuscular control (Aartsma-Rus & Putten, 2014). The Rota-Rod is a rotating cylinder two inches in diameter separated into six lanes (Figure 2-1). The protocol was composed of a five-minute warm-up protocol, which included time on the machine and time to rest. Following the warmup, the mice were placed back onto the machine, and the test began when the surface began rotating at a speed of six revolutions per minute (1 m/min). Mice that fell within ten seconds were returned to the surface to restart the protocol. The Rota-Rod score was determined based on the time it took for the mice to fall (minimum ten seconds) to the nearest second. After this test, a six-minute break was given before the next test.

2.3.6.2 Hanging Test – Isometric Muscle Strength

The static muscular wire hang test required a Kondziela's wire screen (a 45 cm by 30 cm wire screen) to hang the mice upside-down, and the time before falling was recorded to the closest second (Figure 2-1) (Aartsma-Rus & Putten, 2014). The timer started as soon as the surface was flipped, then the screen was placed about 70 cm from the ground over a bin filled with paper bedding to cushion the fall. Mice that fell within 30 seconds of the start were given a second chance. A maximum of three chances were given if they fell before 30 seconds, and the best of the three attempts was taken as the score. Holding impulse was calculated by multiplying the time

spent hanging with the body weight at that time (Hoffman & Winder, 2016; Chen et al., 2021). This allowed us to normalize the data to the variations in body weight between mice.

2.3.6.3 Maximal Exercise Test – Maximal Exercise Capacity

The maximal exercise test was performed on the custom-built rodent treadmill (Figure 2-1) (Bouganim & Bergdahl, 2017). At stage 0, the treadmill was ten m/min for three minutes. Following this, the speed was increased in increments of 3.3 m/min for each consecutive stage. Wire brushes were used as a humane form of tactile motivation to encourage the mice to run. The maximal exercise score and maximal running speed were determined based on the stage and the running speed the mice attained once they reached exhaustion. The mice were considered to have reached exhaustion once they stopped running or remained behind the fatigue line: 15 cm from the wire brush for ten consecutive seconds.

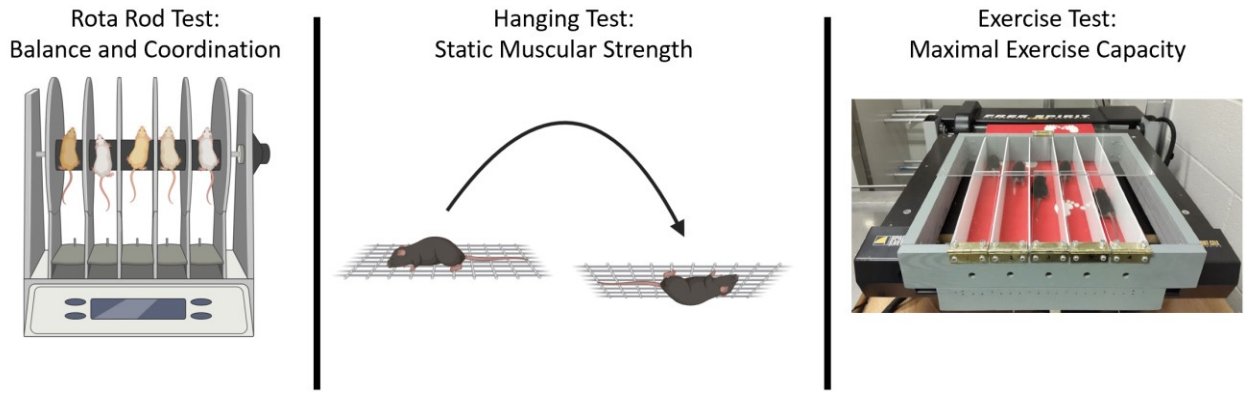

Figure 2-1 - Functional tests.

2.3.7 High-Intensity Interval Training (HIIT) Protocol

The HIIT was performed according to the protocol outlined in the paper published by Caru and colleagues in 2019 (Caru et al., 2019). A custom-built treadmill with five lanes housing individual mice was used (Figure 2-1) (Bouganim & Bergdahl, 2017). The exercising groups (E and TE) performed this exercise five days per week (Monday – Friday). The protocol began with a 12-minute warmup procedure where the treadmill was set at five m/min for one minute, at 10 m/min

for one minute, and 15 m/min for 10 minutes. Following the warmup, the mice performed five cycles at high and low intensity speeds. The protocol had a one-to-two ratio for time spent between the high-intensity and low-intensity speeds, meaning they spent one minute at 75% of their maximal running capacity and two minutes at 50% of their maximal running speed. The treadmill was set at a 12-degree incline for all runs (Bouganim & Bergdahl, 2017). After completing ten cycles, the mice ran 10 m/min for 10 minutes to cool down.

2.3.8 Mitochondrial Physiology

2.3.8.1 Euthanizing and Tissue Collection

After the last week of the HIIT exercise (week 10), the mice were euthanized (using CO_2 asphyxiation). The following tissues were extracted: blood plasma, left ventricular heart tissue, right kidney, urine, and the right vastus lateralis muscle. These samples were stored at -80ºC for protein content analysis.

2.3.8.2 Tissue Preparation

The heart was transported in a chilled physiological salt solution vial to preserve tissue viability. A portion of the apex was cut off and gently pulled apart to increase myocardial fibre surface area without damaging the cells. The tissue was then placed in a five μM solution of saponin diluted in BIOPS to permeabilize the cell membranes for 30 minutes and placed on a shaker as previously described (Kuznetsov et al., 2008). Following the permeabilization, two consecutive 10-minute wash cycles were completed, where the tissue was placed in two new MIRO-5 buffer solutions. The resulting tissue was then dried with light pressure on paper, and 1.8 – 2.5 mg of tissue were weighed and placed into the Oxygraph well containing 2 ml of Miro-5 buffer. The chamber was then hyper-oxygenated and left to calibrate oxygen levels for 30 minutes.

2.3.8.3 Substrates

Changes in energy metabolism within the cardiomyocytes, were measured using the Oroboros Power O2K-Respirometer – commonly referred to as an Oxygraph, which is a high resolution respirometry device (O2k, OROBOROS Instruments, Innsbruck, Austria). The substrates added were pyruvate (5µl) + malate (4µl), ADP (20µl), Cytochrome C (5µl), succinate (20µl), oligomycin (1µl), FCCP (1µl), and antimycin (1µl), respectively.

2.3.9 Plasma TMAO Concentration

Blood collected from the heart's right ventricle was centrifuged at 3000 rotations per minute (RPM) for eight minutes. The plasma was stored at -80ºC. Ultra-performance liquid chromatography-tandem mass spectrometry was used to establish the concentration of TMAO, TMA and choline in the collected plasma.

Ultra-high performance liquid chromatography – tandem electrospray ionization mass spectrometry (UHPLC – ESI - MS/MS or UPLC-MS/MS) is the gold standard for measuring plasma TMAO, TMA and choline levels. The protocol used in this study was aligned with the protocol outlined in a previously published paper (Iglesias-Carres et al., 2022). There are two main parts to this technique: the liquid chromatography and the mass spectrometry.

2.3.10 Statistical Analysis

All analyses were performed using SPSS-25 (IBM). Each outcome was first screened with a two-way ANOVA (Sex × Group). Where a significant main effect of Sex or Sex×Group interaction was detected, the outcome was analyzed sex-separately (i.e., group effects tested within each sex independently); otherwise, sexes were pooled.

Normality was assessed using the Kolmogorov-Smirnov test. For normally distributed outcomes, a one-way ANOVA was performed followed by Tukey's Honestly Significant Difference (HSD)

post-hoc test for pairwise comparisons. Tukey HSD was chosen because it controls the family-wise error rate across all pairwise comparisons, thereby correcting for multiple comparisons. For outcomes that violated normality, a Kruskal-Wallis test was used for between-group comparisons. This general approach was applied to plasma metabolite concentrations (primary aim), cardiomyocyte mitochondrial respiration data (secondary aim), and body weight, food, and water consumption data (Appendix A).

For functional capacity outcomes (tertiary aim), percent improvement from baseline (week 1) was calculated for balance (Rota-Rod), static muscular strength (hanging test), and maximal exercise capacity. A mixed-model ANOVA with Tukey HSD post-hoc was used for normally distributed data. Non-parametric alternatives included the Friedman test with Wilcoxon signed-rank test for within-subject effects and the Kruskal-Wallis test for between-group effects. Significance was set at $\alpha = 0.05$ for all tests.

2.4 Results

2.4.1 Primary Aim: Plasma TMAO Concentration

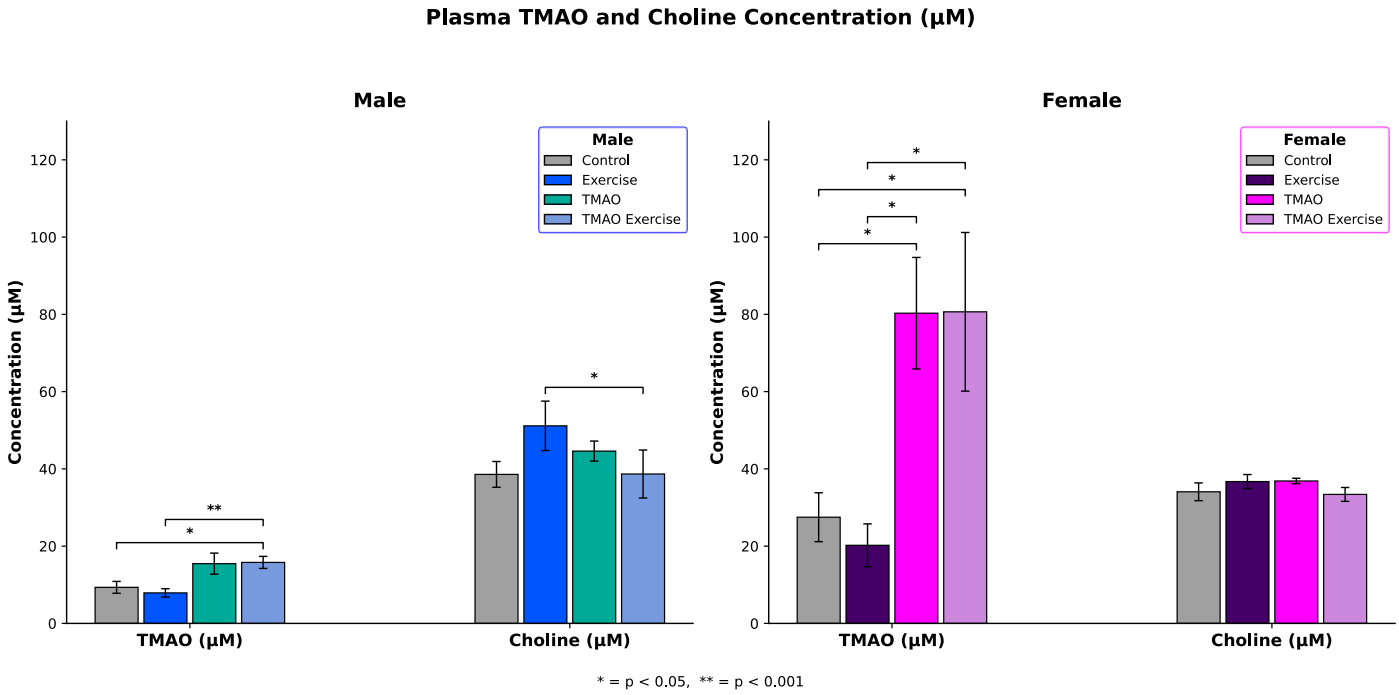

*Figure 2-2- Plasma TMAO and Choline Concentration for male and female mice. *= <0.05, **=<0.001, Error bars as SEM*

There was a slightly larger concentration of plasma [TMAO] in the male TE group compared to group C and E ($P<0.05$, $P<0.001$) (Figure 2-4). In the female groups, there was a significantly larger [TMAO] in the plasma of the groups T and TE compared to groups E and C ($P<0.05$). There was less choline found in male TE than E ($P<0.05$). In the female groups, there were no differences between the groups for plasma choline concentration.

2.4.2 Secondary Aim: Myocardial Mitochondrial Respiration

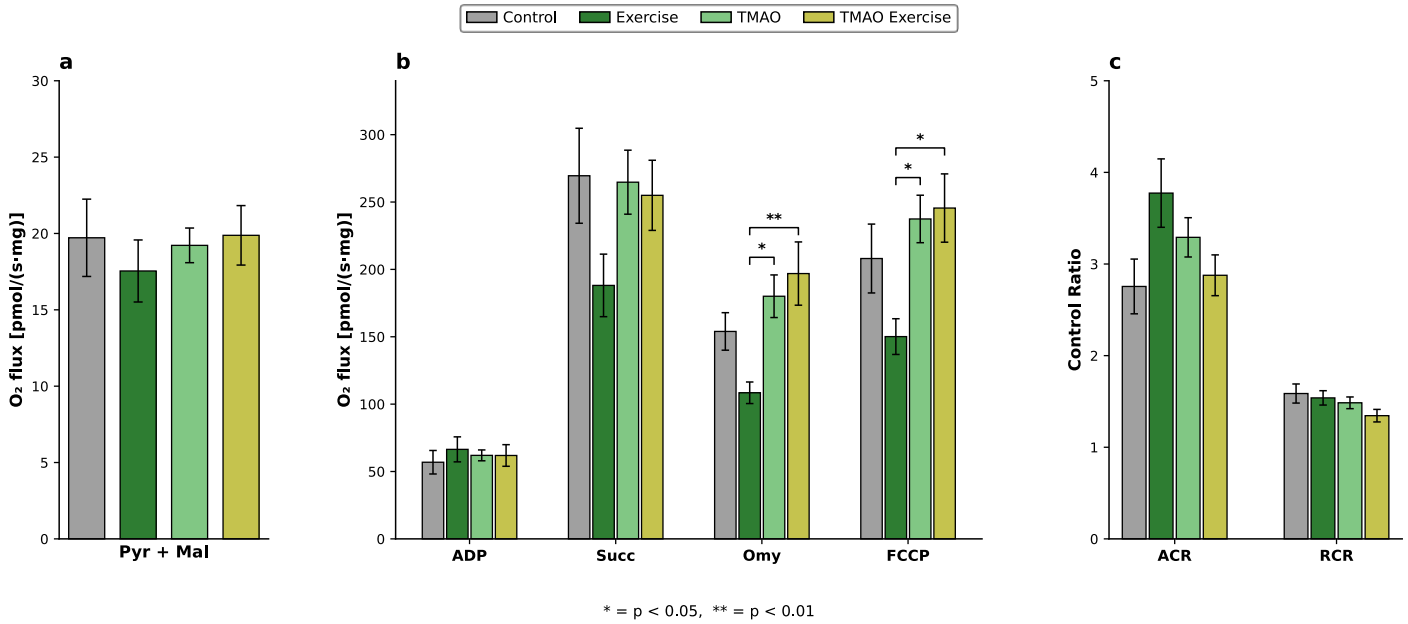

*Figure 2-3- Respirometric Measurements - Sex Combined. * < 0.05, **< 0.01. Error bars as SEM*

Due to the lack of statistically significant differences across sexes, the data were pooled together for respirometry measurements (Figure 2-3). Flux, the rate at which O_2 was consumed was only statistically significantly different between the E and T group ($P<0.05$) as well as the E and TE groups ($P<0.01$) at the addition of oligomycin and FCCP, respectively.

2.4.3 Tertiary Aim: Functional Capacity Measurements

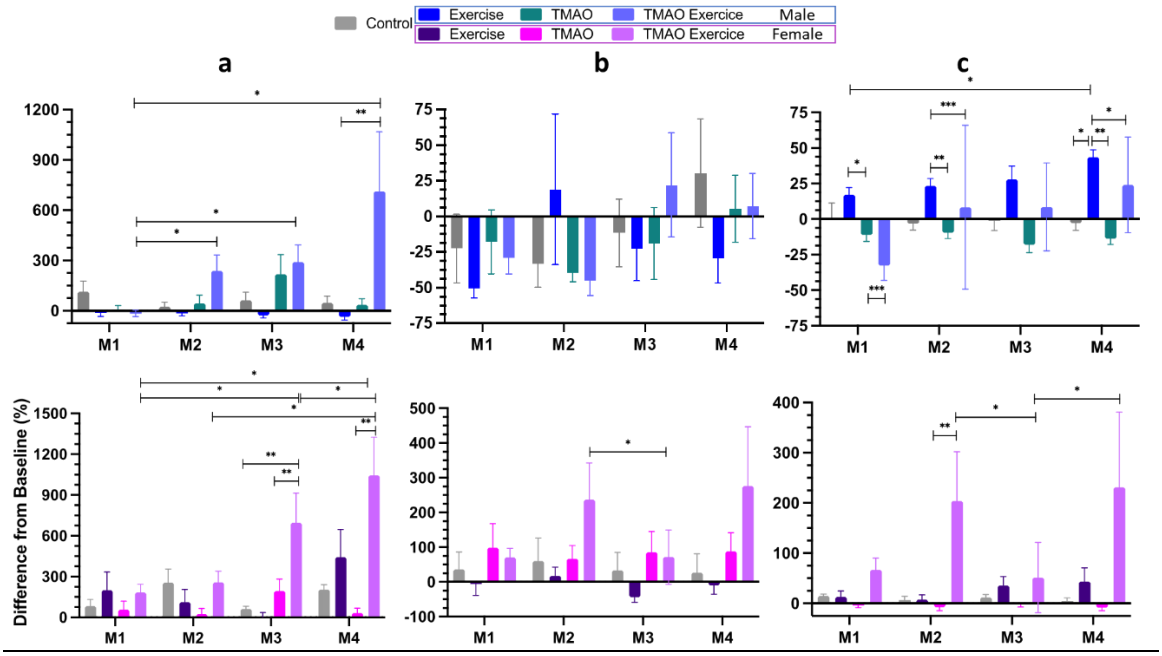

*Figure 2-4 - Functional capacity Measurements over time and change from baseline (M1–M4). (a) Rotarod performance (latency to fall, seconds), (b) wire-hang strength (holding impulse, s×g), and (c) maximal exercise capacity (time-to-exhaustion, seconds). *= <0.05, **=<0.001 Y-axis for all panels: percent change from baseline (week 1), %. X-axis: follow-up time points labeled with both the week and index – Week 3 (M1), Week 5 (M2), Week 7 (M3), Week 9 (M4). Bars show ± SEM; Groups: C (control), T (TMAO), E (exercise), TE (TMAO exercise)*

2.4.3.1 Rota Rod

All male rota rod groups remained near baseline except for the TE group (Figure 2-4a). They showed statistically significant increases in percent difference from baseline between weeks 3, 5, 7, and 9 ($P<0.05$). At week 9, there was a statistically significant difference between the male E and TE groups ($P<0.001$). The improvement made by week 9 was over 600% for the male TE which is much higher than the improvements made by the other groups. The female mice of the TE group improved throughout the 9 weeks with statistically significant differences in balance between week 3 and 7 and 9 ($P<0.05$), week 5 and 9 ($P<0.05$), and week 7 and 9 ($P<0.05$). At week 7, there was a statistically significant difference in improvement between C and TE as well as T and TE ($P<0.001$). At week 9, there was a significant difference between T and TE ($P<0.001$).

2.4.3.2 Hanging Impulse

No statistically significant differences were found in the male Hanging Impulse measurements (Figure 2-4b). All animals decreased in performance from their baseline values during the 9 weeks. In the female groups a statistically significant decrease in performance was observed between week 5 and 7 of TE ($P<0.05$) in the Hanging Impulse measurements. In general, all animals, except for E, improved from their baselines during the 9 weeks. All groups tended to maintain around the same amount of improvement, except for TE, which had an increasing trend of improvement, except for week 7, where there was a reduced improvement compared to week 5 ($P<0.05$) and 9.

2.4.3.3 Maximal Exercise Test

The largest magnitude of improvement in the maximal exercise test over 9 weeks was observed in the male mice in group E relative to the other functional tests ($P<0.05$) (Figure 2-4c). Male mice in group E showed a statistically significant increase in percent improvement from week 3 to 9 ($P<0.05$). The mice of TE showed progressive improvement over time without any statistically significant differences. The mice in group T showed progressively decreasing in maximal exercise performance relative to baseline values without any significant differences. At week 3, the male mice in group E had improved more than those in group T ($P<0.05$), and those in group TE had a larger magnitude in decreased performance relative to male mice in group T ($P<0.0001$). At week 5, there was a statistically significant difference between E and T as well as E and TE ($P<0.001$; $P<0.0001$). At week 9, there was a significant difference between E and C, T and TE ($P<0.05$; $P<0.001$; $P<0.05$). In general, the female mice in C retained consistent improvement, those in E slightly improved over time, and those in T retained consistent deterioration, all without significance. Those in TE had improvements over time except for week 7, where there was a reduced improvement compared to weeks 5 and 9 ($P<0.05$).

2.4.4 Water, Consumption, Food Consumption and Body Weight

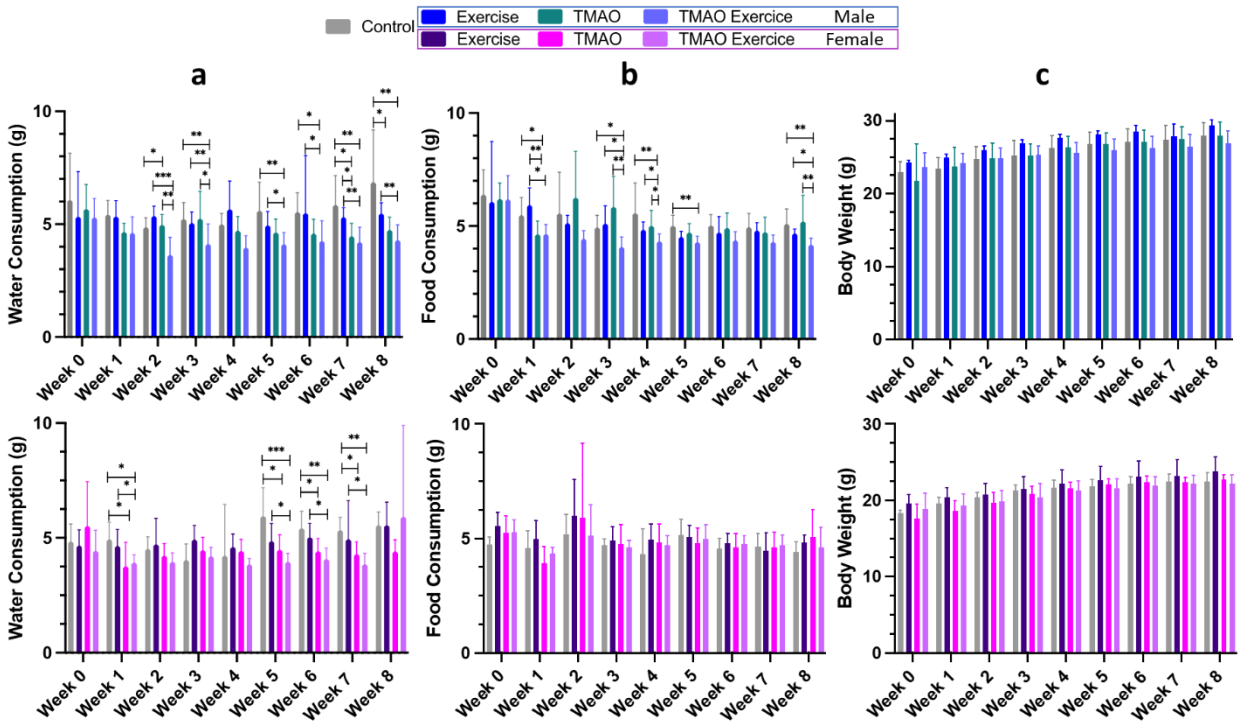

Figure 2-5 - Daily Water Consumption
(a) Daily Food Consumption (b) and Weekly Body Weight (c) for male and female mice from Week 0 to Week 8
= <0.05, **=<0.001, *=<0.0001, Error bars as SEM*

2.4.4.1 Water Consumption

Male C group mice generally consumed more water than other groups, though intake did not differ significantly at weeks 0, 1, and 4 (Figure 2-5a). The maximum difference between male groups was <3 g/day. No clear pattern was observed in females, although significant group differences were detected at weeks 1, 5, 6, and 7, all <2 g/day.

2.4.4.2 Food consumption

The TE group consumed the least amount of food, but did not demonstrate significance at weeks 0,2,6, and 7 (Figure 2-5b). There was only a gap of around 2 g daily. There was no significant difference between the female groups.

2.4.4.3 Body weight

There was no significant difference in body weight for both sexes as seen in Figure 2-5c.

2.5 Discussion

The objectives of this study were to identify if HIIT could be used as an effective method of reducing plasma TMAO, reducing the adverse effects of TMAO on cardiomyocytes and functional capacities. The results showed that the HIIT protocol did not help reduce plasma TMAO concentrations in the mice. Based on previously published papers, we hypothesized that functional capacities and cardiomyocyte respiration would be reduced in TMAO-consuming groups, which would be corrected or improved by the exercise intervention.

2.5.1 Functional Capacity

Our functional capacity findings highlight sex-dependent effects of TMAO in the context of exercise. In males, TMAO supplementation impaired maximal exercise capacity, and HIIT partly mitigated this, consistent with our initial hypothesis of a deleterious effect of TMAO on performance. By contrast, females in the TE group showed improvements, exceeding both female E mice and all male groups. These sex differences suggest that TMAO may not uniformly impair performance and may even potentiate adaptations in females.

Our most prominent finding, which met our expectations, was that of the last functional capacity measured: maximal exercise capacity. There was no previously published data on the impact of high serum TMAO concentration on functional capacity measurements. However, in the assumed context of reduced cardiac bioenergetics, we expected to find a decreased balance on the rota rod machine, strength in the hanging test, and reduced exercise capacity in the T group compared to C and an increase in these metrics from T to TE. The results demonstrated the expected trend for the male mice. The E group showed a progressive increase in maximal capacity while the C group remained constant near 0% improvement. This is because the HIIT exercise was an adequate protocol for improving maximal exercise capacity as it met the specificity criteria. The T group showed worsening in exercise capacity, while the TE group began with a decrease and

progressively improved, but remained behind the E group. This demonstrates that TMAO decreased maximal exercise capacity, reducing their ability to perform, and that HIIT was a valuable tool to help reduce the negative impact of TMAO. In the Female model, this worsening of the T group and the close to 0% improvement of the C group was also observed. However, the TE group showed an improvement, almost double that of the E group. It is also worth noting that the female mice in TE performed more than twice as well as the male mice in their same or even in the E group. This is likely because female mice are known to have higher endurance exercise capacity compared to males (Holcomb et al., 2022).

Figure 2-4a shows percent improvement in rotarod performance from baseline for male and female mice. Counterintuitively, the E group showed the worst balance outcomes in males, suggesting that HIIT alone was not an appropriate stimulus for improving motor coordination. The T group performed comparably to C, indicating that TMAO supplementation did not impair balance. However, unexpectedly, we showed that the TE group of both sexes have improved their balance and coordination significantly over time, week over week. Unexpectedly, the TE group of both sexes demonstrated progressive, week-over-week improvements in balance and coordination, reaching over 600% improvement from baseline in males by Week 9. The superior performance of the TE group, significant in males at Weeks 5, 7, and 9, and in females at Weeks 7 and 9, suggests that TMAO may potentiate exercise-induced neuromotor adaptation through a mechanism that may involve antioxidant signaling. One plausible pathway involves TMAO-mediated activation of the Nrf2 antioxidant signaling pathway, which Zou et al. (2024) demonstrated reduces skeletal muscle oxidative stress and improves exercise performance in male C57BL/6J mice. Attenuated reactive oxygen species accumulation during repeated HIIT bouts could thereby preserve neuromuscular function and facilitate the postural micro-corrections required for rotarod

performance. However, Nrf2 pathway activity was not measured in the present study, and this interpretation remains speculative. Finally, the larger percent improvement observed in females compared to males is consistent with prior reports of superior motor coordination and faster balance acquisition in female mice. (Kovács & Pearce, 2013; Tucker et al., 2016).

Figure 2-4b shows the changes in hanging impulse, a weight-standardized measure of isometric endurance. In males, all groups declined from baseline over the 9 weeks, with no significant differences between groups. This was unexpected, as HIIT has been reported to improve grip strength in both humans and murine models(Seldeen *et al.*, 2018*a*; Defi *et al.*, 2021). The lack of improvement in the male T group also suggests that TMAO alone did not affect isometric endurance in males. In females, the pattern differed considerably. The TE group showed significantly greater hanging impulse than other groups at Weeks 5 and 9 (M2 and M4, $p < 0.05$), while the Exercise group declined. This sex-specific response mirrors the rotarod findings and is consistent with evidence that female mice exhibit greater fatigue resistance under isometric conditions. As proposed by Zou et al. (2024), TMAO-mediated activation of the Nrf2 antioxidant pathway may reduce oxidative stress during exercise, which could preserve muscular endurance; however, why this effect was limited to females in the present study remains unclear and may relate to sex differences in FMO3-mediated TMAO metabolism or hormonal influences on muscle recovery.

2.5.2 Plasma TMAO Concentration

At euthanasia, plasma [TMAO] was significantly higher in the TE group compared with both C and E, indicating that HIIT did not lower TMAO despite previous reports that exercise can reduce circulating levels (Zhang *et al.*, 2023). In females, higher TMAO in C and E compared with males is consistent with elevated hepatic FMO3 expression in females (Wang *et al.*, 2011; Romano *et*

al., 2015; Randrianarisoa *et al.*, 2016; Xu *et al.*, 2017). However, this sex-based enzymatic difference cannot fully explain the much larger rise between T and TE in females. Elevated TMAO in the TE groups may also relate to functional outcomes: as Zou et al. proposed, higher TMAO could improve exercise performance by reducing ROS formation in skeletal muscle, though their study was limited to male C57BL/6J mice (Zou *et al.*, 2024).

For plasma [choline], only male TE mice showed a significant reduction compared with E. This may reflect increased choline utilization during high-intensity exercise (Penry & Manore, 2008), though sex-based microbiome differences in choline metabolism cannot be excluded. Given the shared housing and genetic background of our mice, the precise reason for this reduction remains unclear.

2.5.3 Mitochondrial Respiration

There is limited literature on mitochondrial respiration in the context of TMAO exposure in murine models. Our study employed a chronic supplementation model, similar to those by Makrecka-Kuka et al. (2017) and Videja et al. (2021), who reported reduced state 1 respiration with pyruvate + malate, and suppression of fatty acid-dependent respiration (Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021). Makrecka-Kuka et al. observed that acute TMAO administration immediately before respirometry further decreased mitochondrial activity. In contrast, Naghipour et al. (2023) found that acute TMAO exposure increased respiration in the presence of pyruvate, malate, and glutamate (Naghipour *et al.*, 2023). To our knowledge, these represent the only published mitochondrial respiration studies involving TMAO in animal models. We expected a general reduction in mitochondrial function in the TMAO-supplemented groups based on prior findings. However, in our study, significant differences were only observed at state 1 (after oligomycin) and

state 3 (after FCCP), where the Exercise group exhibited significantly lower respiration compared to both the TMAO ($p < 0.05$) and TMAO Exercise ($p < 0.01$) groups.

This pattern suggests that chronic exercise alone promotes mitochondrial adaptations that reduce proton leak and uncoupled respiration, consistent with tighter coupling and improved respiratory efficiency (Perry *et al.*, 2010; Zhang *et al.*, 2017; Harper *et al.*, 2021). The elevated oxygen consumption at oligomycin and FCCP in the TMAO and TMAO Exercise groups indicates greater uncoupling, which may reflect increased reactive oxygen species involvement. Notably, young ApoE$^{-/-}$ mice already exhibit tighter basal mitochondrial coupling than wild-type C57BL/6 controls (Rocha *et al.*, 2013), and TMAO supplementation in this dyslipidemic background may impose additional oxidative stress on an already constrained bioenergetic system. This interpretation aligns with reports that TMAO compromises mitochondrial structure and function (Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021). However, the relationship between mitochondrial uncoupling and functional performance appears paradoxical. Zou et al. (2024) demonstrated that TMAO supplementation enhanced swim exhaustion time and reduced skeletal muscle oxidative stress through Nrf2 pathway activation in male C57BL/6J mice. Our functional data align with these findings: TMAO Exercise males showed progressive improvement in maximal exercise capacity, and TMAO Exercise females showed the largest improvements across all functional tests. This suggests that two concurrent processes may be occurring: altered myocardial mitochondrial coupling at oligomycin and FCCP, alongside enhanced antioxidant capacity in skeletal muscle that preserves or improves exercise performance. Whether Nrf2-mediated antioxidant signaling also operates in the myocardium of TMAO-supplemented mice was not tested in the present study and represents an important direction for future investigation.

An important consideration is the use of the ApoE$^{-/-}$ dyslipidemic model. These mice exhibit altered baseline mitochondrial bioenergetics compared to wild-type C57BL/6 controls, including lower Complex I linked basal flux but tighter overall coupling (ACR approximately 12 vs 5 in C57BL/6; Rocha et al., 2013). This pre-existing bioenergetic profile may have influenced the magnitude and direction of TMAO's effects on cardiac mitochondria. Specifically, the elevated uncoupled respiration observed at oligomycin and FCCP in the TMAO groups may represent an amplified response in a system already operating under lipid-related metabolic stress. Whether wild-type mice would show the same pattern of TMAO-induced changes at these respiratory states remains unknown. Additionally, the ApoE$^{-/-}$ background predisposes mice to atherosclerosis and altered lipid handling, which may interact with TMAO's established pro-atherogenic effects in ways that would not be observed in a normolipidemic model. Future studies comparing TMAO supplementation across both genotypes would help distinguish TMAO-specific effects from those attributable to the dyslipidemic phenotype.

2.5.4 Food, Water, Body Weight

Food, water, and body weight measurements showed no consistent or biologically relevant effects of TMAO supplementation or HIIT. Although some week-to-week differences reached statistical significance, they were small (<3 g/day for water; <1 g/day for food), inconsistent between sexes, and likely attributable to random variation or measurement error. Body weight increased normally in all groups, with no clear impact of TMAO or HIIT on growth.

2.5.5 Future directions

Since many of the results of this study go against the published literature, it would be important to see if they are reproducible. It is possible that there were acute variations in the presence and

impact of TMAO, which were not picked up in our experiment due to bad timing between the last administered exercise and euthanasia. It is also possible that not enough time was given to see deteriorations in functional capacities. Furthermore, as research with female rodents is often avoided in cardiovascular research, it is important to intentionally include them in future studies to see how they differ from the male model and to better understand female physiology, which, according to our study, seems to show differences compared to their male counterparts. It would also help to track the estrous cycle to ensure this is accounted for and see if it has an impact.

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3 Chapter 3 – Second Manuscript

Investigating the effects of TMAO on functional capacities and bioenergetics of liver, aorta, and skeletal muscle in mice performing voluntary running exercise.

Kevork H. Atamian, Shreya Ramdhooneea, Andreas Bergdahl

Contribution of authors

Kevork H. Atamian: animal handling, surgeries, respiration measurements, statistical analysis, preparation of manuscript.

Shreya Ramdhooneea: animal handling, preparation of manuscript.

Andreas Bergdahl: Editing of manuscript.

3.1 Abstract

Trimethylamine N-oxide (TMAO) is a liver-oxidized molecule from trimethylamine (TMA) produced by the gut microbiome during the digestion process and has been implicated in cardiovascular disease (CVD) pathogenesis, prompting investigations into its metabolic consequences (Wang et al., 2011). There is a paucity of research in the literature about the bioenergetics linked to TMAO's presence in the body. This study investigated the effects of chronic TMAO supplementation on functional capacity and mitochondrial respiration in the liver, aorta, and skeletal muscle tissues of mice engaged in voluntary wheel running. Male and female mice were randomized into control and TMAO groups and monitored over 9 weeks. Functional performance was assessed using Rota-Rod (balance), Hanging Impulse (strength), and maximal treadmill exercise testing. Mitochondrial respiration was analyzed via high-resolution respirometry using the Oroboros Power O2K-Respirometer. Contrary to our initial hypothesis, TMAO-treated mice showed improved balance and aerobic capacity performance, particularly in males. Mitochondrial analyses revealed a significant reduction in ADP-stimulated respiration and acceptor control ratio (ACR) in the liver of TMAO-treated mice. At the same time, no differences were observed in aortic or skeletal muscle tissues. These findings suggest that liver mitochondria may be especially susceptible to TMAO's metabolic effects, whereas peripheral tissues are either resistant or compensating functionally.

This study reveals complex, tissue-specific responses to chronic TMAO exposure, with paradoxical improvements in physical performance despite evidence of impaired hepatic mitochondrial efficiency. Further research is needed to elucidate underlying mechanisms and potential sex-specific vulnerabilities.

3.2 Introduction

Cardiovascular diseases (CVDs), which affect millions of people annually, are responsible for significant reductions in quality of life (QoL) and mortality worldwide. Active lifestyles can improve QoL and reduce mortality (Schrader et al., 2022). While pharmaceutical interventions remain the primary approach to managing CVDs, they are often associated with side effects and risks. In contrast, lifestyle modifications such as regular exercise and dietary changes offer significant, sustained benefits without adverse effects. Animal research involving exercise interventions can be done through forced or voluntary protocols. It has been shown that the former can cause stress in animals, leading to a negative physiological environment such as increased plasma cortisone levels (Ke et al., 2011; Li et al., 2014). Voluntary physical activity has gained popularity in recent years due to the countless benefits of an active lifestyle. In 2014, it was shown that the running wheel is an effective tool for administering voluntary exercise in murine models since wild mice used running wheels left in an open forest at the same frequency as captive mice (Meijer & Robbers, 2014). The vastus lateralis is a muscle from the quadriceps group commonly used in running. This makes it excellent as a tissue to analyze for changes in muscular tissue linked to exercise.

The human body is host to a complex ecosystem of microorganisms collectively known as the microbiome. This intricate community, primarily within the gut, is pivotal in various physiological processes. They have many roles, such as aiding with digestion and immune function. In 2011, the microbiome was linked to heart disease for the first time (Wang et al., 2011). Dietary carnitine and choline are digested by bacteria in the gut to produce trimethylamine (TMA). This bacterial metabolite is then shuttled to the liver, where it is oxidized to trimethylamine N-oxide (TMAO), a quaternary amide. In the systemic circulation, TMAO concentrations are associated with reduced

cardiac function, atherosclerosis, systemic inflammation, and increased all-cause mortality (Yang et al., 2019; Zhang et al., 2021).

Many research projects have investigated the underlying mechanisms in the pathogenesis of CVDs linked to TMAO. One area of research lacking evidence and overall investigation is the bioenergetic perspective. Makrecka-Kuka and colleagues have shown that state 1 respiration is inhibited in the mitochondria of cardiomyocytes by the supplementation of TMAO in the drinking water of mice (Makrecka-Kuka et al., 2017). Videja and colleagues also showed that chronic supplementation of TMAO reduced fatty acid oxidation. However, in a right ventricular dysfunction model, the metabolite attenuated the decline in cardiac energy metabolism (Videja et al., 2021). More recently, Naghipour and colleagues found that acute exposure to TMAO increased respiration linked to complex I and II (NADH dehydrogenase and succinate dehydrogenase, respectively) (Naghipour et al., 2023). These results show contradicting evidence, highlighting the importance of better understanding the pathways involved.

Further exploration of these phenomena is important to see if similar phenomena occur elsewhere in the body or if it, they are limited to cardiac tissue. The aorta contains smooth muscle cells whose mitochondria are affected in the occurrence by atherosclerotic plaque or similar physiological stressors (Shi & Chen, 2018). Since TMAO is oxidized in the liver and considering its metabolic load, changes in hepatic mitochondrial activity can also be detrimental. Finally, skeletal muscles (SM) are imperative to movement and, since CVDs are often paired with reduced exercise capacity, SM mitochondrial function should be investigated in organisms with high plasma TMAO concentrations.

This experiment aims to identify if the supplementation of TMAO impacts functional capacity measurements and the mitochondrial physiology in the aorta, liver and vastus lateralis muscle. We

hypothesize that TMAO will worsen functional capacity measures by suppressing cellular respiration in the aforementioned tissues.

3.3 Methods

Animals

For this study, 20 dyslipidemic mice (Apolipoprotein E; Apo E -/-) between six and eight weeks old were used from the Concordia University breeding colony (Makrecka-Kuka *et al.*, 2017; Videja *et al.*, 2021; Brunt *et al.*, 2021*b*). The animals were kept in a temperature-controlled room at 22°C with a 12-hour light cycle. Animals were housed in individual cages that contained a running wheel where activity data was collected (ClockLab) and kept in circadian cabinets (Phenome Technologies) where environmental conditions were set by Actimetrics ClockLab software. The animals comprised an equal number of males and females. Since no significant observations were made between sex and plasma TMAO levels in previous experiments, this assumption was held in the development of the methodology (Argyridou et al., 2020). The procedures followed the guidelines of the Canada Council on Animal Care and were approved by the Animal Ethics Committee of Concordia University (#30000259). Using stratified randomization, the 20 animals, composed of equal parts male and female, were assigned to two groups (Control and TMAO) and placed individually in clear acrylic cages. All animals consumed regular mouse chow. The control groups consumed regular water, whereas the treatment group had TMAO supplemented to their water at 120g per kg body weight per day. The required TMAO was purchased as a pure crystal from Cayman Chemical Company, Michigan, USA.

3.3.1 Balance and Coordination

The Rota-Rod was used to test balance and coordination. The protocol started with three minutes of warmup and a two-minute resting period in their cages. The mice were returned to their

respective lanes, and a timer was started at the same time as the rod. The time before falling was recorded to the nearest second; the longer they stayed on the rod, the more balance they had.

3.3.2 Isometric Strength

A wire screen measuring of 45 cm by 30 cm was used to test for isometric muscle strength, where the mice hung upside down (Figure 3-1) (Aartsma-Rus & Putten, 2014). The procedure was performed by placing the mouse in the center of the screen and flipping it. The time before falling was measured to the nearest second. To normalize the data to the variations in body weight, holding impulse was calculated by multiplying the time spent hanging by the body weight at that time (Hoffman & Winder, 2016; Chen et al., 2021).

3.3.3 Maximal Exercise Testing

To test the maximal exercise capacity, a custom-built rodent treadmill was employed following a previously published protocol (Figure 3-1) (Bouganim & Bergdahl, 2017). After a three-minute warmup stage at a speed of ten meters per minute, the speed was increased by three m/min until the mice either stopped running or stayed longer than 10 seconds behind the line of fatigue (15 cm from the wire brush) (Caru et al., 2019).

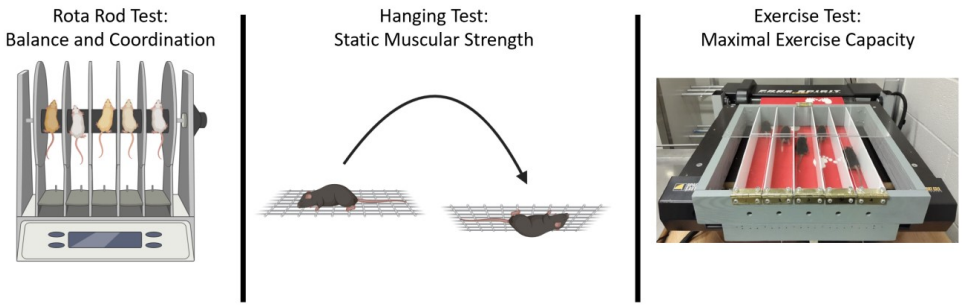

Figure 3-1 - Methods used to establish Functional Capacity Measurement

3.3.4 Tissue Collection and Preparation

At the end of the ninth week, the mice were euthanized using CO_2 asphyxiation and cervical dislocation. Aorta, liver and the vastus lateralis tissues were collected and stored in chilled physiological salt solution for mitochondrial respirometric analysis. Liver tissue was cut into small

cubes and placed into a MIRO-5 buffer solution until it was weighed. The aorta were cleaned, under microscope, of adipose tissue, cut open, endothelial tissue rubbed off using a sponge, and shredded as illustrated in Figure 3-2. Skeletal muscle was cut into small portions and pulled apart to increase the surface area of the tissue, all while ensuring that the cellular integrity was not damaged. In order to permeabilize the aorta and skeletal muscle tissue, a protocol from Kuznetsov and colleagues was followed (Kuznetsov et al., 2008). The muscle and artery were placed in their respective containers in a five μM solution of saponin diluted in BIOPS and placed on a shaker plate for 30 minutes. Following this step, they were placed into MIRO-5 buffer for ten minutes which was repeated. The liver tissue is permeable; thus, this process is not performed for this tissue. After the wash cycles, the aorta, muscle, and liver tissues were placed on filter paper to absorb wet substances. Light pressure was applied to help. 2 – 5 mg, 3.5 – 4.5 mg, and 3 – 4 mg were weighed and placed in the chambers in 2ml of MIRO-5 buffer.

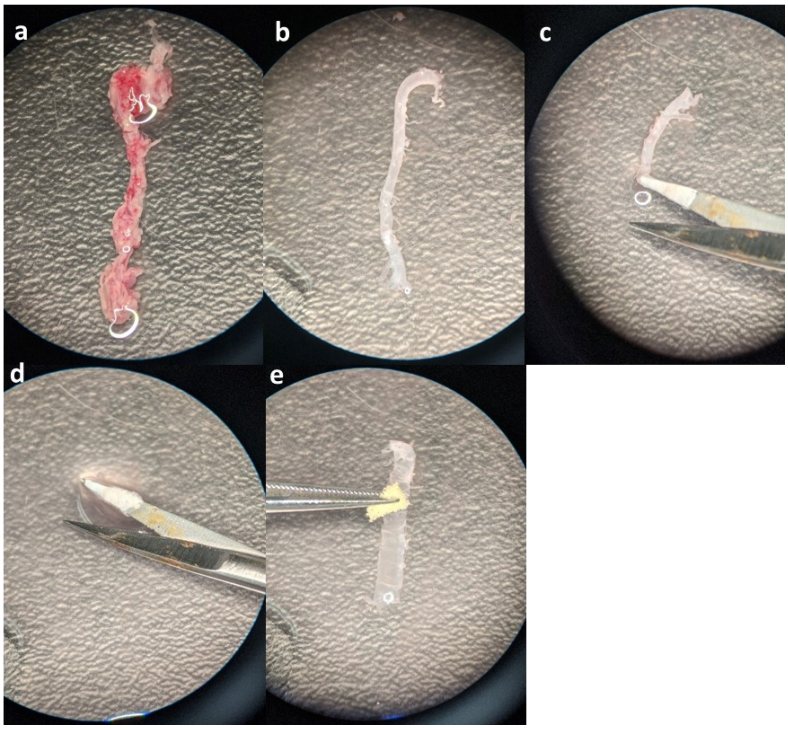

Figure 3-2 - Cleaning of the mouse aorta under microscope. a) Aorta as extracted from the mouse. b) Aorta after being cleaned up. c) Aorta being placed onto a scissor to be cut. d) Aorta entirely placed onto a scissor before being cut along its length. e) Cleaning the inside of the aorta by rubbing the epithelial cells with a sponge.

3.3.5 Mitochondrial Respiration Measurement

The Oxygraph was used to measure oxygen consumption by adding substrates (O2k, OROBOROS Instruments, Innsbruck, Austria). The chambers were capped, and oxygen was injected to hyper-oxygenate the solution and calibrate oxygen levels for 30 minutes. Malate (4µl), glutamate (10µl), pyruvate (5µl) were used first, followed by ADP (20µl), cytochrome C. (5µl), succinate (20µl), oligomycin (1µl), FCCP (1µl), and antimycin (1µl).

3.3.6 Statistical Analysis

All analyses were performed using SPSS-25 (IBM). Each outcome was first screened with a two-way ANOVA (Sex × Group). Where a significant main effect of Sex or Sex×Group interaction was detected, the outcome was analyzed sex-separately, that is, males and females were analyzed independently without pooling, and group effects were tested within each sex. Otherwise, sexes were pooled for between-group comparisons.

Normality was assessed using the Kolmogorov-Smirnov test. For normally distributed outcomes, a one-way ANOVA was performed followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise comparisons. Tukey HSD was chosen because it controls the family-wise error rate across all pairwise comparisons, thereby correcting for multiple comparisons. For outcomes that violated normality, a Kruskal-Wallis test was used. This approach was applied to mitochondrial respiration measurements and to weekly averages of body weight, food consumption, and water consumption.

For functional capacity outcomes, percent improvement from baseline (week 1) was calculated to account for large interindividual variability. A mixed-model ANOVA with Tukey HSD post-hoc was used for normally distributed data. Non-parametric alternatives included the Friedman test with Wilcoxon signed-rank test for within-subject effects and the Kruskal-Wallis test for between-group effects. Significance was set at $\alpha = 0.05$ for all tests.

3.4 Results

Functional Capacity Measurements

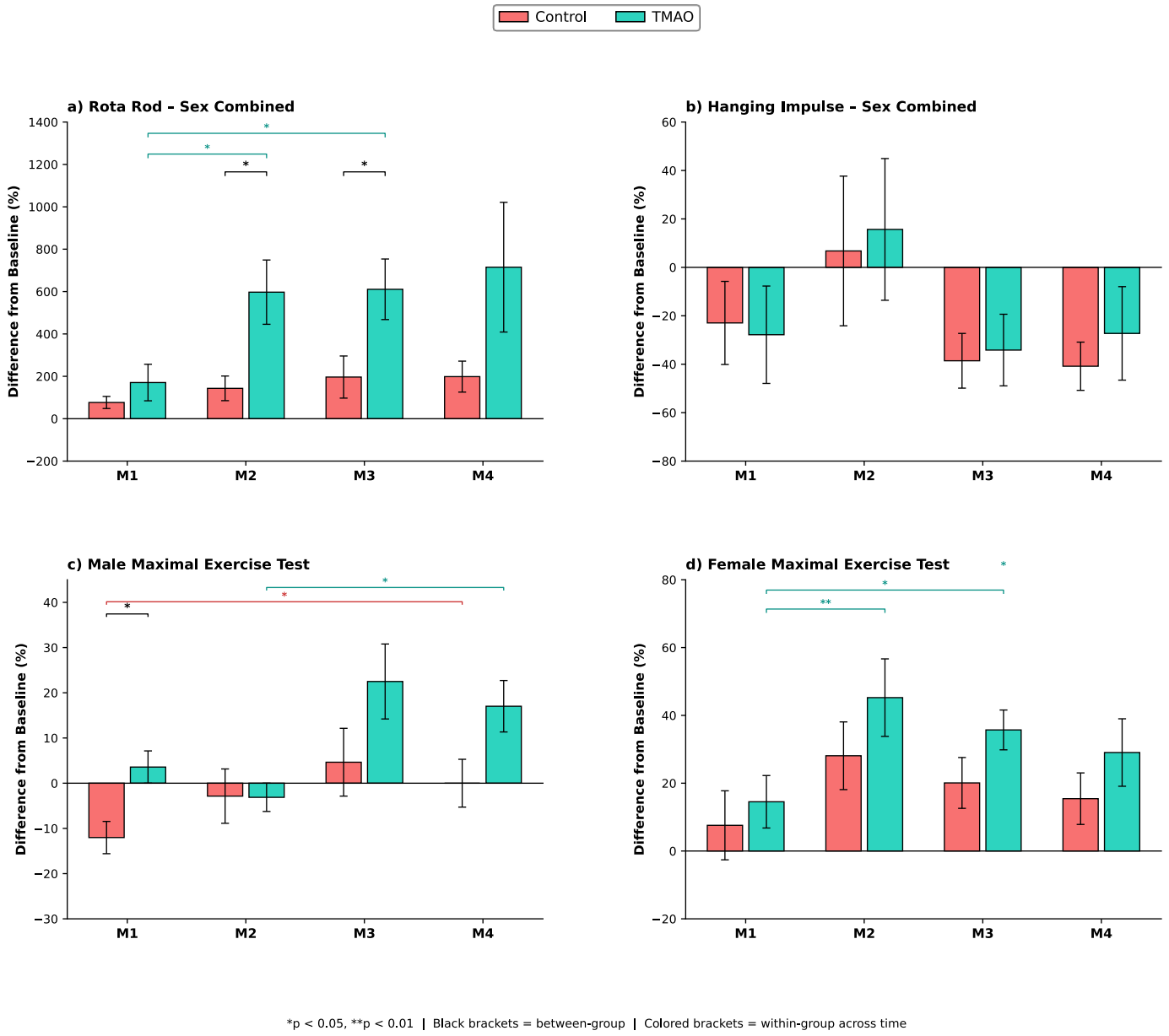

Figure 3-3 - Functional Capacity Measurements.
*a) Rota-Rod – Sex Combined, b) Hanging Impulse – Sex Combined, c) Male and d) Female Maximal Exercise Test. *p <0.05, Error bars as SEM*

3.4.1.1 Rota Rod

Since no significant main effect of sex or Sex×Group interaction was observed, a sex-combined analysis was used. The mixed-model ANOVA revealed a significant main effect of Group ($F(1,13) = 4.949$, $p = 0.044$), indicating that the TMAO group demonstrated greater improvement in balance than Controls across the study. The main effect of Time approached significance after Greenhouse-Geisser correction ($p = 0.057$), and the Group×Time interaction was not significant

(p = 0.168). Between-group comparisons at each timepoint showed that the TMAO group had significantly greater improvement than Controls at Week 5 (p = 0.019) and Week 7 (p = 0.034), but not at Week 3 (p = 0.325) or Week 9 (p = 0.136). Within-group paired comparisons showed that the TMAO group improved significantly from Week 3 to Week 5 (p = 0.020) and from Week 3 to Week 7 (p = 0.039), whereas the Control group showed no significant changes across any timepoint (Figure 3-3a).

3.4.2 Hanging impulse

Since no significant main effect of sex or Sex×Group interaction was observed, a sex-combined analysis was used. The mixed-model ANOVA revealed no significant main effect of Group ($F(1,15) = 0.022$, $p = 0.884$), Time (GG-corrected $p = 0.103$), or Group×Time interaction ($p = 0.746$). Between-group comparisons at each timepoint showed no significant differences (all $p > 0.54$). Both groups declined from baseline at most timepoints, and neither group demonstrated consistent improvement or deterioration across the 9-week period (Figure 3-3b).

3.4.3 Maximal exercise test

Due to a significant Sex×Time interaction, the analysis was performed separately for male and female mice.

In males, the mixed-model ANOVA revealed a significant main effect of Time (GG-corrected $p = 0.023$), indicating that performance improved across the study regardless of group. The main effect of Group was not significant ($p = 0.111$), nor was the Group×Time interaction ($p = 0.328$). Between-group comparisons showed that the TMAO group had significantly higher percent improvement than Controls at Week 3 ($p = 0.018$), while Week 9 approached significance ($p = 0.060$). Within-group comparisons showed that male Controls improved significantly from Week

3 to Week 9 (p = 0.028), while the male TMAO group improved significantly from Week 5 to Week 9 (p = 0.013) (Figure 3-3c).

In females, the mixed-model ANOVA revealed a significant main effect of Time (GG-corrected p = 0.004), indicating that both groups improved over the study period. The main effect of Group was not significant (p = 0.251), nor was the Group×Time interaction (p = 0.716). No between-group differences reached significance at any timepoint (all p > 0.14). However, within-group comparisons revealed that the TMAO group improved significantly from Week 3 to Week 5 (p = 0.003) and from Week 3 to Week 7 (p = 0.010), whereas the Control group showed no significant within-group changes at any comparison. Additionally, the TMAO group showed a significant decline from Week 5 to Week 9 (p = 0.024), suggesting that the initial improvement was not sustained (Figure 3-3d).

3.4.4 Water, Consumption, Food Consumption and Body Weight

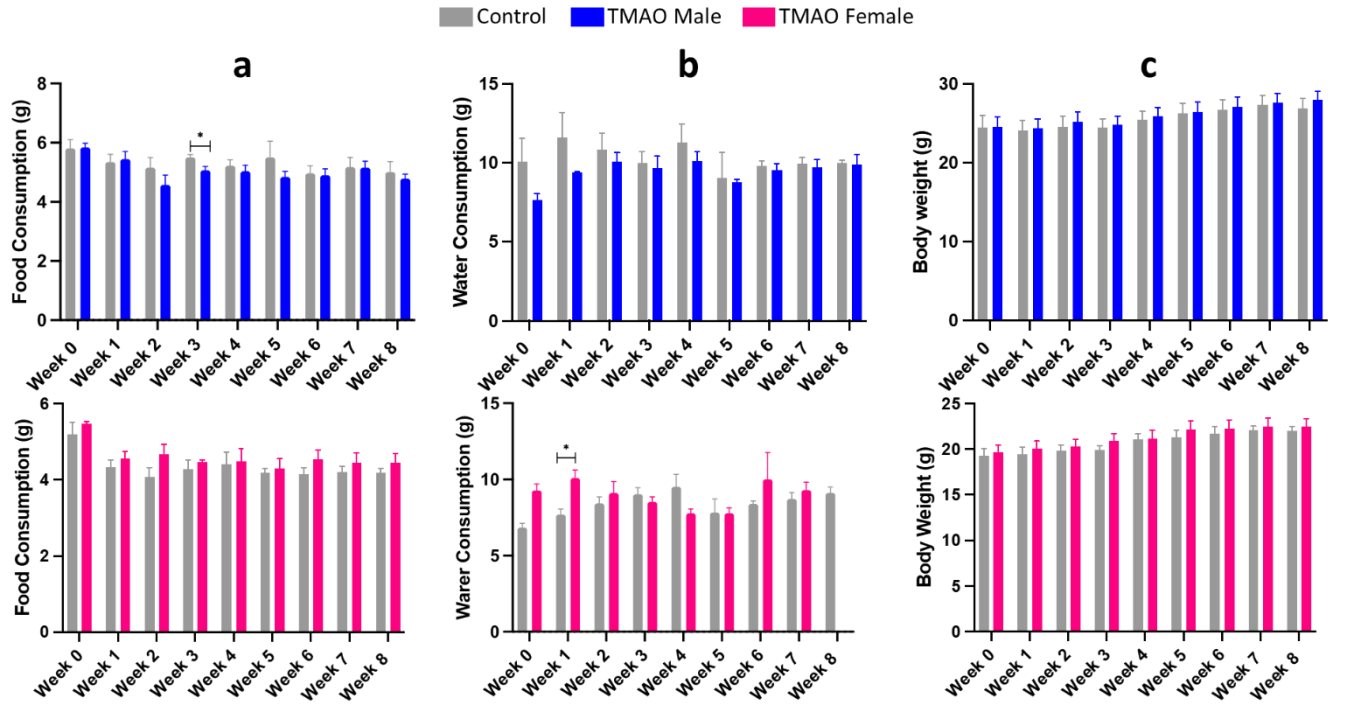

Figure 3-4 - Physical Characteristics from Week 0 to Week 8.
*a) Daily Food consumption, b) Daily Water consumption, c) Weekly Weight Gain. * p<0.05, Error bars as SEM*

3.4.5 Food consumption

In general, both male groups consumed a very similar quantity of food daily in all weeks, but there was a significant difference between groups for week 3 of 0.4356g ($P<0.05$) (Figure 3-4a). Both groups of female mice consumed similar food throughout the experiment.

3.4.6 Water Consumption

Although no significance was found, the male control group drank more water between weeks 0 and 4, with the largest difference at week 0 of 2.2283g. The female TMAO group consumed slightly more water in weeks 0, 1, 2, 6 and 7; however, only week 1 showed a significant difference of 1.5929g ($P<0.05$). (Figure 3-4b)

3.4.7 Body weight

There was no significant difference in body weight measured for both sexes; however, there were trends of increasing weight from week 0 to 8 in line with increasing body size with age (Figure 3-4c).

3.4.8 Mitochondrial Respiration

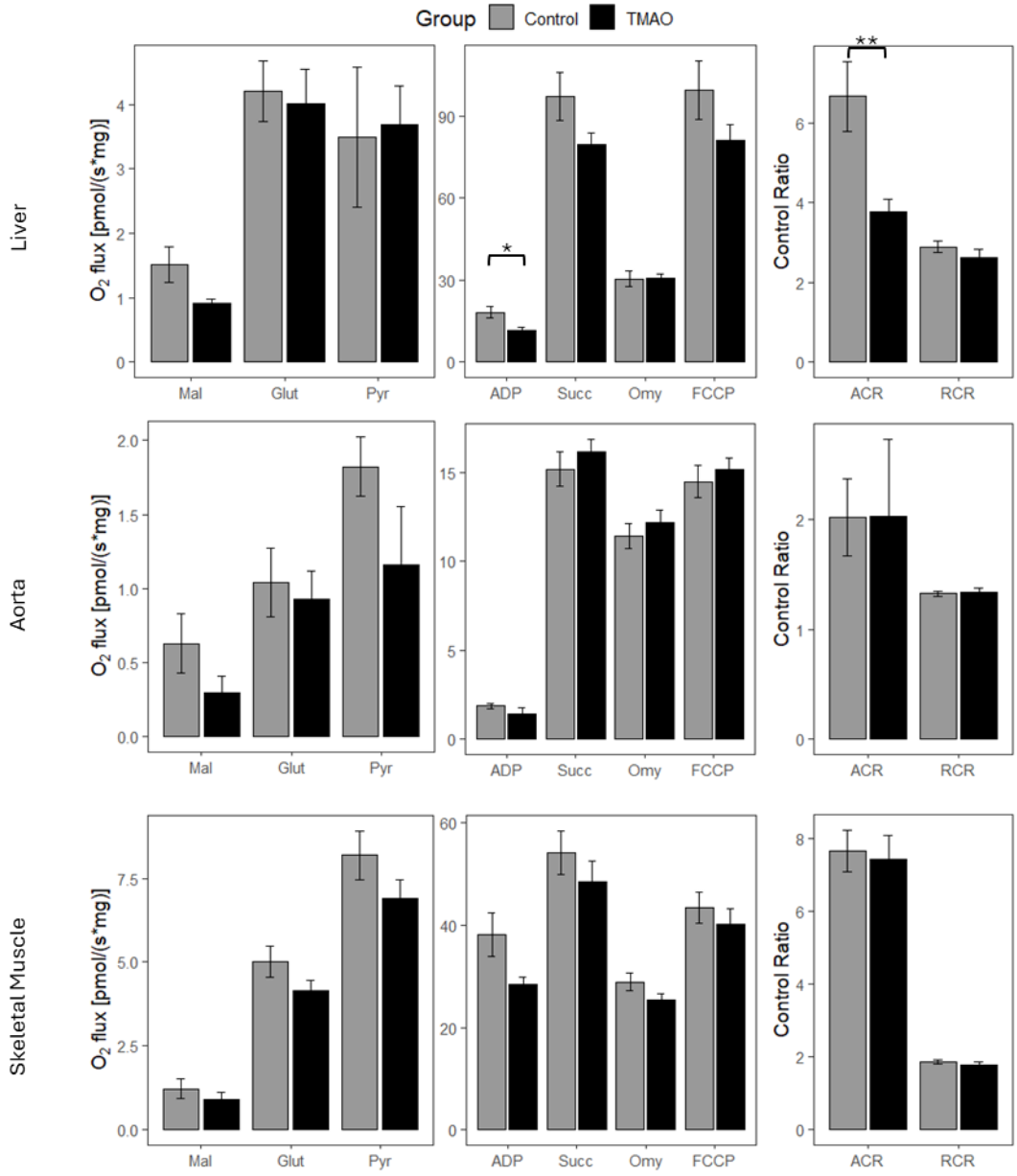

*Figure 3-5 - Mitochondrial Respiration for Liver, Aorta. and Skeletal Muscle. *p <0.05, **p<0.01, Error bars as SEM*

3.4.8.1 Liver

In the liver, upon addition of ADP, the TMAO group showed a significantly lower flux relative to the Exercising Control group ($p < 0.05$). No group differences were observed following succinate, oligomycin, or FCCP stimulation (Figure 3-5). Notably, the acceptor control ratio (ACR) was

significantly reduced in the TMAO group ($p < 0.01$), reflecting the reduced ADP induced flux, while the respiratory control ratio (RCR) remained unchanged between groups.

3.4.8.2 Aorta

No statistically significant differences in oxygen flux were observed in the aortic tissue between the TMAO and Exercise groups for any of the substrates or mitochondrial modulators (Figure 3-5). Although the TMAO group demonstrated numerically higher flux for the complex I substrates (malate, glutamate, pyruvate), these trends were not significant. Similarly, ACR and RCR values did not differ between groups.

3.4.8.3 Skeletal Muscle

All substrate-driven respiration rates in skeletal muscle trended higher in the TMAO group, particularly for glutamate and pyruvate (Figure 3-5). A trend toward higher ADP-stimulated respiration was observed ($p = 0.055$), although this did not reach statistical significance. No differences were found for succinate, oligomycin, or FCCP-driven flux. Both ACR and RCR were comparable between groups.

3.5 Discussion

This study aimed to identify if chronic TMAO supplementation impacted functional capacity measurements and the mitochondrial respiration of liver, skeletal muscle and aortic smooth muscle cells. Based on previous results from studies on cardiomyocytes, we believed that this type of supplementation would have a harmful effect to respiration (Makrecka-Kuka et al., 2017; Videja et al., 2021). Our results are in a different direction from previously published studies; however, they are similar to our previous study investigating the effects of forced exercise on TMAO-consuming mice (Chapter 2).

3.5.1 Mitochondrial Respiration

To our knowledge, this is the first time that liver, smooth muscle, and skeletal muscle mitochondrial bioenergetics have been analyzed in the context of chronic TMAO supplementation. Contrary to our hypothesis, TMAO had no significant effect on mitochondrial respiration in aortic smooth muscle or skeletal muscle, suggesting that these peripheral tissues are either resistant to TMAO's metabolic effects or can compensate functionally.

In contrast, liver tissue showed a significant reduction in ADP-stimulated respiration and a lower acceptor control ratio (ACR) in the TMAO group. Two interpretations of this finding merit consideration.

The first is that TMAO impairs hepatic mitochondrial function upstream of the electron transport system. Previous studies have demonstrated that TMAO reduces pyruvate dehydrogenase activity and pyruvate flux, limiting acetyl-CoA availability and impairing downstream oxidative phosphorylation (Makrecka-Kuka et al., 2017). Since the liver is the primary site of TMA oxidation via FMO3 and is directly exposed to circulating TMAO, it may be particularly susceptible to TMAO-induced metabolic disruption. Chronic exposure may result in substrate limitation or mild mitochondrial damage, consistent with observations of hepatic oxidative stress and inflammation following prolonged TMAO administration(Florea *et al.*, 2024).

An alternative interpretation, however, is that the reduced hepatic respiration reflects a physiological adaptation rather than impairment. Under normal conditions, the liver actively converts TMA to TMAO via FMO3, a process that consumes molecular oxygen and requires ATP. When TMAO is supplemented directly in the drinking water, exogenous TMAO may bypass this enzymatic step and reduce the metabolic demand on hepatic FMO3. The resulting decrease in liver energy expenditure would lower the requirement for ADP-stimulated oxidative phosphorylation,

which would manifest as reduced ADP-linked respiration and a lower ACR, not due to mitochondrial dysfunction, but due to a reduced need for electron coupling with ATP production. This interpretation is consistent with the observation that no differences were found at FCCP-uncoupled respiration, which reflects maximal electron transport capacity independent of ATP demand. If TMAO were directly damaging the electron transport system, one would expect reductions in uncoupled respiration as well.

These two interpretations are not mutually exclusive; chronic TMAO exposure may simultaneously reduce FMO3-related metabolic demand while also exerting subtle oxidative stress on hepatic mitochondria. Distinguishing between these mechanisms would require direct measurement of hepatic FMO3 activity and expression, which was not performed in the present study and represents an important direction for future research.

3.5.2 Functional Capacity Measurements

In the RotaRod test**,** both sexes in the TMAO group showed early improvements in balance and coordination. These results suggest that, rather than impairing neuromotor function, TMAO may facilitate adaptations related to balance and coordination, at least in the early phases of exposure.

The hanging impulse test did not reveal significant group differences, although all mice generally declined from their baselines over time. These results indicate that TMAO did not have a measurable impact on isometric muscular endurance.

In contrast to our original hypothesis, TMAO supplementation was associated with improvements in the maximal exercise test, particularly in male mice. The TMAO group significantly outperformed controls at the first measurement, with a similar trend observed by week 9 without significance. These findings partially mirror our previous study. In the present study, the male

control group showed minimal change in performance, whereas the TMAO group improved, suggesting a potential ergogenic effect of TMAO on aerobic capacity.

The magnitude of these functional improvements warrants consideration of their biological relevance. In the rotarod test, the TMAO group averaged over 500% improvement from baseline by Week 5, compared to approximately 140% in Controls. This represents a several-fold increase in time-to-fall, reflecting a meaningful enhancement in postural control on an accelerating surface, not merely a marginal statistical difference. In the maximal exercise test, female TMAO mice improved by approximately 45% from baseline at Week 5, compared to 28% in Controls. While this between-group difference did not reach statistical significance due to sample size ($n = 5$ per group), the consistent direction of effects across all timepoints and the significant within-group improvement in the TMAO group (Week 3 to Week 5, $p = 0.003$; Week 3 to Week 7, $p = 0.010$) suggest a real effect that the study was underpowered to detect between groups. These improvements are comparable in magnitude to those reported in murine HIIT studies using larger sample sizes (Seldeen *et al.*, 2018*a*).

As with Manuscript I, the use of ApoE$^{-/-}$ mice introduces a baseline metabolic context that may influence the interpretation of these results. The liver in dyslipidemic mice already handles an elevated lipid burden, and the addition of exogenous TMAO may interact with pre-existing alterations in hepatic lipid metabolism. The significant reduction in ADP-stimulated respiration observed in the liver may therefore reflect a compounding of metabolic load rather than a TMAO-specific effect that would be seen in wild-type animals. Similarly, the absence of effects in aortic and skeletal muscle tissue may indicate that these peripheral tissues in the ApoE$^{-/-}$ model have

already adapted to chronic lipid stress, potentially masking additional TMAO-induced changes. Comparative studies using C57BL/6 controls would be needed to resolve this.

3.5.3 Future directions

Our research focused on a severely underexplored part of the search to elucidate the underlying physiological role of TMAO in reduced cardiac function. Because of this, we could not anticipate tests to run to explain specific outcomes. As such, plasma TMAO concentrations were not measured. Since there were male-female differences in outcomes, it would have been important to track the estrous cycle to see if some differences could be attributed to this or if they could be shown not to be associated with it. It would be important to perform protein blotting to determine differences in expression, which could explain TMAO-based or sex-based differences.

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4 Chapter 4

4.1 Concluding Remarks

This thesis investigated the effects of the gut-derived metabolite TMAO on mitochondrial physiology and physical performance in a dyslipidemic murine model, with two complementary studies examining forced (HIIT) and voluntary (running wheel) exercise paradigms.

In Manuscript I, HIIT did not reduce plasma TMAO concentrations, contrary to our first hypothesis and to findings from dietary intervention studies (Erickson et al., 2019; Zhang et al., 2023). This is consistent with evidence that renal excretion, not exercise-mediated clearance, is the dominant elimination route for TMAO (Taesuwan et al., 2017). Regarding cardiac mitochondrial respiration, TMAO did not impair basal substrate-driven flux (pyruvate + malate, ADP, or succinate). However, significant differences emerged at oligomycin-stimulated and FCCP-uncoupled states, where the Exercise group exhibited lower respiration than both the TMAO ($p = 0.033$ and $p = 0.032$, respectively) and TE ($p = 0.005$ and $p = 0.014$, respectively) groups. This pattern suggests that chronic exercise promotes tighter mitochondrial coupling and reduced proton leak, whereas TMAO may disrupt these exercise-induced adaptations. Functional capacity results were sex-dependent: in males, TMAO impaired maximal exercise capacity and HIIT partially mitigated this effect; in females, the combination of TMAO and HIIT produced marked improvements in balance and exercise capacity that exceeded those of exercise alone.

Manuscript II extended the bioenergetic analysis to liver, aortic smooth muscle, and skeletal muscle using a voluntary running wheel model. TMAO had no significant effect on mitochondrial respiration in aortic or skeletal muscle tissue. However, hepatic mitochondria showed a significant reduction in ADP-stimulated respiration and acceptor control ratio in TMAO-treated mice,

indicating either impaired mitochondrial coupling or a physiological downregulation of hepatic energy demand secondary to reduced FMO3 activity when TMAO is supplied exogenously. Functional capacity results were consistent with Manuscript I: TMAO supplementation was associated with improved balance (significant overall Group effect, $p = 0.044$), and in females, the TMAO group demonstrated significant within-group improvements in maximal exercise capacity from baseline to Week 5 ($p = 0.003$) and Week 7 ($p = 0.010$), whereas Controls showed no significant change over time.

Integrating Functional, Metabolic, and Mitochondrial Findings

A central question arising from these results is why TMAO-supplemented mice, particularly females, demonstrated improved functional performance despite the metabolite's established association with cardiovascular disease. Synthesizing across the three data streams provides a partial answer. Cardiac mitochondrial function was largely preserved in TMAO-supplemented animals: the significant differences at oligomycin and FCCP were limited to comparisons with the Exercise group and did not indicate impairment relative to Controls. This preservation of cardiac bioenergetics is consistent with the absence of exercise intolerance in the TMAO groups. In the liver, the reduction in ADP-stimulated respiration may reflect decreased FMO3-related metabolic demand rather than dysfunction, as maximal (uncoupled) respiratory capacity was unaffected. In peripheral tissues (aortic smooth muscle and skeletal muscle) no mitochondrial differences were observed, suggesting that the cellular machinery supporting locomotion and muscular endurance remained intact. Together, these mitochondrial data indicate that chronic TMAO supplementation at the dose used (120 mg/kg/day) did not produce the systemic bioenergetic impairment that would be expected to limit physical performance, which is consistent with the functional results.

Sex Differences: Plasma TMAO and Functional Performance

The most consistent finding across both manuscripts was the sex-dependent response to TMAO, evident at both the metabolic and functional levels. Plasma TMAO concentrations were substantially higher in females than males across all groups in Manuscript I. In the T and TE groups, females had approximately five-fold higher plasma concentrations than their male counterparts, with relatively small error bars suggesting a consistent biological difference rather than random variation. This sex difference is consistent with the known higher hepatic FMO3 expression in female rodents, which results in more efficient conversion of TMA to TMAO (Wang et al., 2011; Romano et al., 2015; Xu et al., 2017). Paradoxically, despite these higher circulating TMAO levels, females in both manuscripts demonstrated equal or superior functional performance compared to males. In Manuscript I, female TE mice showed over 600% improvement in balance from baseline by Week 9, more than double that of the male TE or female E groups, and their maximal exercise capacity improvement exceeded that of all other groups. In Manuscript II, the female TMAO group showed significant within-group improvements over time while female Controls did not. The magnitude of these improvements is not only statistically significant but physiologically substantial, representing a several-fold increase in time-to-fall on the rotarod. This dissociation between high plasma TMAO and improved performance challenges the assumption that elevated TMAO is uniformly harmful and raises the possibility that TMAO, or its downstream effects, may interact with sex-specific physiological pathways to enhance exercise adaptation under certain conditions. Whether this interaction involves sex hormones, sex-linked differences in skeletal muscle fiber composition, or other mechanisms remains to be determined.

4.2 Future Directions

Several limitations of this work point toward productive avenues for future research. First, the present studies supplemented TMAO directly in drinking water, bypassing the gut microbiome. It

remains unclear whether diets rich in TMAO precursors (choline, carnitine) would produce comparable effects, as microbiome-dependent TMA production introduces additional variability. Comparing direct supplementation with precursor-rich diets could clarify whether TMAO itself, or the broader metabolic milieu associated with its production, drives the observed effects.

Second, the sex-specific differences in functional capacity and plasma TMAO concentrations observed across both manuscripts warrant targeted mechanistic investigation. Measuring hepatic FMO3 protein expression, tracking the estrous cycle, and examining sex hormone interactions with TMAO metabolism would help determine whether the superior performance of female TMAO-supplemented mice reflects hormonal influences, differences in TMAO clearance, or other sex-linked physiological factors. Future studies should include sex as an explicit variable in study design and hypotheses rather than treating it as an incidental finding.

Third, the finding that hepatic ADP-stimulated respiration was reduced in TMAO-treated mice could reflect either mitochondrial impairment or a reduced metabolic demand on the liver when TMAO is provided exogenously. Direct measurement of FMO3 enzymatic activity alongside mitochondrial respirometry would distinguish between these interpretations.

Fourth, protein expression analyses (e.g., Western blot for mitochondrial complex subunits, PGC-1α, and Nrf2 pathway components) were not performed in the current studies. Such data would provide mechanistic insight into whether TMAO alters mitochondrial biogenesis, antioxidant signaling, or respiratory chain composition, and would help explain the tissue-specific pattern of effects observed across heart, liver, aorta, and skeletal muscle.

Fifth, the small sample sizes in certain subgroup analyses (n = 4–5 per group in sex-separated functional tests) limited statistical power. Several comparisons showed large effect sizes but did

not reach statistical significance, suggesting real biological differences that this study was underpowered to detect. Future studies should be adequately powered based on the effect sizes reported here.

Finally, translation to human models, using controlled dietary interventions alongside structured exercise programs, will be necessary to determine the clinical relevance of these findings. Given the sex-specific outcomes observed in mice, particular attention should be paid to recruiting balanced sex proportions and stratifying analyses by sex in human trials investigating TMAO and exercise.

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