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Rīga Stradiņš University

Melita Ozola (Vidējā)

Regulation of Trimethylamine N-oxide in Treatment of Cardiometabolic Diseases

Doctoral Thesis for obtaining the scientific degree
"Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Basic Medicine
Sub-Sector – Pharmaceutical Pharmacology

Riga, 2023

Melita Ozola (Vidējā)

ORCID 0000-0002-3453-0099

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Supervisor of the Doctoral Thesis:

Dr. pharm., Professor **Maija Dambrova**,
Latvian Institute of Organic Synthesis, Rīga Stradiņš University, Latvia

Scientific Advisors:

Dr. pharm., **Marina Makrecka-Kūka**, Latvian Institute of Organic Synthesis

Dr. pharm., **Jānis Kūka**, Latvian Institute of Organic Synthesis

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Abstract

Trimethylamine N-oxide (TMAO) is a gut microbiota-derived metabolite synthesised in host organisms from specific food constituents, such as choline, carnitine and betaine, that are highly abundant in products of animal origin. During the last decade, elevated TMAO level has been proposed as biomarker to estimate the risk of cardiometabolic diseases. In addition, elevated concentrations of TMAO have been associated with type 2 diabetes, heart failure, incidence of major adverse cardiovascular events and all-cause mortality. However, there is still no consensus on the exact role of TMAO in the pathogenesis of cardiovascular diseases, since regular consumption of TMAO-rich seafood, is considered beneficial for the primary prevention of cardiovascular events. Therefore, the objective of the present Thesis was to study TMAO-mediated signalling pathways in the pathophysiology of cardiometabolic diseases and to identify possible intervention options.

To assess this aim, 3 preclinical studies and 1 clinical study were conducted. First, the impact of short-term treatment with high TMAO concentrations (up to 1 mM) on cardiac and vascular functionality was evaluated in *ex vivo* and *in vivo* rodent models. Second, the effects of long-term TMAO supplementation (120 mg/kg, 14 weeks) on cardiac functionality and energy metabolism were assessed in a monocrotaline-induced right ventricular heart failure model in rats. Next, the effect of metformin (250 mg/kg daily), the most widely prescribed oral antidiabetic drug worldwide, on TMAO reduction was tested in experimental model of type 2 diabetes in mice. Additional experiments with bacterial monocultures were performed to test the possible TMAO regulatory mechanisms of action of metformin. Lastly, a fasting mimicking diet was tested for 5 days in healthy volunteers to observe the potential changes in plasma TMAO levels and general markers of metabolic health.

An acute 2.5-fold increase in TMAO did not affect vascular functionality, but a higher input of fatty acid oxidation was observed in vascular energy metabolism. In cardiac tissue, acute elevation of the TMAO level did not affect cardiac function. However, in detrimental conditions of right ventricular heart failure, long-term administration of TMAO surprisingly prevented impairment of cardiac mitochondrial energy metabolism and preserved right ventricular function. Regarding potential approaches to reduce TMAO levels, metformin was able to decrease plasma concentrations of TMAO in the type 2 diabetes model in mice nearly twofold; these effects can be attributed to the impact of metformin on the composition and activity of intestinal microbiota. In the dietary intervention study, a 5-day cycle of fasting mimicking diet with limited animal-derived protein intake and caloric restriction was effective in reducing TMAO levels and improving overall metabolic health of the volunteers.

To summarise, the obtained results indicate that a short-term increase in TMAO concentrations does not activate detrimental signalling pathways in cardiac and vascular tissues. Meanwhile, long-term elevation of TMAO levels can even serve as a preconditioning factor and protect cardiac function in the right ventricular heart failure model. Furthermore, the results of the present thesis provide evidence on the possibility of targeting TMAO levels using pharmacological approaches, namely metformin, and lifestyle approaches.

Keywords: TMAO, cardiometabolic diseases, mitochondrial functionality, metformin, gut microbiota, fasting mimicking diet

Anotācija

Trimetilamīna N-oksīda regulācija kardiometabolo slimību ārstēšanā

Trimetilamīna N-oksīds (TMAO) ir zarnu trakta mikrobioma metabolīts, kas tiek producēts saimniekorganismā no tādām ar uzturu uzņemtām vielām, kā karnitīns, holīns un betaīns. Šie TMAO prekursori ir plaši sastopami dzīvnieku izcelsmes produktos. Pēdējā desmitgadē paaugstināts TMAO līmenis ir izvirzīts kā biomarķieris kardiovaskulāro un metabolo saslimšanu risku novērtēšanai. Turklāt paaugstināta TMAO koncentrācija ir saistīta ar 2. tipa diabētu, sirds mazspēju, būtisku nevēlamu kardiovaskulāro notikumu biežumu un mirstību. Tomēr joprojām nav vienota viedokļa par TMAO lomu sirds un asinsvadu slimību patoģenēzē, jo augsti TMAO līmeņi ir sastopami arī jūras veltēs un zivīs. Šo produktu regulāra lietošana tiek uzskatīta par primāro sirds un asinsvadu slimību profilaksi. Tāpēc šīs disertācijas mērķis bija pētīt TMAO ietekmētos signālceļus kardiometabolo slimību patoģenēzē, kā arī identificēt potenciālās iespējas TMAO līmeņa regulācijai.

Lai sasniegtu šo mērķi, tika veikti 3 preklīniskie pētījumi un 1 klīniskais pētījums. Pirmkārt, tika izvērtēti TMAO (līdz 1 mM) akūtie efekti uz sirds un asinsvadu funkcionalitāti *ex vivo* un *in vivo* grauzēju eksperimentālajos modeļos. Otrkārt, tika novērtēta ilgtermiņā paaugstināta TMAO līmeņa ietekme (120 mg/kg, 14 nedēļas) uz sirds funkcionalitāti un enerģijas metabolismu monokrotalīna inducētā labā kambara sirds mazspējas modelī žurkām. Treškārt, 2. tipa diabēta eksperimentālajā modelī pelēm tika pārbaudīts TMAO līmeņa samazināšanas potenciāls metformīnam (250 mg/kg dienā), kas ir visplašāk lietotais pretdiabēta medikaments. Papildus tika veikti eksperimenti ar baktēriju monokultūrām, lai noteiktu iespējamos metformīna darbības mehānismus TMAO līmeņu regulācijai. Visbeidzot, tika pārbaudīta piecu dienu badošanos imitējošas diētas kā uztura stratēģijas ietekme uz TMAO līmeni un vispārējo metabolo veselību raksturojošo biomarķieru koncentrācijām veselos brīvprātīgajos.

Akūts TMAO pieaugums (2,5 reizes) neietekmēja asinsvadu funkcionalitāti, bet asinsvadu enerģijas metabolisma procesā tika novērots augstāks taukskābju oksidācijas īpatsvars. Sirds audos akūts TMAO līmeņa paaugstinājums neietekmēja sirds funkciju. Taču labā kambara sirds mazspējas patoloģijas apstākļos ilgstoša TMAO lietošana pārsteidzoši pasargāja no traucējumiem sirds enerģijas metabolismā un palīdzēja saglabāt labā kambara funkciju. Izvirzoties kā iespējama farmakoloģiska stratēģija TMAO līmeņa regulācijai, metformīns divkārt samazināja TMAO koncentrāciju plazmā pelēm 2. tipa diabēta modelī; šo efektu var skaidrot ar metformīna ietekmi uz zarnu mikrobioma sastāvu un specifisku baktēriju aktivitāti. Savukārt, uztura intervences pētījumā, kurā tika pētīta badošanos imitējoša diēta, kas

ietver atteikšanos no dzīvnieku izcelsmes olbaltumvielu uzņemšanas un kaloriju ierobežojumu, tika parādīts, ka šāda uztura stratēģija ir efektīva, lai veseliem brīvprātīgajiem samazinātu TMAO līmeni un uzlabotu vispārējo metabolo stāvokli.

Iegūtie rezultāti liecina, ka īslaicīgs TMAO koncentrācijas pieaugums neaktivizē kaitīgus molekulāros signālceļus sirds un asinsvadu audos, kamēr ilgstoši paaugstināts TMAO līmenis var pat kalpot kā prekondicionējošs faktors, aizsargājot pret labā kambara sirds mazspēju. Šīs disertācijas rezultāti kopumā sniedz pierādījumus par TMAO līmeņu regulācijas iespējām, izmantojot gan farmakoloģisku līdzekli – metformīnu, gan dzīvesstila pieeju.

Atslēgvārdi: TMAO; kardiometabolās slimības; mitohondriālā funkcija; metformīns; zarnu trakta mikrobioms; badošanos imitējoša diēta

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Abbreviations used in the Thesis

Ach	acetylcholine
ADP	adenosine diphosphate
ALT	alanine aminotransferase/glutamate pyruvate transaminase
AmA	antimycin A
ANOVA	analysis of variance
AS	atherosclerosis
ATP	adenosine triphosphate
BMI	body mass index
BNP	brain natriuretic peptide
cDNA	complementary deoxyribonucleic acid
CKD	chronic kidney disease
CoA	coenzyme A
CRP	C-reactive protein
CVD	cardiovascular disease
eGFR	estimated glomerular filtration rate
ERK1/2	extracellular signal-regulated kinase 1/2
ESR	erythrocyte sedimentation rate
F	fatty acid oxidation-dependent pathway
FAO	fatty acid oxidation
FMD	fasting mimicking diet
FMOs	flavin-containing monooxygenases;
FOXO1	forkhead box protein O1
GBB	gamma-butyrobetaine
HDL	high density lipoprotein
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IGF-1	insulin-like growth factor-1
ISO	isoproterenol
K-H	Krebs–Henseleit
K. pneumoniae	*Klebsiella pneumoniae*
LDL	low density lipoprotein
LEAK	substrate-dependent state
LVDP	left ventricular developed pressure
MACE	major adverse cardiovascular events
MCT	monocrotaline
MetS	metabolic syndrome

N	NADH pathway
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NYHA	New York Heart Association
OCT2	organic cation transporter 2
OXPHOS	oxidative phosphorylation
P	pyruvate
P. mirabilis	*Proteus mirabilis*
PC	palmitoylcarnitine
PERK	protein kinase R-like endoplasmic reticulum kinase
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
Rot	rotenone
ROX	residual oxygen consumption
RV	right ventricular
S	succinate
SD	standard deviation
SEM	standard error of the mean
SNP	sodium nitroprusside
T2D	type 2 diabetes
TGF-β1	transforming growth factor- β1
TMA	trimethylamine
TMAO	trimethylamine N-oxide
UPLC/MS/MS	ultra-performance liquid chromatography-tandem mass spectrometry
VEG	reference group in the dietary intervention study subjected to increased vegetable intake

Introduction

Cardiometabolic diseases, which include cardiovascular diseases and metabolic disorders such as obesity and diabetes, remain a leading cause of morbidity and mortality worldwide. In addition to various biomarkers identified previously, studies in the last decade have pointed to a potential association between elevated levels of trimethylamine-N-oxide (TMAO) and the development of atherosclerosis (Wang et al., 2011; Koeth et al., 2013), type 2 diabetes (Dambrova et al., 2016 a), heart failure severity (Tang et al., 2014), and death due to major adverse cardiovascular events (Trøseid et al., 2015). TMAO is a gut microbiota-derived metabolite. Initially, dietary precursors such as carnitine and choline are metabolised by intestinal bacteria, leading to the production of trimethylamine (TMA), which is further oxidised to TMAO in the host liver.

In preclinical studies, TMAO has been shown to promote inflammation (Seldin et al., 2016; Sun et al., 2016) and oxidative stress (Li et al., 2017; Ke et al., 2018). Furthermore, it impairs reverse cholesterol transport (Koeth et al., 2013) and increases the risk of thrombotic events (Zhu et al., 2016). However, the exact molecular mechanisms affected by TMAO and its causal role in the pathogenesis of cardiometabolic diseases are still under debate. Therefore, understanding the signalling pathways through which TMAO exerts its effects and identifying potential therapeutic targets for the regulation of TMAO levels can provide new insights into the prevention and treatment of cardiometabolic diseases.

Although several preclinical studies have suggested that elevated TMAO levels may contribute to the development of cardiometabolic diseases, some controversial results have also been reported (Zeisel and Warrier, 2017). For example, some studies have failed to demonstrate an association between elevated TMAO levels and cardiovascular disease risk in patients (Andraos et al., 2021; Koay et al., 2021), while others have even reported protective effects after chronically increased TMAO levels in preclinical models (Huc et al., 2018; Gawrys-Kopczynska et al., 2020). Additionally, there is still much to be understood about the complex interplay between TMAO, gut microbiota, and host metabolism (Silke et al., 2021) in the development and progression of cardiometabolic diseases.

Further research is required to clarify these discrepancies and provide a more comprehensive understanding of the role of TMAO in cardiometabolic health as well as to investigate potential intervention strategies to target TMAO levels.

Aim of the Thesis

To study TMAO-mediated effects on signalling pathways in the pathophysiology of cardiometabolic diseases and to identify potential interventions.

Objectives of the Thesis

In order to achieve the aim of the Thesis, the following objectives have been set:

1. To investigate the acute effects of increased TMAO bioavailability in *ex vivo* and *in vivo* experimental models of cardiac and vascular function;
2. To investigate the effect of long-term elevation of TMAO in an experimental model of right ventricular heart failure;
3. To assess the potential of metformin to regulate TMAO levels in an experimental model of advanced type 2 diabetes;
4. To test the effectiveness of a fasting mimicking diet in reducing TMAO levels.

Hypotheses of the Thesis

- Elevated TMAO levels activate detrimental pathways that may contribute to the development of cardiovascular and metabolic disorders.
- Circulating TMAO levels can be modified using dietary and pharmacological approaches.

Novelty of the Thesis

The link between TMAO and cardiovascular diseases was first introduced in 2011 and has rapidly gained scientific interest, with more than 300 articles published annually in recent years. Although initially almost all studies reported clearly detrimental association between elevated TMAO levels and cardiovascular and metabolic health, a different theory has recently emerged, attributing some protective effects to TMAO as well.

In the present Thesis, we have attempted to distinguish between the effects of acute and long-term increase in the concentration of TMAO. The information summarised here indicates that the role of TMAO in the pathogenesis of cardiometabolic diseases depends on specific conditions and organ systems, as well as the duration of exposure.

Despite this, there is a growing interest in intervention strategies targeting TMAO levels, especially through modulation of the intestinal microbiota, which is a crucial component in biosynthesis pathway of TMAO. The results presented in the Thesis provide evidence that metformin targets the composition of the intestinal microbiota and alters the rate of TMA production. As patients with cardiovascular diseases are often advised to introduce some

lifestyle modifications to achieve clinically relevant results, we have also shown how a 5-day dietary intervention, a fasting mimicking diet, can help reduce cardiovascular risks by improving metabolic parameters and decreasing circulating TMAO levels.

1 Literature

1.1 Trimethylamine N-oxide

Trimethylamine N-oxide (TMAO) is an organic compound that belongs to the class of amine oxides. It was first mentioned in the scientific literature in 1940s, in relation to the ability of several bacterial genera to reduce it to form trimethylamine (TMA) (Elrod, 1946; Hitchner, 1946). The biosynthesis pathway of TMAO was first described in 1962 (Baker and Chaykin, 1962). From the 1940s to 1970s, TMAO was studied primarily as a nitrogenous waste product produced by marine animals and excreted by the kidneys (Forster, Berglund, and Rennick, 1958).

Another perspective on TMAO emerged in the late 1970s and early 1980s, after one of the first studies discussing the role of TMAO as an osmolyte was published in 1979 (Yancey and Somero, 1979). Initial studies on the physiological role of TMAO were conducted in marine organisms, where TMAO was described as an osmolyte compound, which exhibits protective properties against destabilising factors in the environment, such as temperature and salinity fluctuations, urea saturation, and hydrostatic pressure changes in deep-sea fish (Weber, 1983; Vieyra and Caruso-Neves, 1993). TMAO also helps to regulate and maintain the appropriate secretory epithelial cell volume under changes in osmotic pressure (Kleinzeller, 1985). TMAO has protein stabilising properties and the ability to counteract the effects of protein denaturation caused by hydrostatic pressure and urea in deep-sea fish, restoring proteins to their native state and preserving enzyme functions. A potential mechanism for this effect could be linked to the amphiphilic nature of TMAO, which allows it to form hydrogen bonds with water and interact with specific functional groups of cellular proteins (Ma, Pazos, and Gai, 2014).

However, in 2011 TMAO gained much greater scientific interest, when an untargeted metabolomics study revealed TMAO as a phosphatidylcholine metabolite associated with unhealthy dietary patterns, gut microbiota, and cardiovascular risks (Wang et al., 2011). Furthermore, this study also provided the first evidence on the mechanistic role of TMAO in the development of atherosclerosis (AS), namely up-regulation of several macrophage scavenger receptors associated with AS, increased accumulation of cholesterol in macrophages, and stimulation of foam cell formation. In the following years, TMAO was extensively studied in various patient populations and in the preclinical setting, resulting in more than 2600 articles on TMAO in PubMed database in July 2023.

1.2 Dietary sources and biosynthesis pathways of TMAO

TMAO is a compound that is predominantly found in animals, but it is also present in plants and fungi, its origin being both exogenous and endogenous. In terrestrial animals and humans, TMAO is produced by gut microbiota enzymes after intake of specific dietary constituents, thus forming TMA which is then oxidised to TMAO in the liver of the host organism (summarised in Figure 1.1).

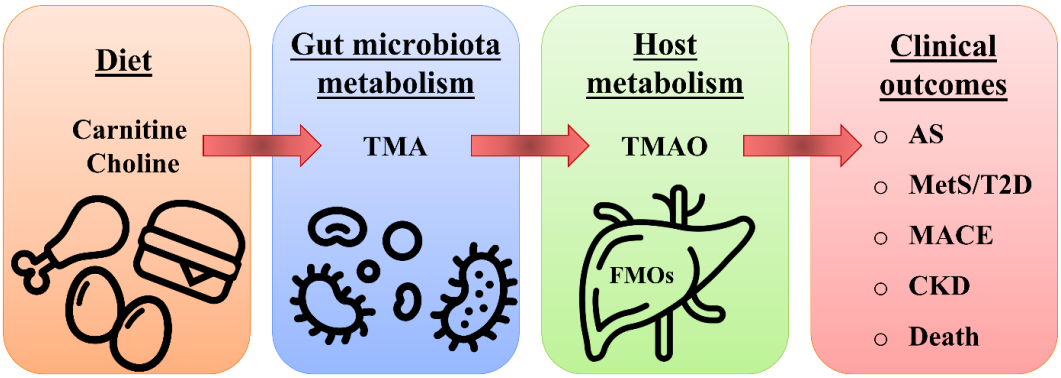

Figure 1.1 **Schematic representation of the biosynthesis of TMAO and the potential clinical outcomes linked to elevated concentrations of TMAO**

TMA, trimethylamine; TMAO, trimethylamine N-oxide, FMOs, flavin-containing monooxygenases; AS, atherosclerosis; MetS, metabolic syndrome; T2D, type 2 diabetes; MACE, major adverse cardiovascular events; CKD, chronic kidney disease.

Plasma TMAO concentration increases after consumption of foods rich in carnitine: red meat, processed foods and high-fat dairy products. The main dietary sources of carnitine are summarised in Table 1.1.

Table 1.1

Carnitine content in common foods

Product	Carnitine (mg/100 g or mg/100 ml)
Veal	78–132
Beef	65–88
Lamb	40
Pork	17–21
Turkey	15–21
Duck	15–26
Yogurt	12
Processed meat	10–66
Chicken	10
Avocado	8
Peas	5
Cheese	2–20
Potatoes	2

Table 1.1 continued

Product	Carnitine (mg/100 g or mg/100 ml)
Fish	2
Milk	2
Nuts	1–2
Butter	1
Eggs	1

Adapted from Demarquoy and colleagues (Demarquoy et al., 2004).

Another dietary precursor of TMAO is choline, which is present in common foods in water-soluble (free choline, phosphocholine, and glycerophosphocholine) as well as lipid-soluble forms (phosphatidylcholine and sphingomyelin). The products containing high amounts of total dietary choline are presented in Table 1.2, with liver, eggs, and meat being the richest in choline.

Table 1.2

Total choline content in common foods

Product	Choline (mg/100 g or mg/100 ml)
Beef liver	431
Eggs	226
Beef	104
Salmon	90
Pork	78
Chicken	62
Nuts	52
Broccoli	40
Beans	31
Potatoes	18
Milk	16
White rice	2

Adapted from Wiedeman and colleagues (Wiedeman et al., 2018).

In general, the precursors of TMAO are more abundant in products of animal origin. In healthy individuals following an omnivorous diet, the plasma concentration of TMAO is around 3 μM, while in vegans and vegetarians it is even lower and can be below 2 μM (Koeth et al., 2013; Zhu et al., 2017). Furthermore, it has been shown that people following a vegetarian or vegan diet for more than one year have a significantly reduced ability to convert carnitine into TMA compared to omnivores, which can be explained by changes in the gut microbiota (Koeth et al., 2013; Wu et al., 2019).

The direct involvement of the gut microbiota in the production of TMA is confirmed by the fact that broad-spectrum antibiotic therapy can reduce plasma TMA and TMAO concentrations to undetectable levels in healthy volunteers even after ingestion of high doses of TMAO precursors, such as carnitine supplements or steak. After several weeks of recovery

of the microbiota, both compounds can be detected again in plasma with TMAO concentrations peaking 24 hours after dietary intake of carnitine (Koeth et al., 2013). Similar studies with antibiotic treatment have been conducted on phosphatidylcholine metabolism, concluding that the increase in plasma TMAO concentration promoted by phosphatidylcholine is also dependent on intestinal microbiota activity (Tang, 2013).

Interestingly, not all microbial species are involved in TMA synthesis. Only a moderate number of bacterial genera have been specifically identified to produce enzyme complexes capable of cleaving the C-N bond in choline or carnitine molecules, resulting in the formation of TMA (Zhu et al., 2014; Romano et al., 2015). TMA-producing bacteria are mostly opportunistic pathogens from phyla *Firmicutes, Pseudomonadota (Proteobacteria), Actinobacteria, Thermodesulfobacteriota, Bacillota* (Craciun and Balskus, 2012; Romano et al., 2015). Moreover, TMA-producing bacteria also differ in their ability to use TMAO precursors, as some are known to metabolise only choline, while others can use both choline and carnitine to generate TMA (Kuka et al., 2014; Wu et al., 2019).

It is known that after TMA is produced in the intestines, it is reabsorbed into the circulation and transported to the liver of the host organism. The final formation of TMAO involves a family of liver flavin-containing monooxygenases (FMOs), with FMO3 being the dominant enzyme isoform for the production of TMAO (ten times more active than FMO1) (Bennett et al., 2013). Consequently, mutations in the FMO3 gene can cause trimethylaminuria, also known as "fish odour syndrome" (Treacy et al., 1998). It is a rare autosomal recessive disorder characterised by the accumulation of TMA in the body. Elimination of TMA occurs through breath, sweat, saliva, and urine, which results in an unpleasant fish-like odour (Treacy et al., 1998). Kidney and liver dysfunction and excessive intake of carnitine and betaine through the diet can cause secondary trimethylaminuria (Mackay et al., 2011).

In marine animals, however, TMAO is synthesised endogenously, and the concentration of TMAO in fish and seafood is much higher than in other organisms. In the muscle tissues of deep-sea animals, TMAO concentrations can reach up to 400 mmol/kg (Yancey et al., 2014) and are directly correlated with the depth below sea level that marine animals inhabit (Laurent et al., 2022). Moreover, in rotten fish, TMAO can be reduced to TMA by bacteria, resulting in a typical spoiled fish-odour. When humans ingest fish or seafood, TMAO undergoes metabolic retroconversion to TMA by gut microbiota (Hoyles et al., 2018) and TMAO is formed again after oxidation in the host liver, resulting in a significant increase in plasma concentrations of TMAO, much higher than those observed after carnitine or choline-rich product intake (Krüger et al., 2017). Interestingly, a recent study reports an increase in TMAO levels already

15 minutes after fish consumption, indicating that TMAO can also be absorbed from fish products without any further processing by the gut microbiota (Cho et al., 2017).

Mammals are not able to further metabolise TMAO, and the vast majority of it (> 95 %) is excreted unchanged by the kidneys (Al-Waiz et al., 1987). Moreover, it has been shown that the increase in plasma levels of TMAO parallels the decline in kidney function (Pelletier et al., 2019). Several potential membrane transporters for TMAO have been identified in recent years. Organic cation transporter 2 (OCT2) is now considered to be the major transporter responsible for the cellular uptake of TMAO in renal tubular cells (Teft et al., 2017). *In vitro* results provide data that the efflux of TMAO occurs via multiple transporters of the ATP-binding cassette family, that are differentially expressed in liver, kidneys and the epithelial cells of the intestines (Teft et al., 2017).

Overall, there are several factors determining the high interindividual differences in plasma TMAO concentration, such as the amount of ingested TMAO precursors, the composition of gut microbiota, FMO3 enzymatic activity, as well as the extent of renal function.

1.3 TMAO as a biomarker of cardiovascular and metabolic diseases

While gut bacteria have been known to play fundamental roles in the pathogenesis of many diseases, such as obesity, diabetes, and cardiovascular disease (CVD), a clear link underlying these associations was missing. A breakthrough in predicting cardiometabolic risks and potential outcomes based on intestinal microbiota composition and dietary patterns came when gut microbiota metabolism of phosphatidylcholine and carnitine with subsequently increased TMAO levels were linked to the development and progression of cardiovascular diseases (Wang et al., 2011; Koeth et al., 2013).

Since then, a positive correlation between the circulating levels of TMAO and the severity of metabolic syndrome (MetS) has been observed with a cut-off value of 8.74 μM (Barrea et al., 2018), and elevated TMAO plasma levels have been observed in patients with type 2 diabetes (T2D) (Lever et al., 2014; Dambrova et al., 2016). Moreover, in patients with heart failure, increased TMAO concentrations correlate with the severity of heart failure, as shown by New York Heart Association (NYHA) classification (NYHA II – 3.5 ± 0.9; NYHA III – 6.0 ± 0.8; NYHA IV – 8.1 ± 1.0 μM, respectively) (Tang et al., 2014, 2015 a; Trøseid et al., 2015; Dong et al., 2020) and heart failure-associated mortality (Suzuki et al., 2016). In heterogeneous and multi-ethnic populations, the correlation between higher plasma TMAO levels and prevalence of cardiovascular disease has been described independently of other factors, for example, intimal medial thickness (Mente et al., 2015).

High intake of TMAO or its precursors has also been shown to promote AS and thus exacerbate CVD risks (Wang et al., 2011; Koeth et al., 2013; Ding et al., 2018). Moreover, clinical studies have identified a TMAO plasma concentration of 6.18 μmol/L as a major adverse cardiovascular event (MACE) risk threshold (Tang et al., 2013). Moreover, TMAO serves as a predictive marker of future coronary artery disease in apparently healthy individuals (Tang et al., 2021). In addition, a dose–response meta-analysis revealed that the relative risk for all-cause mortality increased by 7.6 % per 10 μmol/L increase in TMAO levels (Schiattarella et al., 2017).

In recent years a very strong opinion has formed about the link of increased TMAO concentrations and chronic kidney disease (CKD). It is shown that plasma TMAO levels in CKD patients are significantly higher, compared to non-CKD subjects (Tang et al., 2015 b). Moreover, TMAO levels strongly correlate with CKD stages (CKD 3a-b, 9.35 μM [6.11–14.51]; CKD 4-5, 24.11 μM [13.51–29.26]; CKD 5D, 91.99 μM [54.91–116.10]) (Pelletier et al., 2019), reaching concentrations that are highly above the estimated CVD risk threshold. However, the causal role of TMAO in development of CKD remains elucidated.

Although extensive studies of TMAO in various patient populations clearly demonstrate that TMAO can serve as a biomarker, it remains uncertain whether TMAO is directly involved in the pathogenesis of cardiometabolic diseases.

1.4 The role of TMAO in the pathogenesis of cardiometabolic diseases

In order to clearly identify the role of TMAO in the pathogenesis of cardiometabolic diseases, numerous preclinical studies have been performed over the last decade to clarify the molecular mechanisms and signalling pathways affected by TMAO.

To date, the strongest evidence for the involvement of TMAO in the multifactorial development of AS has been gathered. There is evidence that TMAO activates inflammatory pathways and induces the expression of inflammatory genes such as cyclooxygenase 2 and interleukin 6. This activation occurs through the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor kappa-B p65 cascade, leading to vascular endothelial inflammatory damage and increased leukocyte adhesion (Seldin et al., 2016). TMAO is shown to promote thrombosis by enhancing platelet reactivity, increasing platelet endogenous calcium release and promoting phosphorylation of ERK1/2 and Jun N-terminal kinase (Zhu et al., 2016). TMAO also increases the expression of vascular cell adhesion molecule 1 (Witkowski et al., 2022), further contributing to thrombotic potential. In terms of foam cell formation, TMAO stimulates monocyte adhesion to endothelial cells and

upregulates scavenger receptor expression on macrophages, thus facilitating the accumulation of oxidised low-density lipoprotein cholesterol and the formation of foam cells (Wang et al., 2011; Mohammadi et al., 2016). Additionally, TMAO disrupts reverse cholesterol transport and impairs the classical bile acid synthesis pathway, resulting in increased blood cholesterol levels and the progression of atherosclerotic plaques (Koeth et al., 2013).

In the recent years, an interesting relationship between TMAO, insulin and insulin resistance-induced cardiovascular diseases has been elucidated. Thus, it was shown that the expression of TMAO producing enzyme FMO3 is suppressed by insulin (Miao et al., 2015); subsequently, FMO3 knockout mice are protected from the development of hyperglycaemia, hyperlipidaemia and AS due to the suppression of Forkhead box protein O1 (FOXO1), a transcription factor that is the main target of insulin signalling. Very recently, it was shown that the protein kinase R-like endoplasmic reticulum kinase (PERK) acts as a receptor for TMAO; thus, TMAO at physiologically relevant concentrations selectively activates PERK, which in turn induces the transcription factor FOXO1 (Chen et al., 2019) and promotes metabolic dysfunction.

In a rodent model of heart failure, diet supplementation with TMAO or its precursors exacerbated cardiac dilation, leading to reduced ejection fraction and increased cardiac fibrosis (Organ et al., 2016) via transforming growth factor-β1 (TGF-β1)/SMAD3 signalling (Li et al., 2019). In addition, a reduction of circulating TMAO levels resulted in attenuated cardiac remodelling after aortic banding (Organ et al., 2020; Wang et al., 2020). TMAO-induced impairment in cardiomyocyte contractility and calcium handling (Savi et al., 2018; Oakley et al., 2020), as well as T-tubule formation (Jin et al., 2020) were suggested as possible mechanisms that may link TMAO to heart failure. Moreover, it is well known that the mitochondrial energy metabolism is one of the cornerstones in the pathophysiology of heart failure (Rosca and Hoppel, 2013). It has been shown that both acute and chronic TMAO treatment can cause disruptions in energy metabolism in the heart by impairing pyruvate and fatty acid metabolism (Makrecka-Kuka et al., 2017). However, there is no evidence that TMAO-induced metabolic alterations result in impaired cardiac functionality.

Despite this, the observed effects of TMAO on molecular signalling pathways do not provide a clear explanation of all the associations noted in a clinical setting. Moreover, according to the results observed on cardiac energy metabolism, it could be hypothesised that long-term TMAO administration could exert preconditioning-like effects, thus improving cardiovascular outcomes after stress conditions, such as hypoxia, pressure overload and altered energy substrate availability. Therefore, it is crucial to continue studying the molecular

mechanisms affected by TMAO to clarify the exact role of TMAO in the pathogenesis of cardiovascular and metabolic diseases.

1.5 Protective effects of TMAO

The idea of TMAO as a protective agent has also attracted scientific attention and has been widely promoted in recent years. Initially, one of the main driving factors of this idea was the Mediterranean diet, which foresees regular consumption of fish and seafood rich in TMA and TMAO (Cho et al., 2017). Compliance with the Mediterranean diet was inversely associated with fatal coronary heart disease (He et al., 2004). Moreover, it was proposed to be a strategy for preventing and reducing the risk of cardiovascular and metabolic diseases (Tørris, Molin, and Smastuen, 2014; Widmer et al., 2015; Estruch et al., 2018; Jimenez-Torres et al., 2021).

It was recently shown that chronic treatment with low-dose TMAO is associated with preserved cardiac hemodynamic parameters in Spontaneously Hypertensive rats and Spontaneously Hypertensive Heart Failure rats (Huc et al., 2018; Gawrys-Kopczynska et al., 2020). In addition, a diet enriched with carnitine slowed aortic lesion formation in $ApoE^{-/-}$ mice (Collins et al., 2016); and the same effect was observed after phosphatidylcholine treatment in $Ldlr^{-/-}$ mice (Aldana-Hernández et al., 2021), which was explained by an increase in circulating TMAO levels.

The protective role of TMAO was explained by its ability to reduce endoplasmic reticulum stress (Makhija et al., 2014; Zhao et al., 2019), oxidative-nitrative stress and the subsequent vascular and diabetic complications (Lupachyk et al., 2013; Fukami et al., 2015). More recently, some protective effects were attributed to the ability of TMAO to increase diuresis and natriuresis, as well as its widely discussed protein stabilising properties, that can protect the cardiomyocytes against hydrostatic pressure in a failing heart (Gawrys-Kopczynska et al., 2020).

Such controversial results raise the question of whether increased concentration of TMAO plays a detrimental or protective role in the progression of cardiovascular diseases and indicate that the effects of TMAO might vary in different tissues and organ systems.

1.6 Intervention strategies targeted at reduction of TMAO levels

In parallel with studying molecular mechanisms affected by TMAO, intervention strategies targeted at reduction of TMAO levels have also been investigated. The complex biosynthetic pathway of TMAO provides an opportunity to intervene at multiple levels, targeting the composition of intestinal microbiota, specific microbial enzymes responsible for TMA production, host liver FMO3 activity as well as the rate of TMAO excretion.

As gut microbiota is responsible for the initial step of TMAO formation, a vast amount of evidence indicates that antibiotics can markedly decrease circulating TMA and TMAO levels (Wang et al., 2011; Koeth et al., 2013). However, due to risk of antibacterial resistance and various side effects occurring with antibiotic treatment, this has not been considered a viable therapeutic strategy to reduce TMAO levels. As main constituents of gut microbiota involved in TMA synthesis are opportunistic pathogens, enrichment of gut flora with beneficial bacterial strains could also be effective. However thus far this approach has not provided very promising results, moreover, the observed effects are strictly strain-specific (Tripolt et al., 2015; Cantero et al., 2022). Recently, bacterial enzymes responsible for the metabolism of choline (choline-TMA-lyase (Craciun and Balskus, 2012; Kalnins et al., 2015)) and L-carnitine (carnitine monooxygenase (Quareshy et al., 2021)), have been identified. Thus, pharmacological interventions can be targeted directly at specific microbial enzymes, without significantly altering the gut microbiota composition. Structural analogues of choline, 3,3-dimethyl-1-butanol, iodomethylcholine and fluoromethylcholine have been effective in reducing TMA production by gut microbiota (Roberts et al., 2018) and protecting from some of the detrimental effects induced by TMAO (Chen et al., 2017; Brunt et al., 2022). Interestingly, a well-known phytoalexin, resveratrol, is also reported to act as a prebiotic, modulating the gut microbiota composition and thus reducing circulating TMAO levels (Chen et al., 2016). In addition, a famous Latvian cardioprotective drug meldonium (Dambrova et al., 2016 b) is known to reduce plasma TMAO levels through multiple mechanisms, namely by shifting gut microbiota metabolism from TMA production towards gamma-butyrobetaine (GBB) production (Kuka et al., 2014) and facilitating the renal excretion of TMAO (Dambrova et al., 2013).

Several studies have reported the effects of natural compounds on reducing TMAO levels via inhibition of FMO3. Dietary indoles are well known to inhibit FMO activity and subsequently decrease TMAO levels (Cashman et al., 1999; Chen et al., 2019). In addition, the effects of such bioactive ingredients as alkaloids, (Anwar et al., 2018) flavonoids, phenolic compounds, several monoterpene glycosides and ecdysteroid compounds have been studied (Yu et al., 2021) with a potential to reduce plasma TMAO levels. Nevertheless, this TMAO

reduction strategy has not gained high appreciation, as it results in increased rate of malodorous TMA excretion as in case of "fish odour syndrome".

However, thus far there is no evidence that pharmacological inhibition of TMAO production could prevent disturbances in the lipid profile and obesity (Chen et al., 2017), indicating that in addition to pharmacological intervention, further lifestyle changes would still be necessary to maximise the benefits regarding cardiovascular and metabolic health. Although there is some evidence that TMAO levels could be targeted by some types of caloric restriction (Erickson et al., 2019; Washburn et al., 2019; Sun et al., 2020), reduced protein intake (Mafra et al., 2018) or diets supplemented with sources of dietary fibres and unsaturated fatty acids (Zhang et al., 2015; Hernández-Alonso et al., 2017), it has not been thoroughly investigated. Therefore, studying dietary approaches targeting the level of TMAO together with other metabolic parameters would be of great significance.

2 Methods

2.1 Acute effects of TMAO in experimental *ex vivo* and *in vivo* models of cardiac and vascular functionality

2.1.1 Chemicals

TMAO dihydrate was obtained from Alfa Aesar (Kandel, Germany). Sodium pentobarbital (Dorminal) solution was purchased from Alfasan (Woerden, Holland). Heparin sodium was purchased from Panpharma (Fougeres, France). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), and 98 % formic acid was obtained from Fluka (Buchs, Switzerland). [9,10-^{3}H]-Palmitate (5 μCi/ml) or D-[U-^{14}C]-glucose (0.625 μCi/ml) was purchased from Biotrend (Zürich, Switzerland). Adenosine triphosphate (ATP) was purchased from TCI (Antwerp, Belgium). Isoflurane was purchased from Chemical Point (Deisenhofen, Germany). All other reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.1.2 Animals and treatment

Thirty male Wistar rats weighing 200–250 g were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). Twelve male CD-1 mice at the age of 6–8 weeks weighing 25–30 g were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). All experimental animals were housed under standard conditions (21–23 °C, 12-hour light/dark cycle, relative humidity 45–65 %) with unlimited access to food (R70 diet, Lactamin AB, Kimstad, Sweden) and water. Rats (n = 12) were used to determine the levels of TMAO in vascular and myocardial tissue. Six rats were used to assess the effects of TMAO on energy substrate oxidation and vascular reactivity after incubation in TMAO-containing (100 μM) buffer solution, and 12 rats were used in the isolated heart experiments to assess the functionality of the heart and the size of myocardial infarction after perfusion with 1 mM TMAO. Mice (n = 12) were used to study the effects of TMAO administration on heart functionality and the response to adrenergic stimulation.

To obtain cardiac and vascular tissue for *ex vivo* experiments, the rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and heparin (1000 IU/kg). After the onset of anaesthesia, the thorax was opened, and the heart and thoracic aorta were removed and placed into ice-cold Krebs-Henseleit (K-H) buffer solution (composition (in mmol/L): NaCl 118, $CaCl_2$ 2.5, $MgCl_2$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 10.0, and EDTA 0.05; pH 7.4 at 37 °C) until the tissue was further processed.

2.1.3 Determination of TMAO accumulation

Rat hearts were perfused, and aortic rings from each experimental animal were immersed in K-H buffer solution with or without the addition of TMAO (100 μM final concentration). After 1 hour of perfusion or incubation, the tissue samples were washed to eliminate the residues of TMAO-containing buffer solution and further processed for quantification of TMAO accumulated in the tissue.

2.1.4 Energy substrate oxidation

To assess the energy substrate oxidation rate, cardiac tissues were minced with scissors and homogenised with a Turrax homogeniser (IKA, Staufen, Germany). Samples were prepared at a ratio of 1:10 w/v in Isolation Buffer A (composition (in mmol/L): KCl 180, Tris-base 10, EDTA 0.5). Heart homogenates were centrifuged at 1000 × g for 5 min, and the supernatant was used to assess fatty acid and glucose oxidation. The aortic rings remained intact. The reaction mix for fatty acid oxidation contained 1 mM nicotinamide adenine dinucleotide (NAD), 5 mM ATP, 100 μM Coenzyme A (CoA), 1 mM malate, 700 μM carnitine, and [9,10–^{3}H] palmitate (specific activity, 5 μCi/ml) in K-H buffer solution with or without 100 μM TMAO. The glucose oxidation reaction mix consisted of 1 mM NAD, 5 mM ATP, 100 μM CoA and D-[U-^{14}C] glucose (specific activity, 0.625 μCi/ml) in K-H buffer solution with or without 100 μM TMAO.

Glucose and palmitate oxidation rates were determined as described previously (Liepinsh et al., 2015). Briefly, glucose oxidation was assessed by measuring the $^{14}CO_2$ released from the metabolism of D-[U-^{14}C] glucose. Palmitate oxidation was determined by measuring the $^{3}H_2O$ released from [9,10-^{3}H] palmitate. Glucose and palmitate uptake in the aorta was calculated from the amount of radiolabelled substrates oxidised and the amount found in aortic tissues when the reaction ended.

2.1.5 Vascular reactivity of conductance and resistance vessels

Vascular reactivity of aortic rings was assessed as described previously (Vilskersts et al., 2015). In brief, the excised thoracic aorta was immersed in ice-cold K-H buffer solution and the surrounding tissues were removed. The vessels were cut into 3- to 4-mm-long rings that were suspended between two stainless steel hooks in a 10 mL organ bath filled with K-H buffer solution saturated with 95 % O_2 and 5 % CO_2, and four parallel samples were prepared from the same animal. The aortic rings were stretched to a resting tension of 2 g and equilibrated to the new conditions for 60 min. During this adaptation, the incubation buffer solution was

changed every 15 min. The maximal contraction force of each ring was determined by adding 60 mM potassium chloride. After washing, TMAO at a concentration of 100 μM was added to half of the aortic rings and incubated for 1 h. Aortic rings were then washed once more with buffer solution and precontracted with phenylephrine to 70 %–80 % of maximal contraction. Endothelium-dependent relaxation was assessed by adding cumulative concentrations of acetylcholine (Ach) (10^{-9} to 10^{-5} mol/L). Endothelium-independent relaxation was assessed by adding cumulative concentrations of sodium nitroprusside (SNP) (10^{-10} to 10^{-5} mol/L). The relaxation of the aortic rings in response to acetylcholine or SNP was expressed as a percentage of the phenylephrine-induced constriction.

Vascular reactivity in mesenteric artery rings was assessed as described previously (Bridges et al., 2011) with modifications. The intestine with the mesenteric arcade attached was excised and transferred to ice-cold physiological salt solution (PSS) with the following composition (mmol/L): NaCl 130, KCl 4.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 14.9, glucose 5.5, and EDTA 0.026. Second-order mesenteric arteries were cleaned and dissected of adjoining fat and connective tissues. The arteries were cut into ring segments 2 mm in length, and four parallel samples were prepared from the same animal. Each segment was mounted in a Multi-Myograph System (Danish Myograph Technology, Aarhus, Denmark) in PSS saturated with a gas mixture of 95 % O_2 and 5 % CO_2. Further assessment of endothelium-dependent and endothelium-independent function after incubation with 100 μM TMAO was performed in a similar manner as for the aorta.

2.1.6 Experimental heart infarction *ex vivo*

The infarction was performed according to the Langendorff technique as described previously (Kuka et al., 2012), with some modifications. For the infarction studies, the hearts were perfused with K-H buffer solution with or without 1 mM TMAO at a constant perfusion pressure of 60 mmHg. The heart rate, left ventricular end-diastolic pressure, and left ventricular developed pressure (LVDP) were recorded continuously. Coronary flow was measured using an ultrasound flow detector (HSE) and PowerLab systems from AD Instruments (Sidney, Australia). The isolated rat hearts were left to adapt for 30 min, and then the left anterior descending coronary artery was occluded for 30 min, followed by 120 min of reperfusion. Further analysis was performed as described by Liepinsh et al. (Liepinsh et al., 2022). In brief, left anterior descending coronary artery was reoccluded and perfused with 0.1 % methylene blue. Afterward, the heart was transversely cut into 2-mm-thick slices, treated with triphenyltetrazolium chloride and photographed. Computerised planimetric analysis was

carried out using Image-Pro Plus v6.3 software (Media Cybernetics Inc., Rockville, MD, USA) to determine the area at risk and the area of necrosis. Each area was then expressed as a percentage of the total left ventricle area. The infarct size (IS) was then calculated as a percentage of the risk area according to the formula IS = AN/AR × 100 %.

2.1.7 Isoproterenol-induced cardiac stress model

The isoproterenol-induced acute cardiac stress model was established as previously described (Rohrer et al., 1999) with some modifications. Prior to the study, experimental animals were randomly divided into two experimental groups (n = 6) and weighed. Mice from both groups were anaesthetised with 5 % isoflurane dissolved in 100 % oxygen. After the onset of anaesthesia, the concentration of isoflurane was reduced to 2.5 %, the experimental animals were placed in a decubitus position, and the chest was shaved. M-mode tracings of the left ventricle were recorded at the papillary muscle level using an iE33 ultrasound machine equipped with a linear L15-7io transducer (Philips Healthcare, Andover, USA). Subsequently, the mice from the first experimental group received isoproterenol (ISO) at a dose of 10 µg/mouse, but the animals from the second group received ISO and TMAO at doses of 10 µg/mouse and 120 mg/kg, respectively. After 30 minutes, the experimental animals were anaesthetised with isoflurane once more to record the cardiac response to acute cardiac stress and the impact of TMAO on the inotropic and chronotropic effects. For the next seven days, the mice in the second group received TMAO together with drinking water at a dose of 120 mg/kg, while the animals from the first group received pure drinking water. After one week of treatment, the experimental animals were anaesthetised, and echocardiography was performed before and after administration of ISO or a combination of ISO and TMAO as described before.

2.2 Long-term administration of TMAO in experimental model of right ventricular heart failure in rats

2.2.1 Experimental animals

Wistar rats (n = 40) weighing 280–380 grams (6–8 weeks old) were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia) and housed under standard conditions (21–23 °C, 12-hour light/dark cycle, relative humidity 45–65 %) for two weeks prior to the start of the experiment. The animals were fed a standard R70 diet (Lantmännen, Stockholm, Sweden) with unlimited access to food and drinking water.

Our previous experiments, in which right ventricular (RV) functionality was assessed, indicated that due to interindividual variability, 8 to 10 animals per group are necessary to obtain significant results; therefore, n = 10 per group was selected. The data from previous experiments in which mitochondrial energy metabolism was studied were subjected to statistical power analysis (GPower software, Düsseldorf, Germany), and the calculations indicated that the mitochondrial respiration assay requires at least n = 5 or 6 per group to produce significant results with a power > 0.95.

2.2.2 Experimental design

The aim of the present study was to investigate the effects of long-term TMAO administration in an experimental rat model of monocrotaline-induced right ventricle heart failure. To mimic the chronic increase in TMAO in plasma and tissues, as observed in cases of regular consumption of seafood, TMAO pretreatment for 10 weeks prior to monocrotaline injection was chosen. The schematic representation of the study design is shown in Figure 2.1. The experimental animals were randomly separated into four groups: control (n = 10), TMAO (n = 10), MCT (monocrotaline) (n = 10) and TMAO + MCT (n = 10). One animal in the control group and 2 animals in the other groups died during the experiment due to reasons not related to the experimental protocol and treatment. The samples from these rats were excluded from further analysis; therefore, the final animal count was 9 rats in the control group and 8 rats in the other groups. The animals in the TMAO group and TMAO + MCT group received TMAO (Alfa Aeser, Kandel, Germany) at a dose of 120 mg/kg in their drinking water daily for 10 weeks. To induce pulmonary hypertension and RV remodelling and dysfunction, a single subcutaneous injection of monocrotaline (MCT) (Sigma-Aldrich, Schnelldorf, Germany) at a dose of 60 mg/kg was administered to the animals in the MCT and TMAO + MCT groups. TMAO treatment was continued in both groups that previously received TMAO until the end of the experiment. The rats were weighed twice a week to monitor their general health condition. Since the time from MCT injection to RV failure onset differs markedly (Hardziyenka et al., 2006), a four-week time point after the administration of MCT was selected for the echocardiographic assessment of right ventricle functionality based on our pilot experiments in this model. In addition, invasive direct RV pressure measurement was performed. After the assessment of cardiac functionality, the animals were sacrificed, and cardiac tissue and plasma samples were immediately frozen and stored at –80 °C for further quantification of TMAO, biochemistry measurements and gene and protein expression analysis.

In addition, a mitochondrial functionality study was performed using permeabilised cardiac fibres of the right ventricle.

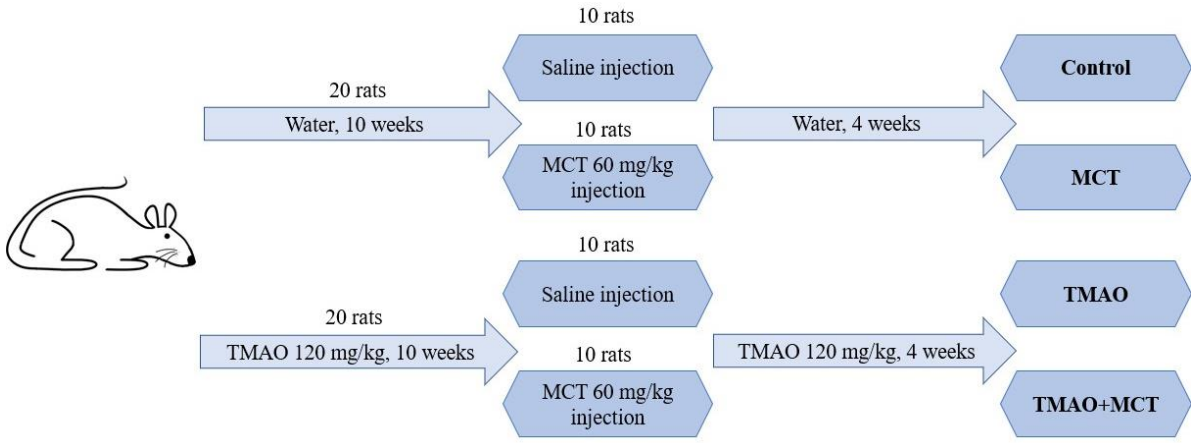

Figure 2.1 **Schematic representation of the study design**

2.2.3 Echocardiography assessment and direct RV blood pressure measurement

The rats were anaesthetised using 5 % isoflurane dissolved in 100 % oxygen. After the onset of anaesthesia, the concentration of isoflurane was decreased to 2.5 %, the experimental animals were placed in a decubitus position, and the chest and upper part of the abdomen were shaved. The animals were connected to a Philips iE33 ultrasound machine (Philips Healthcare, Andover, USA) to record ECG from the II lead. Then, the rat was placed on the left side, and a four-chamber view was recorded from the apical point of view using a Philips (Philips Healthcare, Andover, USA) S12-4 sector array transducer. RV end-diastolic area and RV end-systolic area were recorded. Electrocardiogram was used to determine the exact time of RV systole and diastole. Furthermore, RV end-diastolic area and RV end-systolic area were used to calculate RV fractional area change. The rat was again placed in a decubitus position, and functional parameters of the left ventricle were recorded at the papillary muscle level using a Philips (Philips Healthcare, Andover, USA) linear L15-7io transducer.

After the echocardiographic assessment of ventricular anatomy and functioning, invasive direct RV pressure measurement was performed. The anaesthetised rat was intubated using a 16-G intravenous catheter and mechanically ventilated with 2 % isoflurane dissolved in 100 % oxygen at a tidal volume of 1.5 ml/100 g. The abdominal cavity was opened, and the diaphragm was incised to expose the pleural cavity. The ribs on both sides of the chest were cut to access the heart. An 18-G needle was connected to a pressure transducer (AD Instruments, Sidney, Australia) and inserted into the cavity of the right ventricle through the apex of the heart. The RV pressure was measured until a stable pressure reading was obtained.

2.2.4 Measurements of organ mass

To calculate the organ-to-body weight indexes, the heart and lungs were excised and weighed. Then, the right ventricle (excluding the septum) was separated from the heart and weighed.

2.2.5 Measurements of tissue brain natriuretic peptide

A Rat Brain Natriuretic Peptide (BNP) 45 ELISA Kit (Abcam, Cambridge, United Kingdom, ab108816) was used to test the levels of makers of congestive heart failure in RV tissue extracts. Extract preparation and analysis were carried out according to the manufacturer's instructions.

2.2.6 Isolation of RNA and qPCR analysis

Total RNA was isolated from RV tissues using TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's recommended protocol. First-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The quantitative polymerase chain reaction (qPCR) mix consisted of SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), synthesised cDNA, and forward and reverse primers specific for *VCP*, *BNP*, *αMHC*, and *βMHC*. These genes were selected to characterise heart failure severity and cardiac hypertrophy. The reaction was carried out in an Applied Biosystems Prism 7500 instrument according to the protocol provided by the manufacturer. The relative expression levels of each of the genes of interest were calculated with the ΔΔCt method and were normalised to the expression level of the *VCP* gene. The primer sequences used for the qPCR analysis are represented in Table 2.1.

Table 2.1

Gene accession number and primer sequences of qPCR primers

Gene symbol	Full name	NCBI Accession number	Primer sequence (5'–>3')
VCP	Valosin-containing protein	> NM_053864.2	F- AATATTTGACAAGGCACGACAAG
			R- CCGGTTGGTAGCTCCAATGAT
BNP	Natriuretic peptide type B	> NM_008726.5	F- TAGCCAGTCTCCAGAGCAATTC
			R- TTGGTCCTTCAAGAGCTGTCTC
αMHC	Myosin heavy chain 6	> NM_017239.2	F- CTCCATCTCTGACAACGCCTATC
			R- CTCCGGATTCTCCAGTGATGA
βMHC	Myosin heavy chain 7	> NM_017240.2	F- GGAGCTGATGCACCTGTAGACA
			R-AGTGCGGACACGGTCTGAA

2.2.7 Mitochondrial respiration in permeabilised cardiac fibres

Mitochondrial function was assessed in permeabilised cardiac fibres from the right ventricle that were prepared as previously described (Kuka et al., 2012). The mitochondrial respiration measurements were performed in MiR05 media (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, 0.1 % bovine serum albumin essentially free of fatty acids) at 37 °C using an Oxygraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria). Mitochondrial functionality measurements were performed using a previously described respirometry protocol (Makrecka-Kuka et al., 2020). Briefly, palmitoylcarnitine (PC) and malate (10 μM and 0.5 mM, respectively) were used to measure fatty acid oxidation (FAO)-dependent mitochondrial respiration (F(N)-pathway) in a substrate-dependent (LEAK) state. Then, adenosine diphosphate (ADP) was added to a concentration of 5 mM to initiate oxidative phosphorylation-dependent respiration (OXPHOS state). Next, pyruvate (5 mM, complex I substrate, N-pathway) was added to reestablish FN-pathway-linked respiration. Succinate (10 mM, complex II substrate, S-pathway) was added to reconstitute convergent FNS-linked respiration. Then, rotenone (0.5 μM, complex I inhibitor) and antimycin A (2.5 μM, complex III inhibitor) were added to determine the S-linked respiration and residual oxygen consumption (ROX), respectively.

To determine the contribution of each substrate to the respiration rate, the flux control factor was calculated as follows:

$$1 - \frac{Resp.rate\ before\ the\ addition\ of\ substrate}{Resp.rate\ after\ the\ addition\ of\ substrate}.$$

To determine the mitochondrial mass in the heart, the citrate synthase activity in tissue homogenates was measured spectrophotometrically as previously described (Srere, 1969).

2.3 Effects of metformin on TMAO levels in mouse experimental model of type 2 diabetes

2.3.1 Animals and treatment

Sixteen male db/db (BKS.Cg- + Leprdb/ + Leprdb/OlaHsd) mice and 10 age-matched non-diabetic db/Lean (db/ + (BKS.Cg- + Leprdb/ + /OlaHsd)) male mice (10 weeks old, Envigo (Harlan Laboratories BV), Venray, Netherlands) were housed for two weeks prior to treatment under standard conditions (21–23 ºC, reverse 12-hour light/dark cycle, relative humidity 45–65 %) with unlimited access to water and food (R70 diet from Lantmännen, (Stockholm, Sweden)).

Db/db mice were randomly divided into two experimental groups and given daily oral doses of water (db/db control group, n = 8) or 250 mg/kg metformin (db/db metformin group, n = 8) for 8 weeks. Db/Lean mice (n = 10) were used as a control (Figure 2.2 A). Plasma samples were collected after 4 and 8 weeks of treatment.

Another 30 db/db male mice (BKS.Cg- + Leprdb/ + Leprdb/OlaHsd) and 10 age-matched non-diabetic db/Lean (db/ + (BKS.Cg- + Leprdb/ + /OlaHsd)) male mice (10 weeks old, Envigo (Harlan Laboratories BV), Venray, Netherlands) were obtained for the follow-up experiment based on data obtained from the first study. Here the aim was to test the effects of metformin (TCI Europe N.V., Zwijndrecht, Belgium) in case of increased tertiary amine load; choline (TCI Europe N.V., Zwijndrecht, Belgium) was selected as the most common dietary tertiary amine. Db/db mice were divided into three experimental groups and given daily oral doses of water (db/db control group, n = 10), 0.5 % choline in drinking water for 4 weeks to facilitate bacterial TMA and subsequently host TMAO production (db/db choline 4w group, n = 10) or 0.5 % choline in drinking water and 250 mg/kg metformin (db/db choline + metformin 4w group, n = 10). Db/Lean mice (n = 10) were used as a control (Figure 2.2 B). Faecal samples were collected after 4 weeks of choline administration. At the end of the treatment these 40 mice received bolus dose of choline (100 mg/kg) to evaluate the overall capacity to produce TMAO and the ability of metformin to decrease TMAO production after acute substrate load. For this, plasma samples were collected immediately before and 2 h after choline load. Plasma samples were collected from tail veins during the dark cycle; samples were centrifuged, and the plasma was stored at –80 ºC for future analysis. All experiments were performed in a blinded manner.

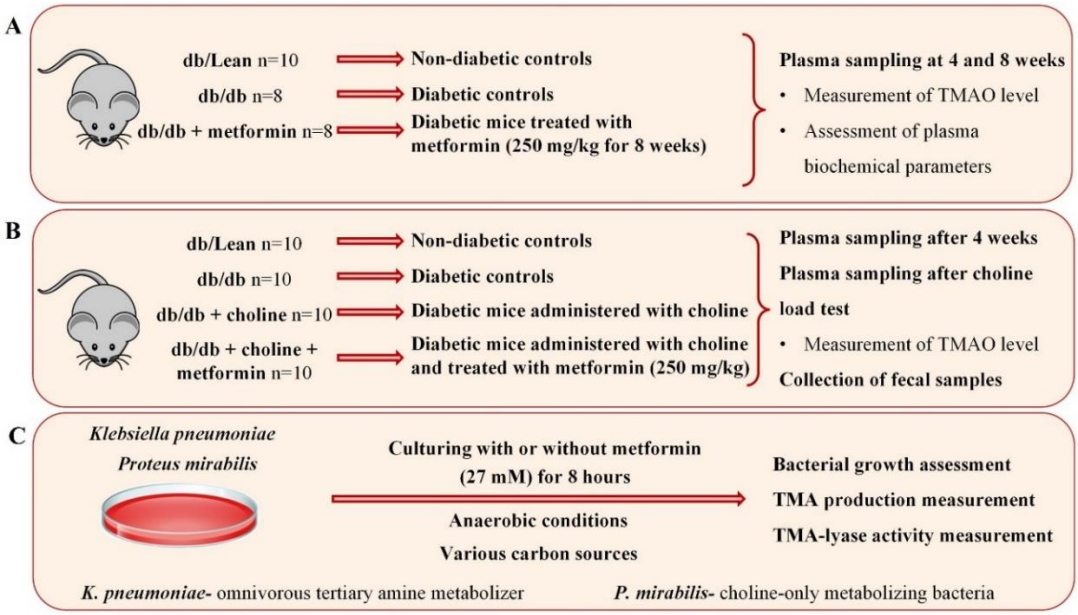

Figure 2.2 **Schematic representation of the study design**

2.3.2 Glucose and insulin measurements

The plasma insulin concentration was determined using a Sensitive Insulin RIA Kit (EMD Millipore, Billerica, MA, USA). Blood glucose was measured using a MediSense Optium glucometer from Abbott Diabetes Care (Maidenhead, UK).

2.3.3 DNA isolation from faecal samples and qPCR analysis

Total DNA from mice faeces was isolated (Ferrand et al., 2014) using FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's instructions. qPCR analysis and relative bacteria quantification was performed by using KAPA SYBR® FAST master mix (Sigma-Aldrich) and MIC qPCR Cycler (Bio-Molecular Systems) and using the following conditions: Polymerase activation 95 °C for 3 min; Touchdown 10 cycles [95 °C for 15 seconds, 65 °C for 15 seconds – 0.5 °C per cycle decrease, 68 °C for 10 seconds]; Cycling 60 cycles [95 °C for 5 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds]. To quantify the relative abundance of *Klebsiella pneumoniae* and *Proteus mirabilis* in the faecal samples, strain specific qPCR primers were selected from literature (Fukumoto et al., 2015) and verified using the Primer-BLAST tool (Ye et al., 2012). The relative bacterial abundance was determined using ΔΔCt method and normalised to the Ct of universal bacteria primer (Fuks et al., 2018).

2.3.4 Microbial cultures, TMA production assay

To test microbial TMA production, two bacterial species were used: *Klebsiella pneumoniae* (*K. pneumoniae*) (obtained from the Microbial Strain Collection of Latvia (MSCL), strain number 535) and *Proteus mirabilis* (*P. mirabilis*) (MSCL, strain number 590). Bacteria were maintained on Lysogeny broth agar plates. A single colony was used as inoculum for each experiment. To test the effect of metformin on choline-dependent TMA production, bacteria were grown in M9 mineral broth supplemented with 0.2 % casamino acids (Merck, Darmstadt, Germany) (Sack et al., 2008) with 27 mM choline as the carbon source, with or without 27 mM metformin addition (Figure 2.2 C). Metformin concentration of 27 mM was selected as it represents levels of a drug that could be achieved in the intestines after administration of high (≥ 850 mg) metformin doses to patients (Bailey, Wilcock, and Scarpello, 2008; Proctor, Bourdet, and Thakker, 2008).

Micro-anaerobic cultivations were performed essentially as described previously (Kuka et al., 2014). Briefly, to ensure anaerobic conditions 2 ml test tubes were filled with broth and covered with airtight caps; the test tubes were kept still in an incubator at +37 °C. Samples were

harvested after 4 and 8 h of cultivation, fixed with formic acid to 5 % final concentration and centrifuged. Supernatants were frozen (−80 °C) and stored until further analyses.

The effect of metformin on bacterial growth was assessed by spectrophotometric recording of *K. pneumoniae* and *P. mirabilis* growth dynamics in anaerobic tubes, as previously described (Seim et al., 1982). Briefly, microbial cells were grown in M9 broth with glucose as the sole carbon source overnight. Cells were harvested by centrifugation, washed and resuspended in fresh M9 broth with different carbon sources, glucose or choline (final concentration of 27 mM) with or without metformin (final concentration 27 mM). Airtight, clear glass high-performance liquid chromatography bottles (vol. 4.5 mL, diameter 1 cm) were top-filled with different microbial suspensions and left to incubate at +37 °C. The absorbance of the microbial suspensions was recorded using a WPA colorimeter (Biochrom, UK) set to 595 nm.

Choline-TMA-lyase enzymatic activity was determined based on a method previously described by Roberts and colleagues (Roberts et al., 2018) with modifications as indicated below. *P. mirabilis* bacterial cells were lysed, and the reaction was carried out in an inflatable glove bag filled with nitrogen gas to ensure anaerobic conditions. An enzymatic activity assay was performed using non-labelled choline, and the conversion of choline to TMA was determined with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS). The reaction mixture contained clarified bacterial lysate in lysis buffer and the necessary co-factors (1 mM S-Adenosyl methionine, 10 mM sodium dithionite, and 2 mM reduced form of nicotinamide adenine dinucleotide (NADH) (Sigma-Aldrich, Schnelldorf, Germany)). Metformin was added to the reaction mixture (27 mM final concentration) to evaluate its effect on choline-TMA-lyase activity. The mixture was then allowed to incubate for 15 minutes before the initiation of the reaction by adding choline (1 mM final concentration). Baseline samples were collected, and the reaction was carried out for 2 h at room temperature in the dark. Afterwards, samples were harvested, fixed with formic acid (1.5 % final concentration) and stored at −80 °C until further analysis.

2.3.5 Choline-TMA-lyase gene expression

To determine if a metformin-induced decrease in TMA production was associated with changes in the transcription of choline metabolism-related genes in gut bacteria, we tested the expression of three *K. pneumoniae* choline-TMA-lyase complex coding genes (*cmcA, cutC, cutD*) (Kalnins et al., 2015). Gene expression was evaluated in *K. pneumoniae* (NCBI Accession number > NZ_CP065838.1) after 4 and 8 h of cultivation with and without 27 mM

metformin. Total RNA was isolated and purified from *K. pneumoniae* cell pellets (stored at −80 °C) using a PureLink™ RNA extraction kit (ThermoFisher Scientific, USA) as recommended by the manufacturer's protocol. First-strand cDNA synthesis and qPCR analysis for genes were performed as described previously (Liepinsh et al., 2016). Relative expression levels for each gene were calculated with the ΔΔCt method and normalised to the expression of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) gene. The primer sequences used for the qPCR analysis are presented in Table 2.2.

Table 2.2

Gene accession number and primer sequences of qPCR primers

Gene symbol	Full name	Primer sequence (5'–>3')
CmcA	Microcompartment structural gene	F- TGTTGATGTCGTTGTGCGGA
		R- CGTCGAGCTTATCGGCTATGA
CutC	Choline utilization protein C	F- TCGGTAACCAGACCCGTAAA
		R- GGCGCGAGTTTTCTCTTCTA
CutD	Choline utilization protein D	F- GATTAACACCGCCGTCGAAA
		R- TCCACCAGCCATTCGAGATT
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F- ACCGTTCGTCTGGAAAAAGC
		R- ACGAAGTTGTCGTTCAGTGC

2.4 Fasting mimicking diet as a lifestyle intervention to target TMAO levels

2.4.1 Volunteers

A total of 44 omnivorous volunteers were subjected to an interventional study. Routine biochemistry tests and blood counts were performed to assess the general health of all volunteers prior to joining the study. The exclusion criteria were as follows: body mass index (BMI) < 18.5 kg/m^2; abnormal levels in any of the blood biochemistry measurements that indicate severe health problems; and taking antibiotics, probiotics or dietary supplements containing TMAO precursors within 2 months before the start of dietary interventions. All volunteers were informed about the aim and nature of this study. The recruitment of the volunteers and study procedures were carried out between December 2019 and June 2021.

2.4.2 Study design

The schematic design of the study is presented in Figure 2.3. Baseline anthropometric measurements and biochemical tests were performed in a fasted state before the planned dietary intervention. All participants were instructed to fast ≥ 10 hours prior the blood sampling, drinking pure water was allowed during the fasting time. As fish consumption could interfere

with the measurement of the TMAO level, volunteers were requested to abstain from sea food consumption for two days prior to sampling. Participants were requested to maintain their usual levels of physical activity throughout the intervention.

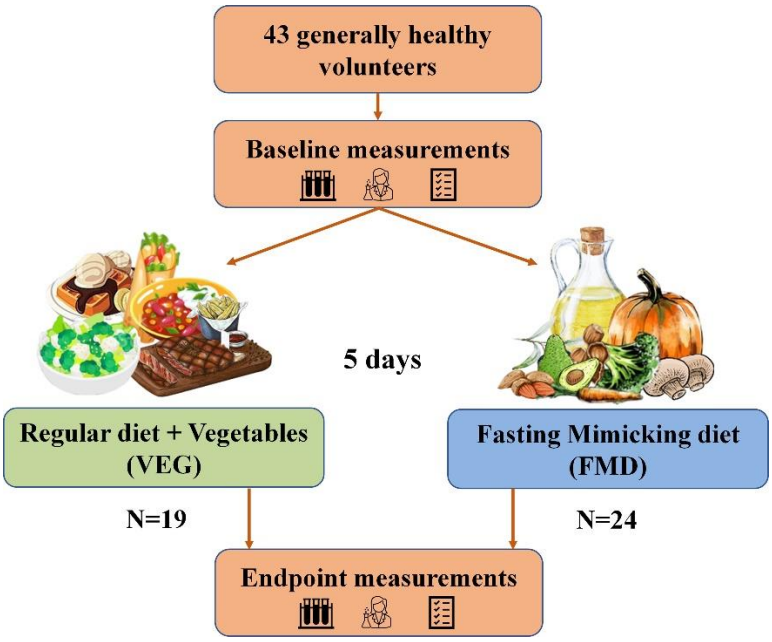

Figure 2.3 **Schematic representation of the study design**

The research was carried out as a parallel arm study, and the volunteers were assigned to either the reference group (VEG) or fasting mimicking diet (FMD) group for 5 days. The baseline characteristics of the participants are presented in Table 2.3. Fasting plasma glucose was selected as the main parameter for randomization of the volunteers.

Table 2.3

Baseline bio-anthropometric and biochemical parameters of the study participants

Baseline characteristics	VEG (n = 19)	FMD (n = 24)	P-value
Age, years	37 ± 3	39 ± 2	0.660
Sex, n (%)			
Men	6 (31.6)	9 (37.5)	–
Women	13 (68.4)	15 (62.5)	–
BMI, kg/m^2	25.2 ± 0.9	28.8 ± 0.9	0.004
Body type (regional fat distribution), n (%)			
Abdominal	8 (42.1)	10 (41.7)	–
Gluteofemoral	11 (57.9)	14 (58.3)	–
Plasma biochemistry			
Haemoglobin, g/L	144.0 ± 3.5	150.3 ± 6.2	0.350
Glucose, mmol/L	4.99 ± 0.13	4.87 ± 0.11	0.470
HDL cholesterol, mmol/L	1.51 ± 0.07	1.49 ± 0.08	0.841
LDL cholesterol, mmol/L	3.33 ± 0.16	3.37 ± 0.19	0.857
Triglycerides, mmol/L	1.44 ± 0.22	1.30 ± 0.09	0.440

Table 2.3 continued

Baseline characteristics	VEG (n = 19)	FMD (n = 24)	P-value
Plasma biochemistry			
Creatinine, μmol/L	75.7 ± 3.8	75.2 ± 6.6	0.941
eGFR, mL/min/1.73 m^2	86.6 ± 5.9	92.7 ± 6.9	0.527
ALT, U/L	21.5 ± 2.7	24.7 ± 3.4	0.478
Total bilirubin, μmol/L	9.7 ± 1.1	10.3 ± 1.5	0.788
Lipase, U/L	41.6 ± 1.9	37.3 ± 1.8	0.149
ESR, mm/h	2.9 ± 0.9	2.3 ± 0.2	0.641
CRP, mg/L	1.26 ± 0.33	1.24 ± 0.22	0.964
TMAO, μmol/L	3.65 ± 0.68	6.22 ± 1.16	0.083
Physical activity, n (%)			
Low	11 (57.9)	15 (62.5)	–
Moderate	6 (31.6)	8 (33.3)	–
High	2 (10.5)	1 (4.2)	–
Meat consumption, n (%)			
> 5 servings per week	10 (52.6)	14 (58.3)	–
3–5 servings per week	9 (47.4)	9 (37.5)	–
< 3 servings per week	0 (0.0)	1 (4.2)	–

Data are presented as the mean ± SEM, unless indicated otherwise. ALT, Alanine aminotransferase/Glutamate pyruvate transaminase; BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, Erythrocyte Sedimentation Rate; HDL, high density lipoprotein; LDL, low density lipoprotein; TMAO, trimethylamine N-oxide.

FMD as a dietary regimen was based on the plan developed by the team of Prof. Valter D. Longo (Brandhorst et al., 2015). Briefly, participants in the FMD group were subjected to a 5-day hypocaloric diet that provides 34–54 % of regular caloric intake (approximately 1100 kcal on the first day and approximately 800 kcal on the four subsequent days). The volunteers in the FMD group were asked to consume primarily complex carbohydrates and unsaturated fat but to limit protein intake (the caloric intake of these macronutrients was distributed as follows: 40–45 %; 45–50 %; 10–15 %, respectively). The meals in the FMD group mainly consisted of vegetables, seeds, nuts and vegetable oils. Legumes were allowed only on the first day as they are considered a protein source.

Volunteers in the VEG group were expected to continue their usual dietary regimen, with an exception that they were asked to incorporate 4 servings (each approximately 100–125 grams) of vegetables into their diet per day. The sizes of the meals, the caloric intake and the macronutrient content of the diet were otherwise unrestricted.

The volunteers subjected to this interventional study were under careful supervision throughout the study. Detailed information leaflets were prepared and distributed to volunteers, containing all the important information about the dietary intervention to which they were assigned and the list of allowed products together with their nutritional value. Volunteers usually ate two identical meals together (breakfast and lunch). For evaluation of dinner a special

WhatsApp Messenger group was created, where volunteers shared photos of their meals prepared at home, which was also used as a measure of volunteer compliance.

After 5 days of dietary intervention, volunteers were weighed, and blood samples in the fasted state (fasting at least 10 hours prior the blood sampling) were taken. One volunteer from the FMD group withdrew from the study due to difficulties adhering to the dietary regimen. Samples previously taken from this volunteer were excluded from further analysis.

2.4.3 Determination of biochemical parameters

Blood sampling was carried out in the fasted state immediately before the start of the dietary intervention and the morning after the 5-day dietary intervention. The samples obtained were stored on ice and delivered to the Limited Liability Company "E. GULBJA LABORATORIJA" (accredited by the Latvian National Accreditation Bureau, accreditation No. M-365) within two hours. The samples were subjected to clinical chemistry analyses. β-Hydroxybutyrate (plasma ketone bodies) was measured using a commercially available enzymatic kit (Biosystems S. A, Barcelona, Spain; Lot 39099) according to the manufacturer's instructions.

2.5 Measurement of TMA and TMAO levels by UPLC/MS/MS

The determination of TMA concentrations is used in experiments with bacterial cultures in Publication III; the determination of TMAO concentrations is used in Publications I, II, III, and IV.

Quantification of TMA was performed by UPLC/MS/MS using positive ion electrospray mode as previously described by Kuka et al. (Kuka et al., 2014). Quantification of TMAO in plasma and tissue samples was also performed by UPLC/MS/MS using positive ion electrospray mode as previously described (Dambrova et al., 2013; Grinberga et al., 2015). In brief, the obtained blood samples were centrifuged at 3.000 g for 5 min at 4 °C to separate plasma. The obtained tissue samples were homogenised with water in OMNI Bead Ruptor 24 (Camlab, Cambridge, United Kingdom) at a w/v ratio of 1:10. The obtained homogenates were centrifuged at 20.000 g for 10 min at 4 °C. Separated plasma and supernatants from tissue homogenates were collected and stored at − 80 °C until further analysis.

The sample preparation was carried out by deproteinization with an acetonitrile–methanol mixture (3:1, v/v). The samples were then vortexed and centrifuged at 13.000 g for 20 min. The supernatant was transferred to UPLC vials and used for UPLC/MS/MS analysis.

MassLynx 4.1. software with a QuanLynx 4.1. module (Waters, Milford, USA) was used for data acquisition and processing.

2.6 Ethics statement

All experimental procedures described here involving laboratory animals (Publications I, II, III) were performed in accordance with the EU Directive 2010/63/EU for animal experiments and local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia. The *ex vivo* experiments described in Publication I were performed in compliance with ethical approval Nr. 82; isoproterenol-induced cardiac stress model was performed in compliance with ethical approval Nr. 84. For Publication II, experiments were performed according to ethical approval. Nr. 105. The experimental procedures from Publication III were performed complying with ethical approval Nr. 84. All studies involving animals are reported in accordance with the ARRIVE guidelines (Percie du Sert et al., 2020).

The interventional study involving human volunteers described in Publication IV was approved by the local Ethics Committee of Rīga Stradiņš University, Latvia (No. 6-2/10/51); the ethics permit is attached as Annex 5.

3 Statistical Analysis

The statistical analysis of the data was performed using GraphPad Prism statistical software (GraphPad, Inc., La Jolla, USA). All data are represented as the mean ± standard error of the mean (SEM), except for data from bacterial cultures (Publication III), which are represented as mean ± standard deviation (SD). The data distribution was determined using the Shapiro-Wilk normality test.

Statistically significant differences in mean values were evaluated using a one-way analysis of variance (ANOVA) or Kruskal-Wallis test based on data normality analysis. If ANOVA or Kruskal-Wallis test provided a p value less than 0.05, Tukey's (Dunnett's in Publication II) or Dunn's post-test was performed, respectively, and differences were considered significant when $p < 0.05$. A t-test was used when only two independent groups were compared, repeated measures t-test or Wilcoxon matched-pairs test, depending on the data distribution, was used when changes over time were compared within one group. The results were considered statistically significant if the p value was less than 0.05. None of the animal samples (Publications I, II, III) or the samples of the volunteers (Publication IV) were excluded from further analysis. After performing ROUT analysis to identify outliers, several samples were excluded from analysis for bacterial composition data in faecal samples (Publication III), and exclusions are indicated in the respective figure legends.

In Publication IV, the calculation of insulin sensitivity and insulin resistance indices was performed using homeostatic model assessment and HOMA2 Calculator (version 2.2.3, available online, developed by the Diabetes Trial Unit, University of Oxford, UK) (Wallace, Levy, and Matthews, 2004).

4 Results

4.1 Acute effects of TMAO in experimental *ex vivo* and *in vivo* models of cardiac and vascular functionality

The effects of increased concentrations of TMAO in incubation buffer on tissue accumulation of TMAO were studied (Figure 4.1). For this, male Wistar rat hearts were perfused, and aortic rings were immersed in Krebs-Henseleit (K-H) buffer solution with or without 100 μM TMAO. After 1 hour of perfusion or incubation, the samples were further prepared for UPLC/MS/MS analysis to assess the tissue content of TMAO. After perfusion with a buffer solution containing TMAO, the TMAO content in cardiac tissue increased 3 times (from 2.0 ± 0.2 to 6.3 ± 1.2 nmol/g tissue) and ~2.5 times in aortic tissue after incubation (from 4.8 ± 0.5 to 12.0 ± 1.2 nmol/g tissue).

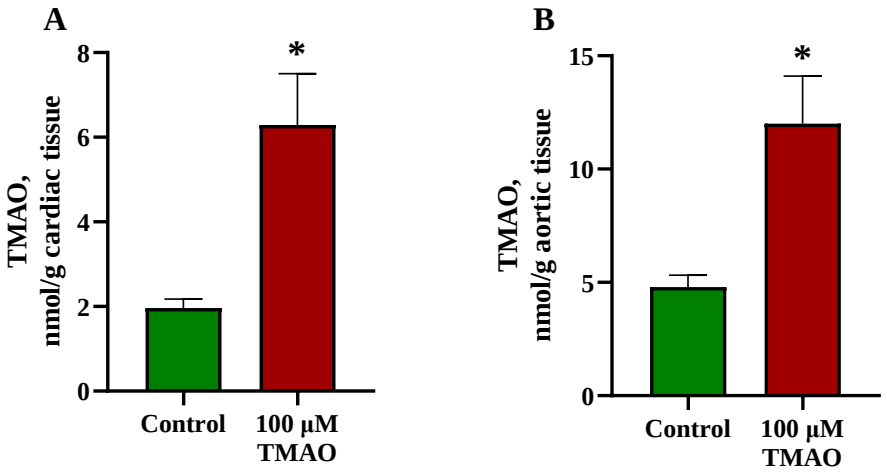

Figure 4.1 **TMAO content in the heart (A) after 1 hour of perfusion and in aortic tissues (B) after 1 hour of incubation in Krebs-Henseleit buffer solution with or without the addition of 100 μM TMAO**

Data are shown as the mean ± SEM of five experiments. * $p < 0.05$ unpaired Student's t-test.

Furthermore, the acute impact of 100 μM TMAO on energy substrate metabolism was assessed by measuring the oxidation of radiolabelled glucose and palmitate and their accumulation in rat aortic rings (Figure 4.2). Incubating aortic rings with 100 μM TMAO increased palmitate oxidation nearly two-fold (from 0.26 ± 0.03 to 0.46 ± 0.03 nmol/(h × mg) ^{3}H-palmitate); however, glucose metabolism was not affected.

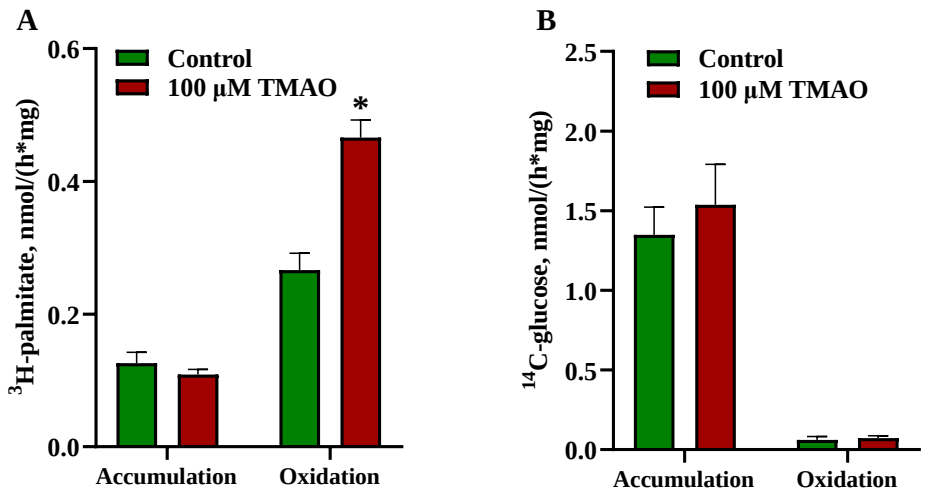

Figure 4.2 **Effects of 100 µM TMAO on the accumulation and oxidation of ^{3}H-palmitate (A) and ^{14}C-glucose (B) in aortic tissues**

Data are shown as the mean ± SEM of five to six experiments. * $p < 0.05$ unpaired Student's t-test.

For cardiac tissue, the reaction was performed in tissue homogenates; therefore, only the oxidation of radiolabelled glucose and palmitate was evaluated (Figure 4.3). TMAO did not affect energy substrate oxidation in heart homogenates.

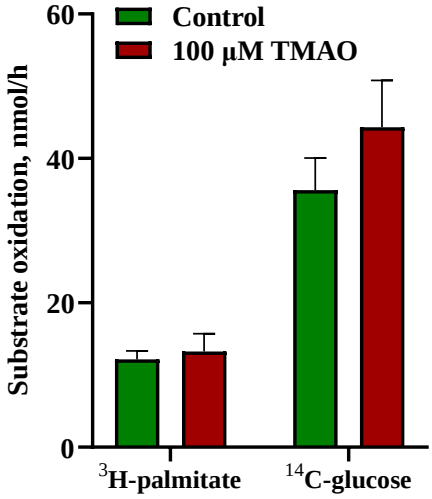

Figure 4.3 **Effects of 100 µM TMAO on the oxidation of ^{14}C-glucose and ^{3}H-palmitate in heart muscle homogenates**

Data are shown as the mean ± SEM of five experiments.

Next, the potency of TMAO to affect the reactivity of conductance and resistance vessels was evaluated. Isolated organ bath experiments were conducted in rat aortic rings submerged in a K-H buffer solution with or without 100 µM TMAO for 1 hour to assess the response to acetylcholine (endothelium-dependent relaxation) and sodium nitroprusside (endothelium-independent relaxation) (Figure 4.4).

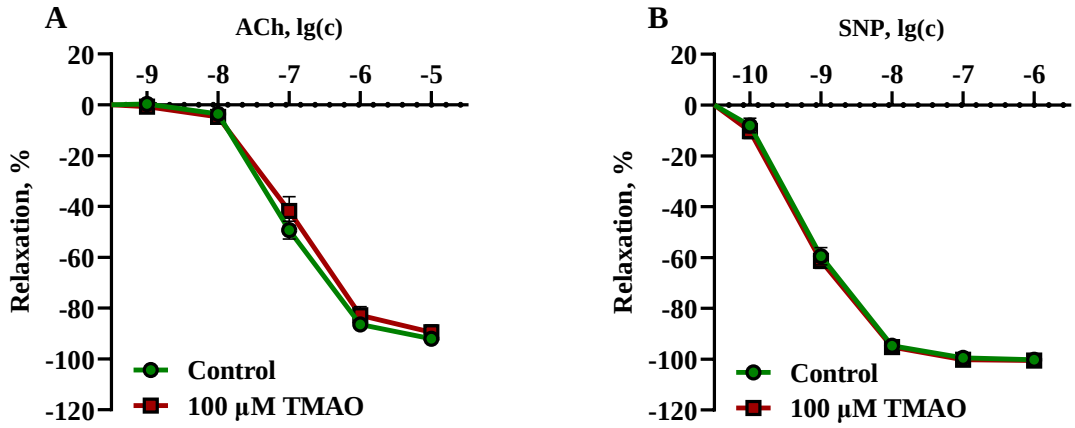

Figure 4.4 **Effects of 100 µM TMAO on endothelium-dependent (A) and endothelium-independent (B) relaxation in aortic rings**

Data are shown as the mean ± SEM of twelve aortic rings. Ach, acetylcholine, SNP, sodium nitroprusside.

A similar procedure was performed on wire myograph with mesenteric arteries (Figure 4.5) to assess whether resistance vessels were affected by TMAO in a similar way as conductance vessels. Incubating the rings of both the aorta and mesenteric artery with 100 µM TMAO did not alter endothelium-dependent or independent relaxation.

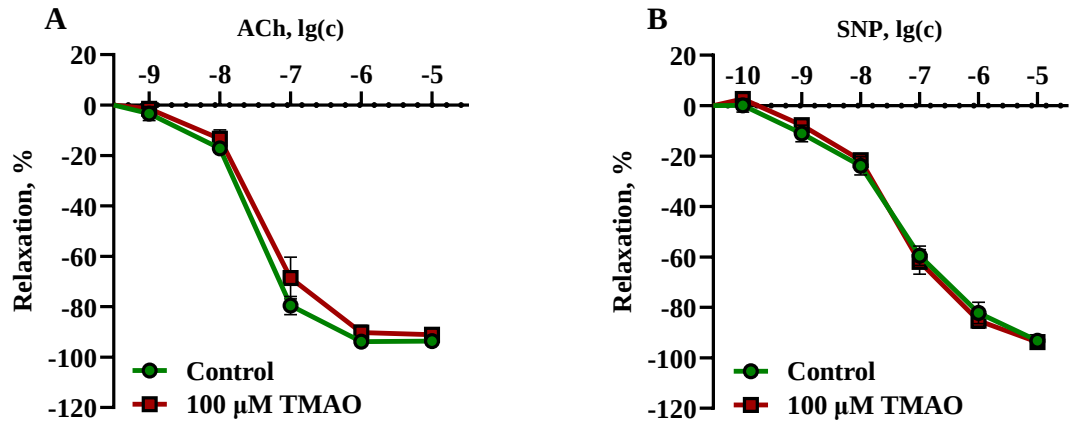

Figure 4.5 **Effects of 100 µM TMAO on endothelium-dependent (A) and endothelium-independent (B) relaxation in mesenteric artery rings**

Data are shown as the mean ± SEM of seven to eight mesenteric artery rings.
Ach, acetylcholine, SNP, sodium nitroprusside.

In addition, the effects of elevated TMAO concentrations on cardiac function were assessed. First, cardiac functional parameters were tested in a Langendorff isolated rat heart model in the presence of 1 mM TMAO in K-H perfusion buffer (Table 4.1).

Table 4.1

Effects of 1 mM TMAO on cardiac function in *ex vivo* model

Measured parameter	Control	1 mM TMAO
Coronary Flow, ml/min	12.4 ± 1.3	11.5 ± 0.8
LVDP, mmHg	155 ± 19	153 ± 17
Heart rate, BPM	219 ± 12	230 ± 15
Contractility, mmHg/sec	5458 ± 347	5419 ± 399
Cardiac Work, kU	33 ± 4	34 ± 3

Results are shown as the mean ± SEM of six hearts.

Next, the effects of elevated TMAO concentrations in the perfusion buffer (1 mM) on overall cardiac functionality during ischemia–reperfusion were evaluated (Figure 4.6). Perfusion of isolated rat hearts with 1 mM TMAO caused no effect on heart function (heart rate, coronary blood flow, contractility, LVDP and cardiac work) at the baseline or during ischemia–reperfusion.

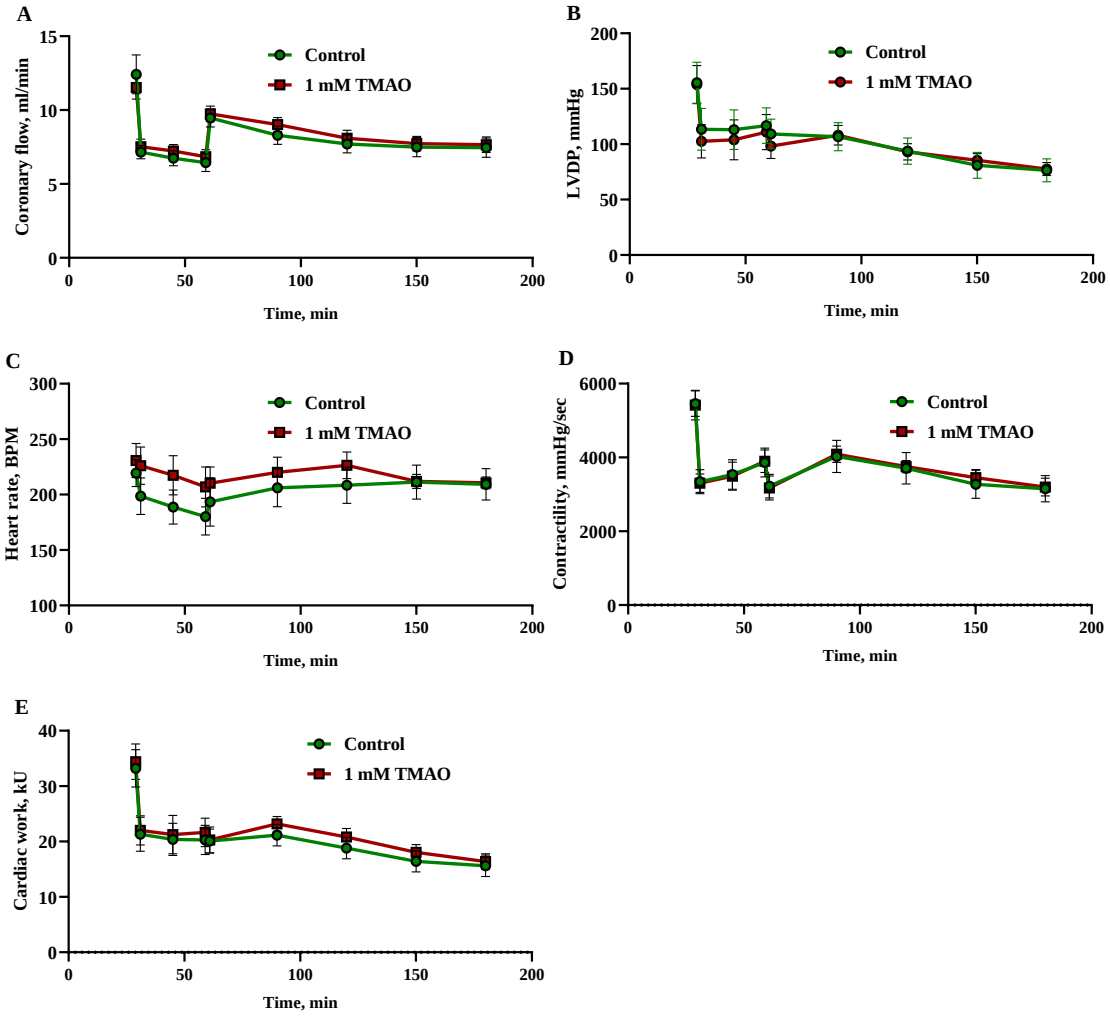

Figure 4.6 **Effects of 1 mM TMAO in the perfusion buffer solution on functional parameters (coronary flow (A), LVDP (B), heart rate (C), contractility (D), and cardiac work (E)) of the isolated rat heart before and during 30 min of occlusion with 120 min of reperfusion**

Data are shown as the mean ± SEM of six hearts.

After this, the size of myocardial infarction was compared in both groups (Figure 4.7). After 30 min of left anterior descending artery occlusion and then 2 h of reperfusion, the area at risk was similar in both experimental groups. Moreover, the necrosis zone or infarct size was nearly identical (~40 % of the risk zone) in both groups.

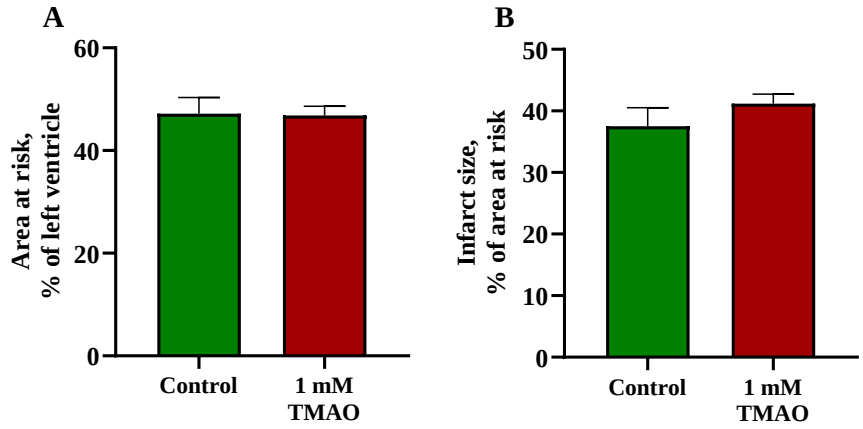

Figure 4.7 **Effects of 1 mM TMAO on the size of myocardial infarction after performing 30 min of occlusion of the left anterior descending coronary artery and then 120 min of reperfusion**

Data are shown as the mean ± SEM of six hearts.

Last, the effects of single and 7-day administration of TMAO at a dose of 120 mg/kg in CD-1 mice on the cardiac response to isoproterenol-induced acute cardiac stress in mice were tested (Figure 4.8). Administration of TMAO at a 120 mg/kg dose caused no effect on the baseline systolic function of the left ventricle. The ejection fractions in the control and TMAO-treated groups were 77 ± 3 % and 79 ± 1 %, respectively. Treating animals with ISO (10 μg/mouse) significantly increased left ventricular ejection fraction, fractional shortening, and heart rate; however, neither single administration nor 7-day administration of TMAO at a dose of 120 mg/kg showed any effect on these parameters.

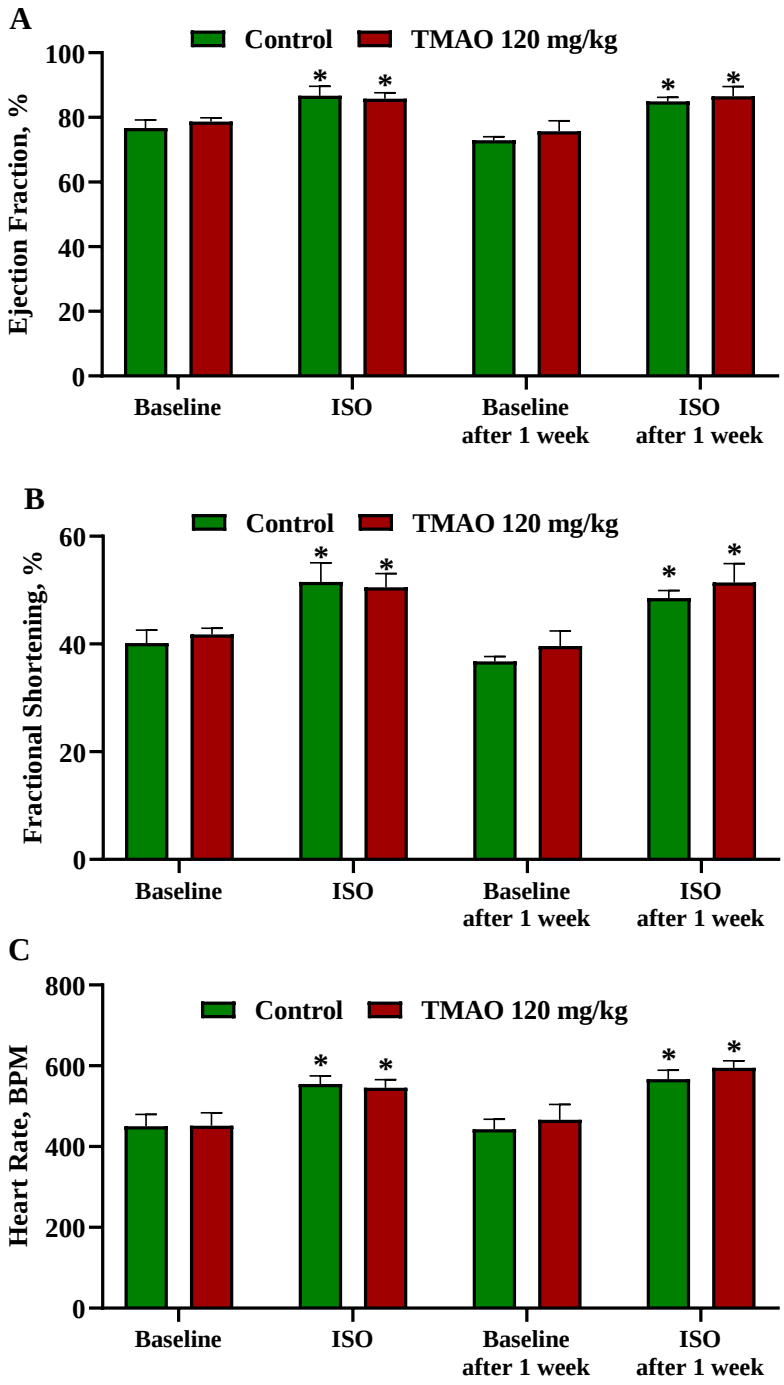

Figure 4.8 **Effects of single and 7-day administration of TMAO at a dose of 120 mg/kg on left ventricular ejection fraction (A), fractional shortening (B), and heart rate (C)**

Results are shown as the mean ± SEM of six animals. * $p < 0.05$ paired Student's t-test, compared to baseline.

4.2 Effects of long-term TMAO administration in experimental model of right ventricular heart failure in rats

4.2.1 Heart failure severity

Administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks resulted in a 6-fold increase in the TMAO plasma concentrations (up to 100 μM) in both the TMAO and TMAO + MCT groups (Figure 4.9A). The analysis of the TMAO content in the

tissues of the right ventricle revealed that treatment with TMAO resulted in a 14-fold increase in the TMAO tissue content (up to 140 nmol/g tissue) in both groups that received TMAO (Figure 4.9B).

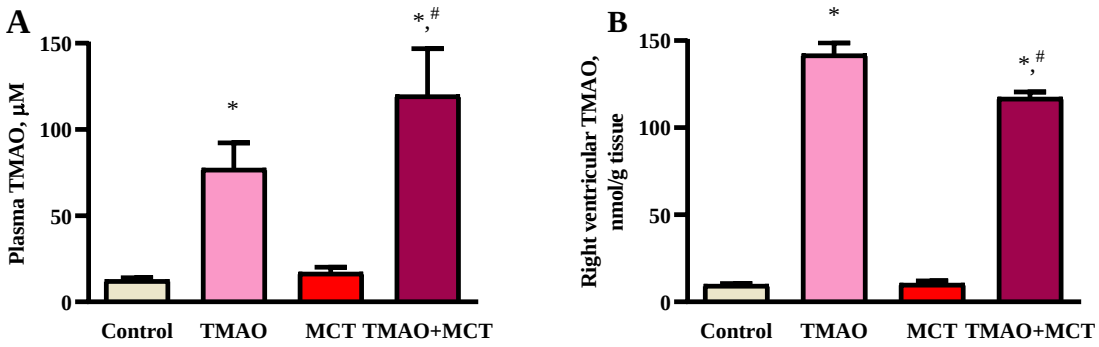

Figure 4.9 **TMAO concentration in plasma (A) and right ventricular tissue (B) after administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks**

Results are presented as the mean ± SEM of 8-9 animals. * indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's posttest), # indicates a significant difference from the MCT group (unpaired t-test), $p < 0.05$.

The echocardiographic assessment did not reveal any significant differences in cardiac function between the control and TMAO groups. Administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks did not affect direct RV pressure, RV systolic and diastolic area or RV fractional area change (Table 4.2). Compared with the control, administration of monocrotaline induced a significant increase (approximately 50 %) in direct RV pressure (Table 4.2). In addition, dilatation of the right ventricle was observed in the hearts of the animals in the MCT group, as indicated by 34 % and 83 % increases in the RV diastolic and systolic areas, respectively. Subsequently, the RV fractional area change was significantly decreased in the MCT group compared to the control group. Compared to those in the MCT control group, the direct RV pressure measurement was decreased by 22 %, the RV diastolic and systolic areas were decreased by up to 27 %, and therefore, the RV fractional area change was increased by 25 % in the TMAO + MCT group. None of the measured parameters in the TMAO + MCT group were significantly different from those in the control group. Overall, these results indicate that long-term TMAO treatment does not affect cardiac functionality. However, in pathological conditions of monocrotaline-induced heart failure TMAO administration preserves myocardial mechanical function.

Table 4.2

Echocardiographic assessment of right ventricle functionality after administration of TMAO at a dose of 120 mg/kg for 14 weeks in a MCT-induced model of right ventricle heart failure

Measured parameter	Control	TMAO	MCT	TMAO + MCT
Right ventricular pressure, mmHg	21.9 ± 2	22.7 ± 1.3	33.5 ± 5.3 *	26.1 ± 1.8
Right ventricular diastolic area, cm^2	0.37 ± 0.02	0.33 ± 0.02	0.5 ± 0.07	0.4 ± 0.04
Right ventricular systolic area, cm^2	0.2 ± 0.01	0.21 ± 0.02	0.36 ± 0.07 *	0.26 ± 0.03
Right ventricular fractional area change, %	46.6 ± 2.6	37 ± 2.8	29.7 ± 4.8 *	37 ± 5

Results are presented as the mean ± SEM of 8-9 animals. *indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's posttest), $p < 0.05$.

To evaluate MCT-induced cardiac and pulmonary remodelling, the organ mass indexes were calculated. Long-term TMAO administration did not impact either the right ventricle (Figure 4.10A) or lung-to-body weight (Figure 4.10B) indexes. Compared to the control group, the MCT group exhibited increased right ventricle hypertrophy and pulmonary remodelling, as indicated by significant increases in the organ-to-body weight indexes by 46 % and 76 %, respectively (Figure 4.10AB). In the TMAO + MCT group, the right ventricle and lung-to-body weight indexes were decreased by 15 % and 11 %, respectively, compared to those in the MCT group (Figure 4.10AB) suggesting that long-term TMAO administration can partially prevent monocrotaline-induced organ remodelling and hypertrophy.

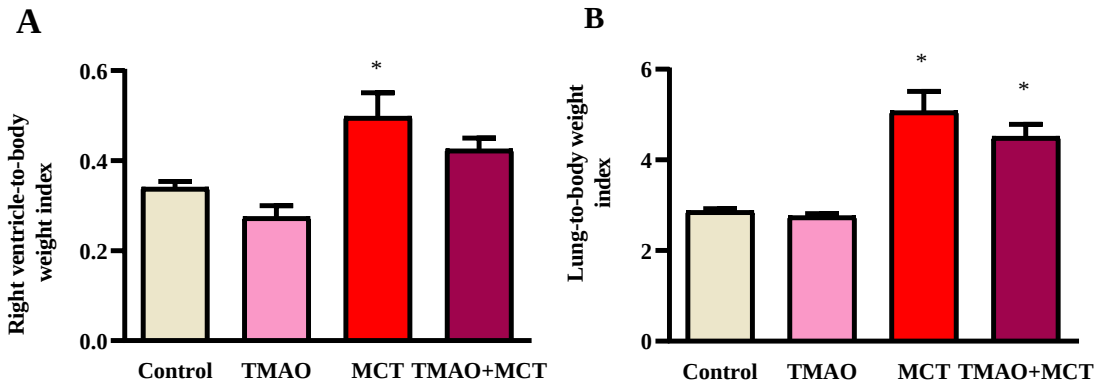

Figure 4.10 **Changes in the right ventricle-to-body weight (A) and lung-to-body weight (B) indexes after monocrotaline injection**

Results are presented as the mean ± SEM of 8-9 animals. * indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's posttest), $p<0.05$.

In addition, long-term TMAO administration did not cause any significant changes in the expression of genes related to heart failure and hypertrophy (Figure 4.11AB) or in the protein expression of BNP45 (Figure 4.11C). In the MCT group, a 3-fold decrease in the *α/β-MHC* expression ratio (Figure 4.11A) was observed, indicating a shift in favour of the *β* isoform caused by right ventricle hypertrophy. In addition, the expression of a maker of heart failure severity, *BNP*, was upregulated by 12-fold in the MCT group (Figure 4.11B). Consistent

with the gene expression results, BNP45 protein expression in cardiac tissue was significantly increased by 10-fold in the MCT group compared to the control group (Figure 4.11C). In the TMAO + MCT group, the *α/β-MHC* expression ratio was 2-fold higher, suggesting less pronounced cardiac hypertrophy compared to that in the MCT group (Figure 4.11A). The gene and protein expression of BNP was lower in the TMAO + MCT group than in the MCT group (Figure 4.11BC).

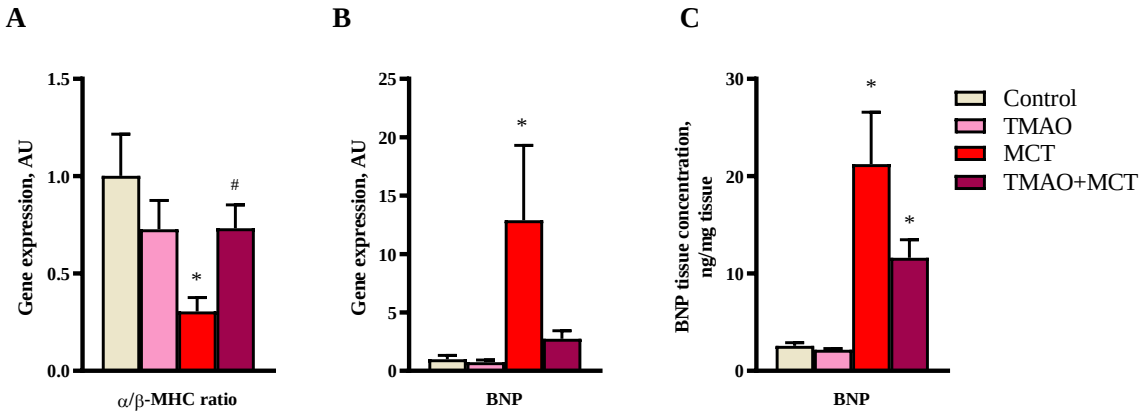

Figure 4.11 **Effect of administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks on heart failure severity-related gene expression (A, B) and BNP protein expression (C) in right ventricular tissue of a rat model of monocrotaline-induced heart failure**

Results are presented as the mean ± SEM of 6–8 animals. * indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's posttest for panel A, Kruskal-Wallis test followed by Dunn's multiple comparison test for panels B and C;), # indicates a significant difference from the MCT group (unpaired t-test), p–< 0.05.

4.2.2 Mitochondrial energy metabolism

To further investigate the effects of long-term TMAO administration on energy metabolism, mitochondrial respiration measurements were performed using permeabilised cardiac fibres prepared from RV tissue samples. Long-term TMAO administration decreased the FAO-dependent respiration rate by 69 % in the OXPHOS state (Figure 4.12A), resulting in an 11 % decrease in the FAO-dependent OXPHOS coupling efficiency (Figure 4.12B). Although pyruvate metabolism input to overall respiration was increased by approximately 44 % in the TMAO group, as indicated by Flux control factor analysis (Figure 4.12B), it was not sufficient to restore FN and FNS pathway-linked mitochondrial respiration in the OXPHOS state (Figure 4.12A). In the MCT group, there was a 75 % decrease in the FAO-dependent respiration rate in the OXPHOS state (Figure 4.12A) and a subsequent 13 % decrease in the FAO-dependent OXPHOS coupling efficiency (Figure 4.12B). Similar to the TMAO group, in the MCT group, pyruvate metabolism input to respiration was increased by 50 % (Figure 4.12B), but this increase was not sufficient to restore FN- and FNS-pathway-linked respiration

in the OXPHOS state (Figure 4.12A). In contrast to the TMAO group, in the MCT group, the flux control factor for rotenone was reduced ($p = 0.06$), indicating partial complex I dysfunction (Figure 4.12B). Moreover, in the TMAO + MCT group, mitochondrial energy metabolism was preserved, as shown by normalised respiration rates (Figure 4.12A), preserved FAO-dependent oxidative phosphorylation efficiency and subsequently decreased pyruvate metabolism input (Figure 4.12B).

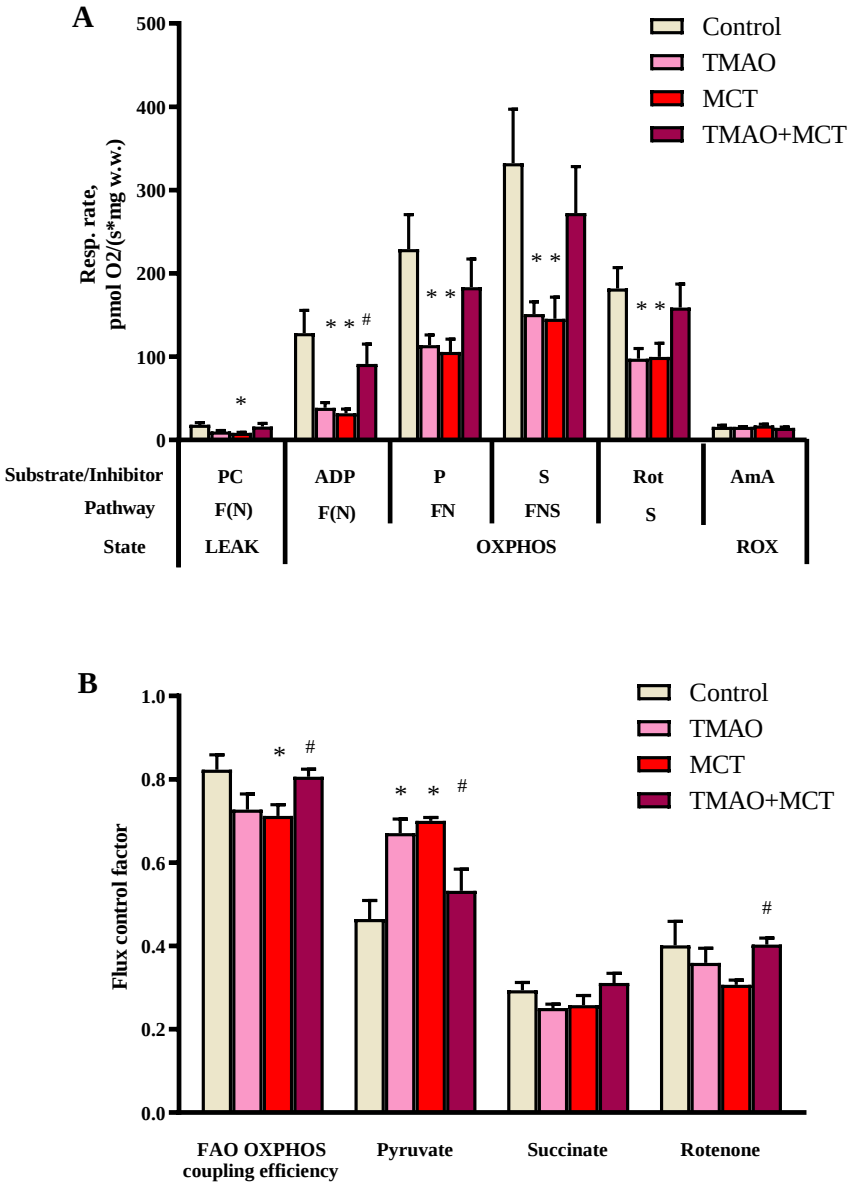

Figure 4.12 **Mitochondrial respiration rate measurements (A) and flux control factors (B) in right ventricular cardiac fibres using different energy substrates after administration of TMAO at a dose of 120 mg/kg for 14 weeks in a model of monocrotaline-induced right ventricle heart failure**

Results are presented as the mean ± SEM of 6 animals. * indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's posttest), # indicates a significant difference from the MCT group (unpaired t-test), $p < 0.05$. Flux control factor, the contribution of each substrate/pathway to the respiration rate; PC, palmitoylcarnitine; ADP, adenosine diphosphate; P, pyruvate; S, succinate; Rot, rotenone; AmA, antimycin A; F, fatty acid oxidation-dependent pathway; N, NADH pathway; LEAK, substrate-dependent state; OXPHOS, oxidative phosphorylation-dependent state; ROX, residual oxygen consumption.

Moreover, the observed effects are independent of changes between groups in the amount of mitochondria in the cardiac samples, because measurements of the citrate synthase activity (Figure 4.13) in RV tissue showed that there were no differences in mitochondrial mass between the experimental groups.

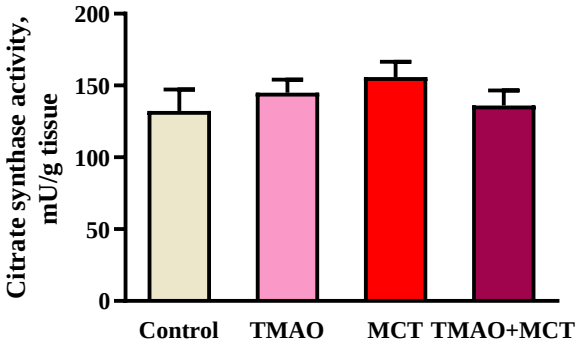

Figure 4.13 **Cardiac citrate synthase activity in right ventricular tissue homogenates**

Results are presented as the mean ± SEM of 6 animals.

Taken together, the obtained results show that long-term TMAO administration induces mitochondrial metabolic preconditioning by causing a switch from fatty acid utilization to pyruvate utilization without affecting electron transfer functionality; moreover, in right ventricle heart failure, TMAO treatment can preserve cardiac mitochondrial energy metabolism.

4.3 Metformin reduces TMAO levels in experimental model of type 2 diabetes

4.3.1 TMAO production in db/db mice

To test metformin as a pharmacological approach to control TMAO levels, it was used in db/db mice model of advanced type 2 diabetes. The TMAO concentration in plasma of db/db control mice fed standard chow (R70) was significantly, up to 13.2-fold, higher than that in db/Lean mice plasma. Metformin administration to db/db mice at a dose of 250 mg/kg for 8 weeks significantly decreased TMAO levels up to 2.0-fold when compared to those of db/db control mice (Figure 4.14A).

Glucose and insulin plasma concentrations were significantly increased in both fed and fasted states, and metformin had no effect in any of these states (Figure 4.14BC). Taken together, metformin treatment had no glucose-lowering and insulin sensitivity-improving effect in db/db mice with type 2 diabetes; thus, the effects observed in this study were independent of glucose and insulin plasma concentrations.

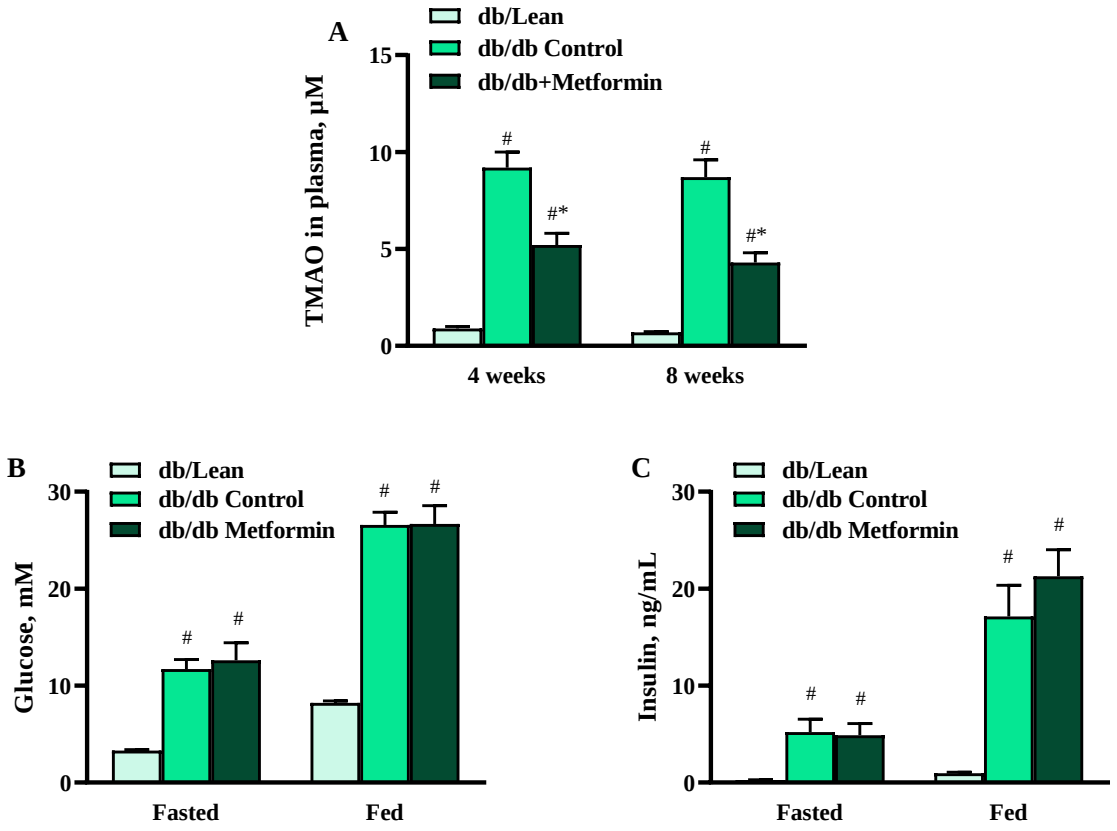

Figure 4.14 **Effects of metformin (250 mg/kg) treatment on plasma TMAO concentrations (A) at 4- and 8-week time points in db/db mice fed a standard laboratory diet. Effects of an 8-week metformin treatment at a dose of 250 mg/kg on blood glucose levels in fasted and fed states (B) and plasma insulin in fasted and fed states (C).**

Results are the mean ± SEM of 8 animals in the db/db and db/db + metformin groups and 10 animals in the db/Lean mice group. * Significantly different from the respective db/db control group, # significantly different from the respective db/Lean group (ANOVA, followed by Tukey's test for A and B, Kruskal-Wallis test followed by Dunn's multiple comparison test for C).

4.3.2 TMAO production in db/db mice supplemented with choline

As TMA and subsequent TMAO production highly depend on diet composition, next we evaluated whether metformin treatment can affect chronic and acute dietary choline load-induced increases in TMAO levels. Based on findings with standard chow, where the efficacy of metformin was similar after 4 and 8 weeks of treatment, administration of metformin for 4 weeks was selected for follow-up studies using a choline supplementation. In this experiment, choline pre-treatment for 4 weeks resulted in a further 1.8-fold increase in basal TMAO levels (36.9 µM) when compared to db/db mice on a standard diet (Figure 4.15A) and 16.8-fold increase when compared to control db/Lean mice. Two hours after acute choline load, TMAO plasma levels increased in all experimental groups, and highest concentrations up to 95–108 µM were observed in the db/db control and choline pre-treated groups. In db/db mice on a diet supplemented with choline, metformin treatment significantly decreased both basal and 2 h post-choline-load TMAO levels (Figure 4.15A).

To assess the population of selected bacteria in the faecal samples (from mice treated with choline or choline in combination with metformin for 4 weeks) we evaluated metformin-induced changes in abundance of *P. mirabilis* and *K. pneumoniae*. While levels of *K. pneumoniae* were too low to be detected, *P. mirabilis* bacteria were detected and we observed tendency for relatively higher *P. mirabilis* bacteria presence in db/db mice that received choline and tendency for metformin to prevent this increase (Figure 4.15B).

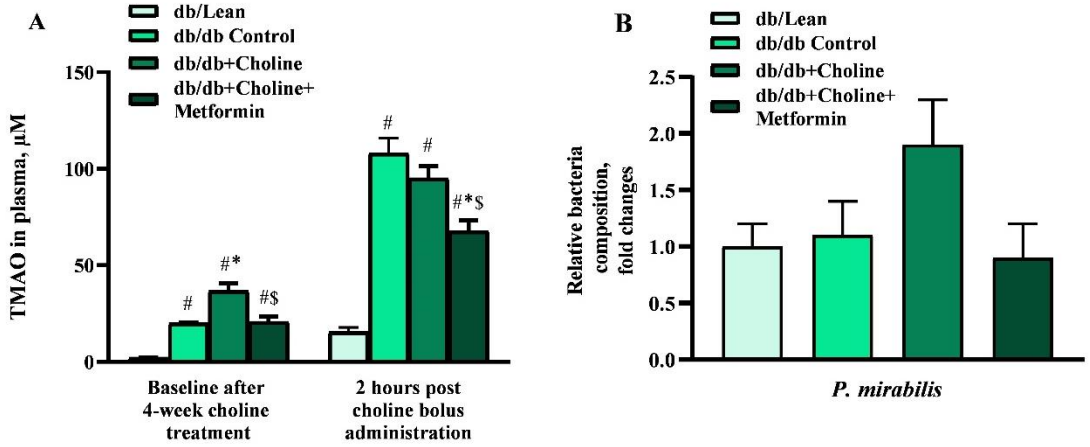

Figure 4.15 **Effects of 4 weeks of metformin (250 mg/kg) treatment on plasma TMAO levels in mice supplemented with choline (0.5 % with drinking water) and after acute choline load (100 mg/kg, bolus dose) (A) and on relative presence of *P. mirabilis* (B) in gut microbiota**

Results are the mean of 10 animals ± SEM for panel A. Results are mean of 9-10 animals for panel B, one sample was excluded in choline group and one in choline + metformin group after identified as outliers by ROUT analysis. * Significantly different from the respective db/db Control group, $^{\#}$ significantly different from the respective db/Lean group, $^{\$}$ significantly different from the respective db/db + Choline (ANOVA followed by Tukey's test; $p < 0.05$).

4.3.3 Bacterial TMA production

Since treatment with metformin significantly decreased TMAO production in diabetic mice, both in normal conditions and after choline load, we tested whether this effect could be related to changes in ability of gastrointestinal tract bacteria to produce TMA, which is required for host liver to produce TMAO. For this we selected human gastrointestinal tract bacteria *K. pneumoniae* and *P. mirabilis* both known to be able to produce TMA (Kuka et al., 2014; Wu et al., 2019). *K. pneumoniae* was chosen because it can produce TMA from all the main precursors – choline (via CutC/D (choline-TMA-lyase complex)) and carnitine/GBB (via CntA/B (Carnitine monooxygenase complex)), while *P. mirabilis* was chosen because it has just CutC/D and can produce TMA only from choline (Kuka et al., 2014; Wu et al., 2019). Metformin significantly decreased TMA production 3.25-fold in *K. pneumoniae* (Figure 4.16A), while bacterial growth of *K. pneumoniae* was not affected (Figure 4.16C) when choline or glucose was used as the sole carbon source for up to 8 h. Metformin significantly decreased

TMA production (up to 26-fold) in *P. mirabilis* (Figure 4.16B). Unlike with *K. pneumoniae*, metformin significantly decreased *P. mirabilis* bacterial biomass growth when choline was used as the sole carbon source (Figure 4.16D). The effect was present, albeit less pronounced when *P. mirabilis* was grown in media with glucose as the sole carbon source.

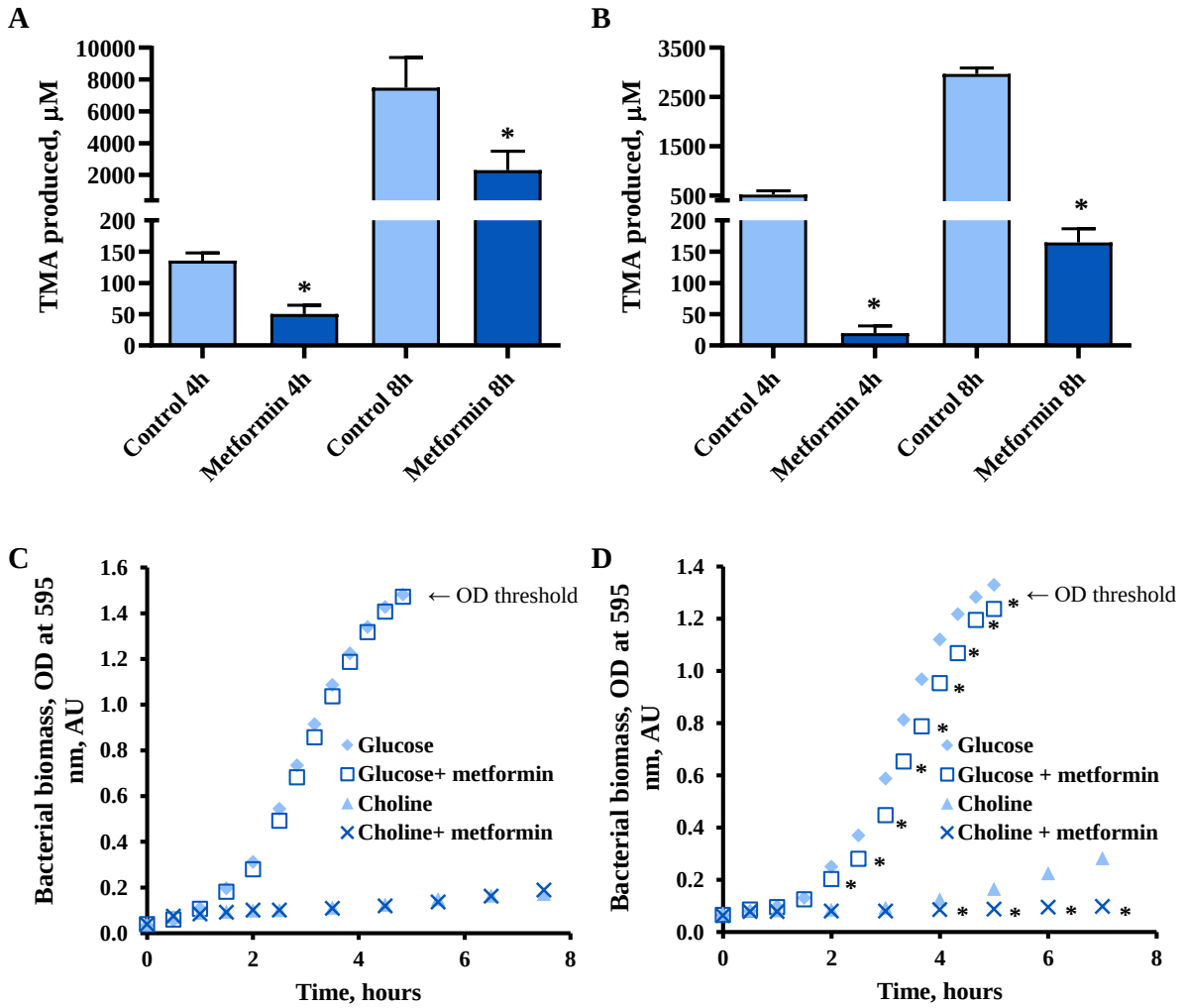

Figure 4.16 **Metformin (27 mM) effects on TMA production from choline (A, B) and bacterial growth (C, D) in *K. pneumoniae* (A and C) and *P. mirabilis* (B and D) under anaerobic conditions**

Results are the mean ± SD of 3 independent replicates for A and 4 for B, C and D.
* $p < 0.05$ vs. respective time point control (t-test). The maximum absorbance value for turbid suspensions in the colorimeter used in the experiments was 1.4.

To determine if the administration of metformin changes the expression of bacterial genes encoding key proteins of choline metabolism, choline lyase enzyme complex subunits *CutC* and *CutD* and enzyme enclosure microcompartment structural protein *CmcA* (Craciun, Marks, and Balskus, 2014; Kalnins et al., 2015), *K. pneumoniae* were cultivated with choline (0.5 % in broth) as the sole carbon source, and metformin was added at the final concentration of 27 mM (0.5 % in broth). Samples for gene expression analysis were extracted after 0, 4 and 8 h of anaerobic cultivation with choline and with or without metformin. The expression of the

genes encoding choline-TMA-lyase complex subunits (*CutC* and *CutD*) and the microcompartment structural gene (*CmcA*) increased over time; however, metformin had no significant effect on the expression of any of the tested genes (Figure 4.17ABC). Moreover, choline-TMA-lyase enzymatic activity was determined in *P. mirabilis* bacterial lysate, and we found no effect of metformin on the TMA production rate (Figure 4.17D).

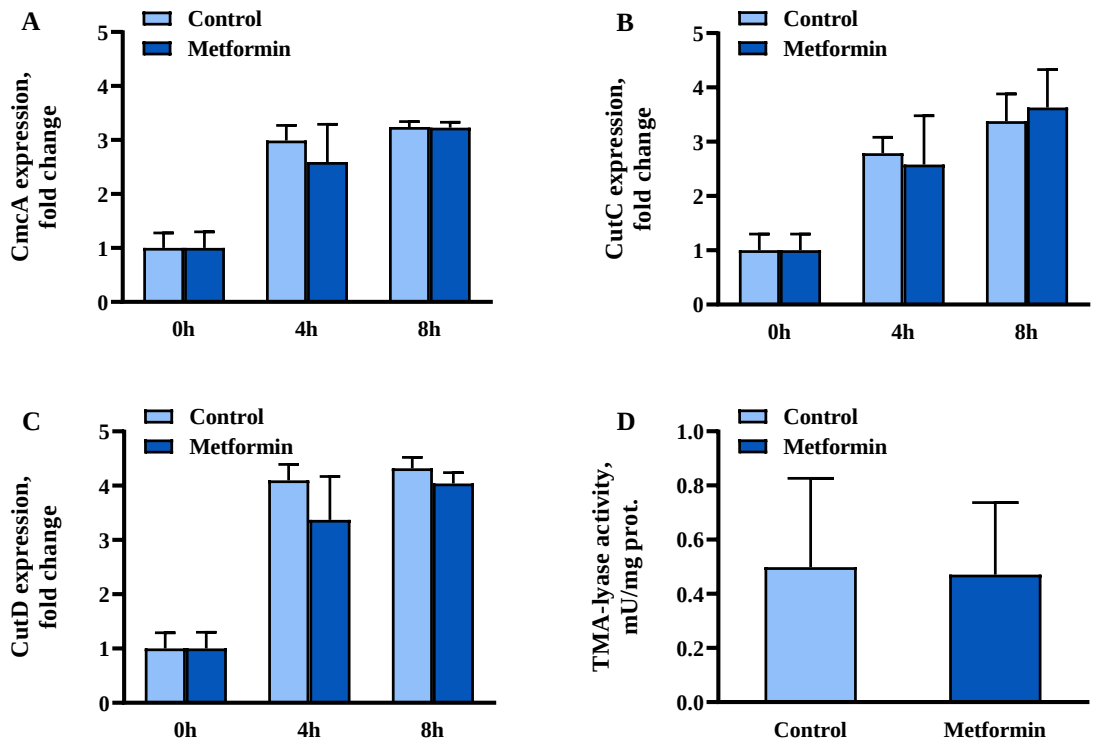

Figure 4.17 **Effects of metformin (27 mM) on the expression of genes that encode enclosure microcompartment structural proteins *CmcA* (A) and choline-TMA-lyase subunit proteins *CutC* (B) and *CutD* (C) in *K. pneumoniae* and on the choline-TMA-lyase activity in *P. mirabilis* bacterial lysate (D) under anaerobic conditions**

Results are the mean ± SD of 4 independent replicates for A, B and C and the mean of 5 independent replicates for D.

Overall, these data provide evidence that metformin can reduce TMA production rate in two selected bacterial strains via at least two different mechanisms. This effect seems to be rather complex, and our data indicate that the effects of metformin do not occur through choline-TMA-lyase.

4.4. Fasting mimicking diet as a strategy to reduce TMAO levels

To test FMD as a possible lifestyle strategy for reduction of TMAO levels, a clinical study was performed in healthy individuals. To distinguish, whether the expected effects are attributed to specific regimen of FMD or to an increase of vegetable intake in an omnivorous diet, it was decided to involve a group of participants that would incorporate an additional

amount of vegetables in their usual diet (4 portions per day). All volunteers recruited in this clinical study were generally healthy, as baseline biochemistry measurements did not indicate any severe health-related conditions of any of the organ systems (Table 2.3). Anthropometric measurements suggested that the volunteers were slightly overweight with a mean BMI of 27.2 ± 0.7 units. The mean TMAO concentration in plasma was 5.08 ± 0.74 μmol/L at baseline.

The interindividual variability of the baseline TMAO levels was high (ranging from 1.3 μmol/L to 24.8 μmol/L) in our volunteers. The measurement of plasma TMAO levels after the dietary interventions revealed that 5 days of the regular diet supplemented with 4 servings of vegetables per day (VEG) did not result in significant changes in plasma TMAO levels (Figure 4.18), with a mean increase of 0.43 ± 0.70 μmol/L. In 8 out of 19 volunteers, we observed a reduction in plasma TMAO levels after the dietary intervention; however, 11 volunteers experienced an increase in plasma TMAO levels. In contrast, 75 % (18 out of 24) of the volunteers who followed the FMD experienced a notable reduction in plasma TMAO levels. The volunteers from FMD group with higher plasma TMAO levels at baseline exhibited more prominent decrease in plasma TMAO levels after the intervention ($r = -0.9226$, $p < 0.0001$). Moreover, the average plasma level of TMAO in the FMD group at the 2nd visit was by 3.01 ± 1.43 μmol/L lower than that at the 1st visit.

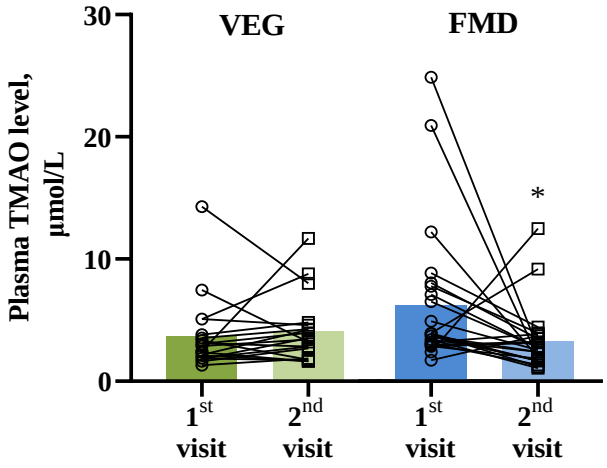

Figure 4.18 **The impact of the 5-day cycle of regular diet supplemented with 4 servings of vegetables (VEG) and fasting mimicking diet (FMD) on the plasma level of TMAO**

Results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

As FMD as a dietary regimen is based on imitating the molecular effects of prolonged fasting, we next evaluated the effects of both diets on plasma ketone body concentrations. As shown in Figure 4.19A, 5 days of the VEG diet resulted in only a slight increase in plasma ketone body levels from 0.11 ± 0.02 mmol/L to 0.16 ± 0.04 mmol/L. In contrast, the FMD

group exhibited a significantly higher increase in plasma ketone body levels by 1.87 ± 0.32 mmol/L (14-fold elevation compared to baseline measurement). We also observed a significant reduction in plasma insulin-like growth factor-1 (IGF-1) concentrations (Figure 4.19B) in the FMD group by 37 ± 8 ng/mL, which was not present in the VEG diet group.

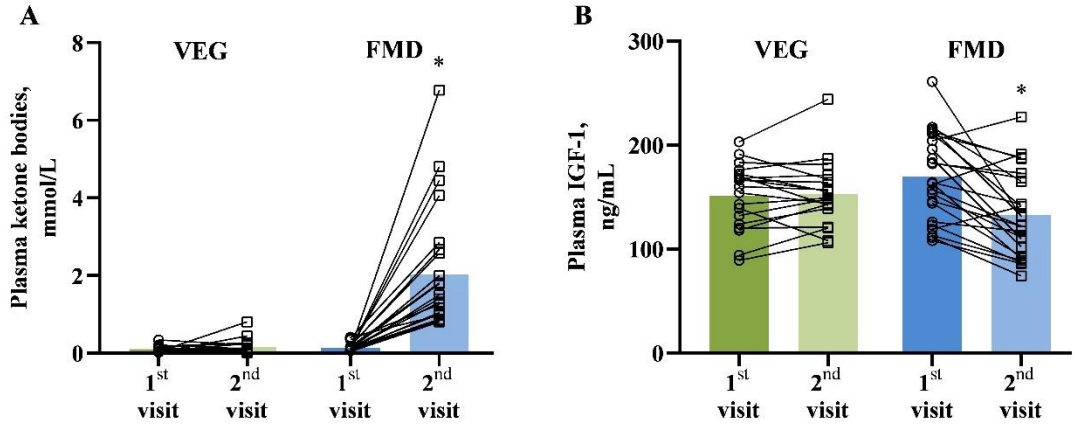

Figure 4.19 **Changes in the levels of plasma ketone bodies (A) and insulin-like growth factor-1 (IGF-1) (B) induced by 5 days of the regular diet supplemented with additional vegetables (VEG) and fasting mimicking diet (FMD)**

Results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

To investigate the contribution of applied dietary strategies to weight loss, volunteers were weighed before the study and after 5 days of the applicable diet. At baseline, volunteers in the VEG group weighed 78 ± 4 kg. The baseline weight of the volunteers in the FMD group was slightly higher (88 ± 3 kg). Only 5 volunteers subjected to the VEG diet experienced a slight weight reduction of an average of 0.28 ± 0.15 kg of body weight (Figure 4.20A). However, each of the volunteers who followed FMD experienced significant weight loss. The average weight loss in the FMD group after 5 days of the dietary intervention was 2.8 ± 0.2 kg of body weight. These changes in body weight resulted in a more pronounced reduction in the BMI (Figure 4.20B) in the FMD group (0.90 ± 0.06 units in the FMD group compared to 0.09 ± 0.05 units in the VEG group).

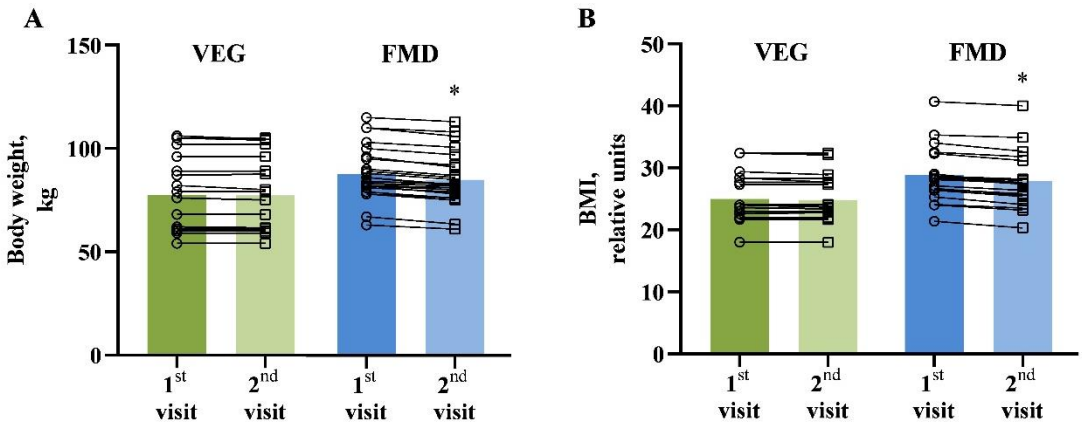

Figure 4.20 **Effects of a 5-day regular diet with additional intake of vegetables (VEG) and fasting mimicking diet (FMD) on weight (A) and BMI (B) in healthy volunteers**

Results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

Next, we evaluated the effects of a 5-day cycle of the VEG diet and FMD on metabolic parameters. Fasting plasma glucose (Figure 4.21A) in the VEG group was reduced by 0.22 ± 0.12 mmol/L. Meanwhile, in the FMD group, the lowering of fasting plasma glucose was 2.7 times more pronounced (a decrease of 0.57 ± 0.11 mmol/L). A similar pattern was observed in plasma C-peptide levels (Figure 4.21B), where the FMD group exhibited a significant reduction in plasma C-peptide compared to the VEG group (a decrease of 0.72 ± 0.11 ng/mL and 0.09 ± 0.11 ng/mL, respectively).

Subsequently, volunteers in the FMD group also had an improved insulin sensitivity index (Figure 4.21C). The increase in insulin sensitivity was 3.8 times greater than that in the VEG group and exceeded the baseline measurement by more than 60 %. The benefits of FMD were even more pronounced when we calculated the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index, which defines the extent of insulin resistance (Figure 4.21D). In the VEG group, we observed a nonsignificant reduction in HOMA-IR by 0.08 ± 0.08 units. In contrast, every volunteer in the FMD group showed a reduced HOMA-IR index, with an average decrease of 0.55 ± 0.08 units.

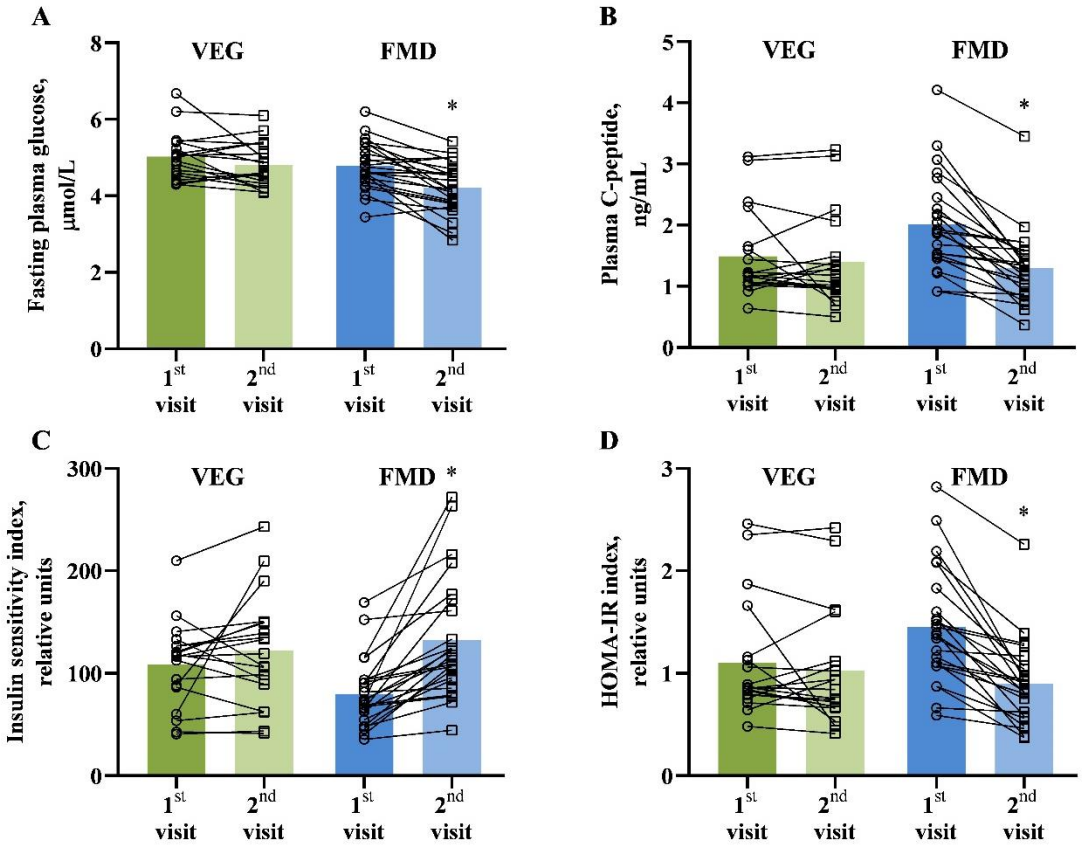

Figure 4.21 **Changes in fasting plasma glucose levels (A), plasma C-peptide levels (B), insulin sensitivity index (C), and HOMA-IR index (D) after the 5-day cycle of regular diet supplemented with additional vegetables (VEG) and fasting mimicking diet (FMD)**

Results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

In the FMD group, we also observed a slight reduction in plasma high-density lipoprotein (HDL) levels after the 5-day dietary intervention. Both diets showed similar effects on plasma triglycerides (a reduction of up to 15 %). However, no other significant changes in the plasma lipid profile were evident in any of the experimental groups (Table 4.3).

Table 4.3

The effects of a 5-day regular diet supplemented with 4 servings of vegetables (VEG) and fasting mimicking diet (FMD) on the plasma lipid profile

Measured parameter	VEG		FMD	
	1st visit	2nd visit	1st visit	2nd visit
High-density lipoprotein, µmol/L	1.51 ± 0.07	1.51 ± 0.07	1.49 ± 0.08	1.30 ± 0.07
Low-density lipoprotein, µmol/L	3.33 ± 0.16	3.32 ± 0.15	3.38 ± 0.19	3.41 ± 0.20
Triglycerides, µmol/L	1.44 ± 0.22	1.22 ± 0.20 *	1.30 ± 0.09	1.10 ± 0.07 *

Results are presented as the mean ± SEM of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

5 Discussion

In the present Thesis, the results indicate that short-term treatment with TMAO does not induce detrimental effects on cardiac or vascular function in *ex vivo* and *in vivo* experimental models. Furthermore, under specific conditions in the rat model of RV heart failure the long-term TMAO treatment exerts preconditioning-like effects and results in preserved fatty acid oxidation capacity with subsequently protected cardiac function. Thus, the first hypothesis of Thesis was not confirmed, as we did not observe any activation of detrimental molecular signalling pathways or impaired cardiac or vascular function after supplementation with TMAO in rodent models. To provide relevant reference values in *ex vivo* and *in vivo* models, the whole data set from Publication I is available open access. In addition, the data presented in the Thesis can be reused for comparative studies aimed at the short- and long-term effects of increased TMAO concentrations on energy substrate metabolism, cardiac functionality, and vascular reactivity. On the contrary, the second hypothesis of Thesis was confirmed, as we demonstrated reduction of circulating TMAO levels by metformin in the animal model of advanced T2D. Moreover, we showed that 5-day FMD serves as a viable lifestyle strategy to reduce TMAO levels and improve overall metabolic health in generally healthy volunteers.

5.1 Increased TMAO bioavailability in experimental models of cardiovascular and metabolic diseases

The data on the role of TMAO in development of cardiometabolic diseases from various patient populations are mostly equivocal, suggesting TMAO as a biomarker and a predictor of nearly all chronic conditions linked to cardiovascular and metabolic health (Li et al., 2022). To some extent the preclinical studies support the idea on the involvement of TMAO in development of CVD. Several studies have found an association between TMAO and the development of AS (Koeth et al., 2013; Geng et al., 2018; Liu and Dai, 2020), which is known to be the leading cause of CVD. Other possible mechanisms linking TMAO to the pathogenesis of CVD include platelet activation, increased probability of thrombosis (Zhu et al., 2016), aggravation of vascular inflammation (Chen et al., 2017; Ma et al., 2017) and prolongation of the hypertensive effect of angiotensin II (Ufnal et al., 2014), as indicated by preclinical research.

However, more recently several preclinical studies in rodents have reported lack of any TMAO-related effects linked to pathogenesis of CVDs even at high concentrations. It has been shown that even a 100-fold increase in circulating TMAO levels (up to 60 μM) in rats did not affect cardiac functionality (Ufnal et al., 2014). Similarly, no effects on cardiac parameters in mice were observed after 3 weeks of administration of 0.12 % TMAO in the chow (Organ

et al., 2016). Although the TMAO concentration in target tissues was not determined in previously mentioned studies, it has recently been shown that TMAO at concentrations up to 10 mM does not affect cell viability, mitochondrial membrane potential or production of reactive oxygen species in rat cardiomyocytes (Querio et al., 2019). In addition, recent studies indicated that TMAO administration does not exacerbate the condition of already present stressors (Querio et al., 2019), such as H_2O_2, which is a major contributor to oxidative stress (Nita and Grzybowski, 2016), and doxorubicin, which is known to cause disturbances in cardiac energy substrate metabolism similar to those caused by heart failure (Wu et al., 2016). Our experimental results from acute administration of TMAO in *ex vivo* and *in vivo* models provide evidence that TMAO should not be considered as a primary cause of cardiac damage, as we did not observe any effects of TMAO on cardiac function neither at baseline, nor during ischemia-reperfusion in rat isolated heart model or in isoproterenol-induced cardiac stress model in mice (Table 4.1, Figure 4.6–4.8).

Of course, we cannot assume that the response of all cell types and organ systems would be the same to such high TMAO doses, and differential effects depending on the dose, duration of treatment and the cell type used should be considered. In fact, previously conducted studies in rodent models of natural and accelerated ageing have shown harmful effects of TMAO (up to 14 μM) on endothelial cells, such as promotion of cellular senescence and induction of oxidative stress (Li et al., 2017; Ke et al., 2018), which are major contributors to vascular ageing. These results also provide a rationale behind the involvement of TMAO in development of AS, as deleterious effects of endothelial cells are one of the instigating mechanisms in pathogenesis of this process. Our results, however, did not reveal any acute effects of TMAO on conductance or resistance vessel endothelium-dependent or endothelium-independent reactivity (Figure 4.4, 4.5), which is in line with previous studies (Matsumoto et al., 2020; Oakley et al., 2020; Florea et al., 2023). These findings could indicate that the impact on endothelial cells is not an acute effect of TMAO, but rather occurs after chronically increased TMAO levels as shown previously (Ke et al., 2018; Brunt et al., 2022).

Our results from right ventricle heart failure model, where TMAO was administrated to rats for 14 weeks with drinking water, indicate that the TMAO level in the cardiac tissue can reach up to 140 nmol/g tissue (Figure 4.9). This provides a rationale behind further dose selection strategies in *in vitro* experiments. Moreover, our results demonstrate that such long-term increase in plasma TMAO levels up to 100 μM for 14 weeks and a subsequent increase in TMAO levels in cardiac tissue do not affect cardiac function (Table 4.2). In our experimental setup, long-term TMAO administration shifted mitochondrial energy substrate utilization from FAO to glucose metabolism, but in contrast to the heart failure group, the TMAO treatment

group did not exhibit altered mitochondrial electron transfer system functionality (Figure 4.12). Since the shift from compensated cardiac hypertrophy to heart failure is usually preceded by respiratory complex I and II dysfunction (Griffiths et al., 2010), unaltered complex I and complex II function could explain our observations of maintained cardiac functionality in case of 14-week TMAO treatment (Figure 4.12). Overall, our findings suggest that despite this shift in mitochondrial energy metabolism, long-term elevations in TMAO levels in plasma and cardiac tissue do not result in detrimental effects on cardiac function in rats.

In the previous studies utilising experimental models of heart failure elevated plasma TMAO levels were reported to worsen cardiac parameters, suggesting that TMAO is a detrimental factor in the pathophysiology of heart failure. Administration of TMAO and its precursor, choline, has been shown to exacerbate left ventricle remodelling and result in rapid loss of cardiac function (Organ et al., 2016). Moreover, withdrawal of dietary TMAO even 6 weeks after aortic constriction reversed these changes, indicating the ability of the heart to recover from detrimental changes caused by TMAO (Organ et al., 2020). Furthermore, a reduction in circulating TMAO levels by 3,3-dimethyl-1-butanol or iodomethylcholine alleviated cardiac hypertrophy and remodelling after aortic banding (Organ et al., 2020; Wang et al., 2020). On the contrary, our results demonstrate that an increase in TMAO levels in plasma and tissues partially prevents the remodelling of the right ventricle and the development of right-sided heart failure. Another study reported reduction of cardiac fibrosis and improvement of cardiac functionality after TMAO treatment in a model of Spontaneously Hypertensive rats (Huc et al., 2018). Consistent with these findings, our study shows that TMAO administration can partially prevent RV hypertrophy, as shown by normalised organ-to-body weight indexes and hypertrophy-related gene expression (Figure 4.10, 4.11). Moreover, in line with our results, protective effects of TMAO were also observed in Spontaneously Hypertensive Heart Failure rats, in which long-term TMAO treatment improved survival and cardiac parameters and lowered plasma levels of NT-proBNP (Gawrys-Kopczynska et al., 2020). Similarly, in our study, TMAO administration preserved RV function, as indicated by normalised direct RV pressure and RV fractional area change (Table 4.2); moreover, TMAO decreased the expression of a marker of heart failure severity, BNP, in RV tissue (Figure 4.11C). Additionally, a recent study showed that administration of betaine, a common precursor of TMAO, attenuated pulmonary artery hypertension (Yang et al., 2018). Interestingly, monocrotaline injection was used to induce pulmonary artery hypertension in the previous study; this is the same method we used in our study to induce right ventricle heart failure. Although approximately 100 times less TMAO is produced from betaine than from choline (Wang et al., 2014), at least to some extent, the observed protective effects of betaine might be explained by the increase in TMAO

concentration, which was not evaluated in this study. In general, previously published and our present observations suggest that long-term administration of TMAO can reduce RV remodelling and improve cardiac function in right-sided heart failure.

In addition to previously reported protective features of TMAO, our study proposes preserved cardiac energy metabolism as a possible mechanism underlying the observed protective effects of TMAO. The heart is capable of adapting to both physiological and pathological stressors by shifting from FAO as a dominant energy source to more pronounced utilization of glucose (Brown et al., 2017). In physiological states, this shift could be considered a preconditioning strategy, since the heart is thus better prepared for future stress conditions, due to higher reliance on more energy-efficient substrates in the case of oxygen deficiency (Karwi et al., 2018). In the present study such metabolic shift was observed, when after long-term TMAO administration FAO was decreased, and pyruvate metabolism was subsequently increased (Figure 4.12) without changes in cardiac functionality (Table 4.2). A long-term increase in TMAO concentration appears to induce this metabolic shift, possibly through direct inhibition of β-oxidation, towards more efficient substrate metabolism under stress conditions, such as hypoxia, thus ensuring preserved energy metabolism and subsequently improving cardiac function recovery after injury. However, such protective effects of TMAO were not observed after short-term elevation in TMAO levels in isolated rat heart experiments (Figure 4.6, 4.7). Overall, maintaining metabolic flexibility and preserving FAO are vital strategies to restore cardiac bioenergetic balance (Kolwicz et al., 2012; Karwi et al., 2018), and our findings indicate that the long-term administration of TMAO demonstrates metabolic preconditioning-like effects in heart failure, utilising both of the strategies mentioned above.

In conclusion, the results presented here provide evidence that acute elevation of the TMAO level cannot be considered as a direct causative factor of cardiac or vascular damage. However, the observed effects of TMAO are duration dependent. Long-term administration of TMAO protects cardiac function by preserving mitochondrial energy metabolism in an experimental model of monocrotaline-induced right ventricle heart failure, where TMAO acts as a preconditioning factor.

5.2 Targeting diet-microbiota-TMAO axis using metformin

Knowledge on the mechanism of the anti-diabetic action of metformin has become increasingly complex over the years: from complex I inhibition in the liver (Owen, Doran, and Halestrap, 2000) to suppression of gluconeogenesis by inhibition of mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014), interaction with intestinal microbiota

(Forslund et al., 2015; McCreight, Bailey, and Pearson, 2016) and the suggested potential iron chelating activity of metformin (Stynen et al., 2018). We now show that metformin can directly inhibit bacterial TMA production and decrease TMAO availability in mice. In this study db/db mice had pronounced hyperglycaemia and obesity; under our setup, treatment with metformin did not affect blood glucose and insulin levels, indicating that metformin-induced changes in TMAO levels are at least partially independent of glucose and insulin plasma concentrations.

TMAO has been shown to cause endothelial dysfunction in vasculature through cellular inflammation, elevation of oxidative stress and suppression of endothelial progenitor cell functions (Al-Obaide et al., 2017; Li et al., 2017; Chou et al., 2019). In turn, in the clinical setting, metformin treatment is beneficial in attenuating endothelial dysfunction in patients with prediabetes, MetS and T2D (Nafisa et al., 2018; Sardu et al., 2019). While decreasing glucose levels and improving insulin sensitivity are largely responsible for the beneficial effects of metformin, considering the present findings, there is a basis for attributing part of the protective effects of metformin on the cardiovascular system to the decrease in circulating pro-atherogenic TMAO levels. Indeed, metformin treatment has been shown to improve the endothelial glycocalyx barrier in db/db mice without changing blood glucose levels (Eskens et al., 2013) and to improve endothelial function in spontaneously hypertensive rats with type 1 diabetes independent of glycaemia control (Hamidi Shishavan et al., 2017).

Previously, it was shown that 12-week-old db/db mice have a significantly elevated TMAO up to 9 μM (Dambrova et al., 2016 a). If translated to human patients, this indicates a significantly increased risk of MACE as the TMAO plasma level is above the risk threshold of 6.18 μM (Tang et al., 2013). Thus, in db/db control mice fed a standard laboratory R70 diet, TMAO plasma levels were well above 6.18 μM (Figure 4.14), while treatment with metformin decreased TMAO levels approximately two-fold and, most importantly, below the threshold associated with major cardiovascular complications. In animals on a diet supplemented with choline metformin treatment significantly decreased TMAO plasma concentration almost 1.8-times (Figure 4.15); however, TMAO plasma levels remained higher than the risk threshold.

Our findings strongly imply that acute effects of metformin result in overall decrease in TMAO availability through decreased bacterial TMA production (Figure 4.16). The effect of metformin on TMA synthesis also appears to be rather complex. Choline degradation to TMA in bacteria occurs in specialised microcompartments that contain choline-TMA-lyase (Herring et al., 2018). Our data imply that other components or processes like co-factor recycling in these microcompartments but not choline-TMA-lyase itself (Figure 4.17) are affected by metformin resulting in overall inhibition of TMA production. The current results prove that metformin

inhibits microbial TMA production from choline (Figure 5.1); metformin is unable to decrease TMA production in *K. pneumoniae* when carnitine instead of choline is used as the sole substrate (unpublished observations). Interestingly, metformin had no effect on anaerobic growth of *K. pneumoniae* (omnivorous tertiary amine metaboliser) but significantly delayed (bacteriostatic effect) growth of *P. mirabilis* (choline-only metabolising bacteria) under the same experimental conditions (Figure 4.16), indicating on at least two different mechanisms of metformin on gut microbiota.

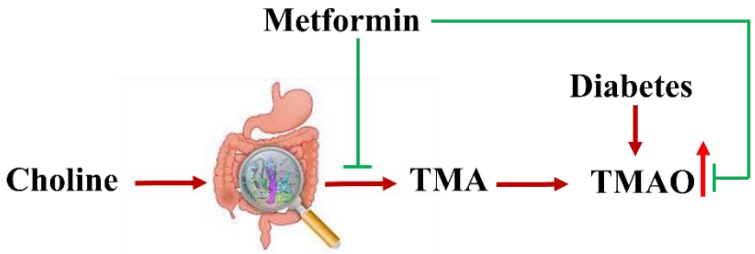

Figure 5.1 **The ability of metformin to reduce the elevated TMAO levels in T2D model**

It must be stressed that we measured effects of metformin on abundance and their ability to produce TMA of only selected intestinal bacteria in mice. However, in line with our findings, a recent study by Su et al. reports effects of metformin on serum TMAO levels in choline-supplemented mice with differential effects on gut microbiota composition *ex vivo* and *in vitro* (Su et al., 2021). Despite this, further studies would be required to show how *in vitro* findings and data from db/db mice model translate to clinical setting under diet-controlled conditions, and how metformin treatment changes the abundance of selected bacteria that produce TMA from tertiary dietary amines in patients.

To conclude, we present evidence that metformin can induce significant changes in TMAO levels likely due to direct non-lethal inhibition of bacterial TMA production. Moreover, these effects are independent of glucose and insulin plasma concentrations and could be an additional mechanism behind the known cardiovascular benefits of metformin therapy. Although metformin can decrease plasma TMAO availability in mice below the risk threshold, a follow-up study in patient population is warranted. Moreover, dietary changes would likely be required to reach clinically relevant endpoints.

5.3 Fasting mimicking diet as a lifestyle strategy to improve metabolic health and decrease TMAO levels

The immense role of dietary choices is undeniable in the regulation of TMAO concentrations. Furthermore, targeting TMAO using pharmacological approaches usually does not normalise other metabolic parameters that characterise cardiovascular and metabolic health.

Thus, it is crucial to study and promote dietary and lifestyle strategies that would be beneficial in terms of regulation of TMAO level and would improve overall metabolic health. In the present Thesis, we demonstrate that 5 days of FMD is a viable dietary strategy to reduce plasma levels of TMAO. Moreover, our data suggest that the reduction in TMAO levels and the improvement in the parameters characterising glucose metabolism and the general metabolic state in healthy volunteers are attributed to intermittent energy restriction and the limitation of animal-derived protein consumption rather than increased vegetable intake.

The baseline characteristics of the volunteers in our study showed that they were slightly overweight and some of the volunteers had extremely high plasma levels of TMAO (Figure 4.18), which are way above the CVD risk threshold (up to 24 μmol/L). Moreover, we also observed high individual variability of TMAO levels, which has previously been described (Kühn et al., 2017). Because of this, fasting plasma glucose, as one of the main parameters characterising metabolic health, was selected as the key criterion for randomisation in our study. However, adherence to FMD resulted in a significant decrease in plasma TMAO levels in 75 % of volunteers (Figure 4.18). At the endpoint, 22 of 24 volunteers had plasma TMAO levels below the CVD risk threshold in the FMD group. A recent study reported that the benefits of FMD are more pronounced in individuals at risk than in those whose metabolic markers are within the normal range (Wei et al., 2017). In our study the same applied to TMAO levels, as the most noticeable reduction in TMAO concentrations was also observed in volunteers from the FMD group with higher baseline plasma concentrations of TMAO.

Although the data from observational studies suggest that increased vegetable intake is also inversely associated with biomarkers of metabolic diseases (Mamluk et al., 2017; Tian et al., 2018), these findings are poorly supported by the evidence from interventional studies (Kuzma, Schmidt, and Kratz, 2017). Our results indicate that a short-term increase in vegetable intake, as in the VEG group, may not be sufficient to reduce plasma TMAO levels and provide noticeable benefits with respect to metabolic health (Figure 4.18, 4.21), as we only observed significant reduction of plasma triglyceride levels in the VEG group (Table 4.3). Moreover, volunteers in the VEG group were expected to proceed with their usual caloric intake and dietary habits in terms of meat consumption, which has been associated with an increased risk of MetS (Kim and Je, 2018; Guo et al., 2021) and T2D (Yang et al., 2020). An alternative to FMD, in terms of limiting the consumption of products of animal origin, would be a vegan diet, which in a recent study displayed promising results and reduced plasma TMAO levels already a week after switching to a plant-based diet (Argyridou et al., 2021). However, the TMAO concentration returned to the previous level after reintroduction of the usual diet (Argyridou et al., 2021), indicating that a vegan diet should be used as a permanent dietary regimen

to sustain TMAO levels within the normal range. This in turn could lead to lowered compliance with the diet (Moore, McGrievy, and Turner-McGrievy, 2015), a problem previously reported with continuous caloric restriction as well (Del Corral et al., 2009; Anastasiou, Karfopoulou, and Yannakoulia, 2015; Dorling et al., 2020). FMD, on the other hand, due to its cyclic nature, is associated with high compliance (Vernieri et al., 2021), which we also observed in our study. Another dietary intervention that highly relies on plant-derived products along with moderate consumption of fish and poultry and limited intake of red meat and processed foods is the Mediterranean diet. This dietary intervention is widely discussed in terms of TMAO regulation, as the consumption of fish and seafood is beneficial for cardiovascular and metabolic health (Estruch et al., 2018; Jimenez-Torres et al., 2021). However, fish intake can also significantly increase TMAO levels (Krüger et al., 2017; Costabile et al., 2021). Nonetheless, the Mediterranean diet has recently been associated with lower TMAO levels, although only when reducing the intake of animal protein (Barrea et al., 2019), especially red meat (Krishnan et al., 2021). The impact of common dietary interventions on regulation of TMAO levels are summarised in Figure 5.2. Overall, previous and present observations emphasise the importance of reduced animal-derived protein consumption and limited calorie intake to achieve beneficial results in terms of TMAO reduction, as in the case of FMD.

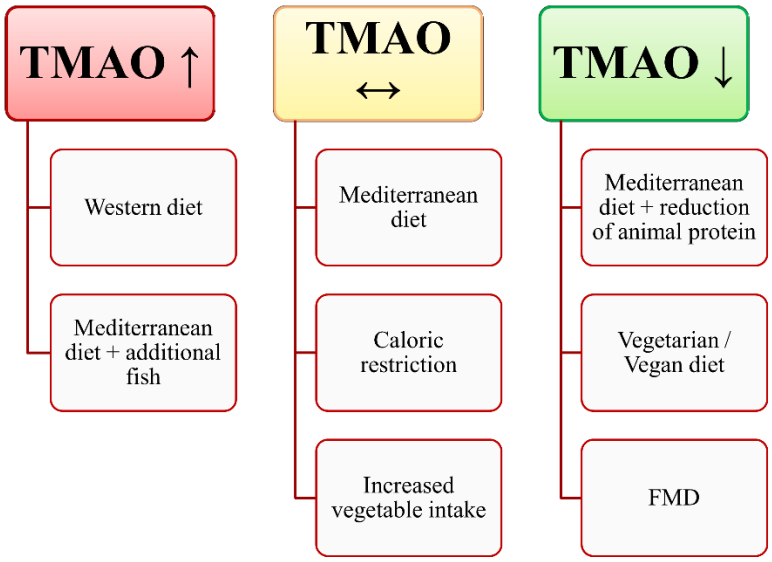

Figure 5.2 **The effects of dietary interventions on circulating TMAO levels in humans**

TMAO, trimethylamine N-oxide; FMD, fasting mimicking diet.

The main limitation of our dietary intervention study is the short-term nature of the interventions (for only a 5-day period) imitating an acute change in diet. However, it has already been reported that such 5-day cycles of FMD could also serve as a long-term strategy if repeated each month (Brandhorst et al., 2015). Since our pilot data indicate that, to some extent, the

reduction of TMAO levels in plasma can also be observed a week after completion of the FMD cycle (data not shown), further research should be done to assess the durability of the beneficial effects of FMD on TMAO levels in the plasma after returning to the usual diet. As the production of TMA is strictly microbiota-dependent, another limitation is that we were not able to collect samples to assess the impact of FMD on gut microbiota. Some studies state that alterations in microbiota composition that favour TMA-producing bacteria are the possible mechanism by which plasma TMAO levels increase in T2D patients (Al-Obaide et al., 2017). However, recent research shows that some of the typical changes observed in gut microbiota composition in patients with T2D (Turnbaugh et al., 2008; Dávila, 2018) or AS (Wang et al., 2015) can be restored by FMD (Wei et al., 2018; Rangan et al., 2019), thus possibly lowering TMA production and reducing CVD risks. Overall, these data suggest that the benefits of FMD are not limited to only the exclusion of dietary sources of TMAO (Koeth et al., 2019; Wang et al., 2019; Wu et al., 2019) but could also be explained through the impact on gut microbiota composition. It has been shown that some of the beneficial effects on gut microbiota composition occur only after continuation of the usual diet (Rangan et al., 2019). Thus, it would also be of great interest to investigate the changes in the abundance of specific TMA-producing bacterial genera after following the FMD cycle and upon reintroduction of the usual diet.

To conclude, our results show that FMD, a vegetable-based, low-calorie variation of intermittent fasting with a strict exclusion of animal-derived protein sources, is an efficient strategy to reduce plasma TMAO levels. Our results add a novel component to the interaction of FMD and the metabolic state of a person, suggesting that TMAO reduction should be considered one of the noteworthy benefits of FMD with respect to improving metabolic health. However, further research is required to assess the potential of compliance to FMD and the effects on TMAO levels after completion of several cycles of the diet, as well as upon the reintroduction of the regular diet.

5.4 General considerations about the role of TMAO in cardiovascular and metabolic diseases

The opinions on the role of TMAO are unequivocal and the studies trying to elucidate the molecular mechanisms affected and outcomes induced by TMAO are contradictive. Several factors must be considered when critically appraising the results of the previous studies.

The first and probably one of the most important factors that must be considered is the range of concentrations observed in a clinical setting where circulating TMAO is detected, and those used in planning and performing mechanistic studies with supplementation of TMAO (summarised in Figure 5.3). The average plasma TMAO level of a healthy person fluctuates

around 3 μM, with some slight deviations because of diet preferences (Wu et al., 2019), age (Li et al., 2018) and gender (Manor et al., 2018). Geographic location and ethnicity are associated with higher variability in TMAO levels (Yazaki et al., 2019, 2020). For example, in the Japanese population, the mean plasma TMAO level is significantly higher (~10 μM) and depends on the area of living with 3-fold higher TMAO levels in those living near the coastal area compared to inland inhabitants (Yano et al., 2018). This can be explained by higher fish intake in Japanese population. In addition, the type of fish or seafood matters, as the increase in TMAO levels differ significantly after consumption of various fish species and products (Wang et al., 2022). Meanwhile, the consumption of a beef burger leads to an increase in TMAO levels of approximately 14 μM, while an intake of a single recommended daily dose of carnitine as a food supplement results in plasma TMAO levels of around 60 μM (unpublished data). Several studies have reported TMAO concentrations peaking approximately 24 hours after the consumption of precursor-rich meals (Koeth et al., 2013; Krüger et al., 2017; Wu et al., 2019), which is in line with our observations. This information stresses the importance of dietary control in the last 2 days before measurements of TMAO, if we want to address the validity question of TMAO as a biomarker. Numerous studies have examined plasma and serum levels of TMAO in different patient populations and conditions, aiming to establish a definitive range of physiologically relevant TMAO concentrations. Thus far the concentrations related to CVD incidence and severity and the risk of MACE are in a low micromolar range, usually around 6–15 μM (Heianza et al., 2017). The highest TMAO levels are present in patients with declining kidney function, especially in those who require dialysis, as these levels can easily reach concentrations over 100 μM (Pelletier et al., 2019). Interestingly, only a few preclinical investigations have been conducted using such low concentrations as those observed in the clinical setting. Many *in vitro* and *ex vivo* studies have utilised significantly higher concentrations, often in high micromolar or even high millimolar range, for various durations up to 60 days (Ke et al., 2018). It is highly recommended that researchers incorporate measurements of TMAO uptake in their studies, both *in vitro* and *in vivo*. This practice is crucial for determining the concentrations of TMAO that elicit specific effects, thus providing important background information. Similarly, in *in vivo* animal models where TMAO or its precursors are supplemented through diet or drinking water, measuring TMAO levels in the target tissues becomes vital. This approach not only adds crucial insights but also helps to develop a valid dose selection strategy for future investigations.

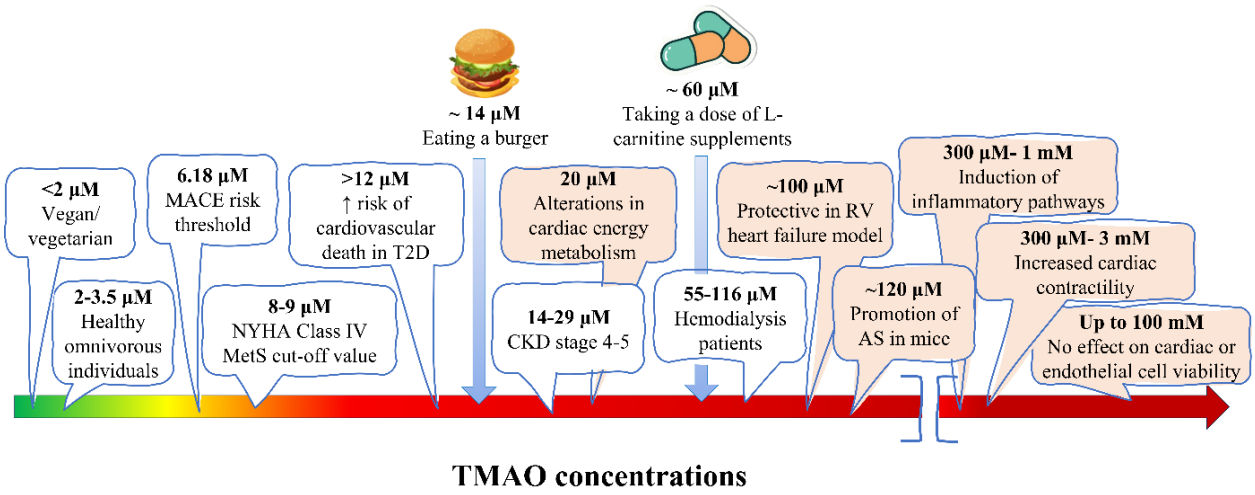

Figure 5.3 **Differential effects observed due to increased TMAO concentrations in preclinical and clinical setting**

Summary of the circulating TMAO levels observed in a clinical setting (white) and concentrations used in preclinical experiments to describe the role of TMAO in the pathogenesis of cardiometabolic diseases (beige). TMAO, trimethylamine N-oxide; MACE, major adverse cardiovascular event; NYHA, New York Heart Association; MetS, metabolic syndrome; T2D, type 2 diabetes; CKD, chronic kidney disease; RV, right ventricle; AS, atherosclerosis.

The available literature suggests that further examination of the relationship between kidney function and TMAO levels requires a more comprehensive evaluation. While it is known that renal clearance of TMAO worsens with declining kidney function (Zeisel and Warrier, 2017), there is currently no clear evidence to suggest that TMAO directly causes or promotes CKD. Most preclinical studies in animal models with increased TMAO bioavailability use supplementation of TMAO precursors, which requires mandatory gut microbiota metabolism (Tang et al., 2015 b; Gupta et al., 2020; Xie et al., 2022). These studies cannot provide conclusive evidence regarding the direct involvement of TMAO in CKD progression. Moreover, it should be noted that elevated TMAO levels in patients with CVD may be attributed to the initial loss of kidney function, as several studies have identified kidney function as a contributing factor (Tang et al., 2015 b; Kim et al., 2016). In addition, measurements of the tissue concentration of TMAO in rodent models have also helped us in terms of future research in this direction, as we have observed significantly higher tissue accumulation of TMAO in the kidneys, with subsequent impairment of mitochondrial function (Videja et al., 2022) and altered expression of TMAO uptake and efflux transporters (unpublished data). This information prompts future investigation to clarify the mechanisms linking higher levels of TMAO and the decline of kidney function, hopefully helping to transfer the findings from preclinical models to a clinical setting.

Considering the recent findings about protective effects of TMAO in some experimental models, many unanswered questions remain: is TMAO a benefactor, a villain or simply a bystander and biomarker with no physiological or pathological role in humans and do we really need to lower our TMAO levels using pharmacological or lifestyle strategies? First, a TMAO-prone diet, with a high abundance of animal-derived products (Lombardo et al., 2022), processed foods and high-fat dairy products, is one that is considered unhealthy due to several crucial factors (Martinez, Leone, and Chang, 2017; Clemente-Suárez et al., 2023). This does not necessarily mean that improvement in cardiometabolic health is possible only by completely switching to a plant-based diet; in terms of TMAO reduction, decreased precursor intake, as in case of FMD (Publication IV) or simply by avoiding carnitine-or choline-containing food supplements, which are extremely popular nowadays, should be effective. Second, to date, there is no solid evidence that low TMAO values would be associated with detrimental health outcomes, putting the physiological role of TMAO under doubt. Of course, there are some specific conditions mentioned in the literature and also described in the present Thesis (Publication II), where TMAO is shown to exert protective effects, however, it is crucial to exercise caution when interpreting the findings. It should be noted that many of the observed protective effects are demonstrated in preclinical animal models, which may not always accurately resemble the disease progression seen in human patients. Although these models provide valuable insights, it is important to consider the limitations and differences in biological processes between rodents and humans. Third, because of the discrepancies regarding the effects induced by TMAO in the preclinical setting and due to several joint pathogenetic factors for various cardiovascular and metabolic diseases, (Zhang et al., 2021; Bu et al., 2022), a more neutral role can be attributed to TMAO as well. The opinion on the role of the intestinal microbiota in the pathogenesis of non-communicable diseases becomes increasingly popular (Byndloss and Bäumler, 2018), and in light of these findings circulating TMAO levels could serve as a valid marker of gut microbiota composition (Silke et al., 2021). This theory could also justify the versatile observations regarding elevated levels of TMAO from the clinical setting.

To conclude, there are several factors to consider when interpreting the overall knowledge on the role of TMAO in the pathogenesis of cardiovascular and metabolic disease. It is crucial to establish a relevant concentration range for studying the molecular mechanisms affected by TMAO, especially to study organ-specific effects, as in case of the kidneys. Moreover, the complex interplay between intestinal microbiota and the cardiometabolic health of the host requires further investigation to establish a clear role of TMAO in the pathogenesis of cardiovascular and metabolic diseases.

Conclusions

1. Short-term exposure to high concentrations of TMAO does not cause detrimental effects on cardiac and vascular function in *ex vivo* and *in vivo* rodent models of cardiovascular health.
2. Supplementation of TMAO in experimental model of right ventricular heart failure exerts preconditioning-like effects by preserving cardiac mitochondrial energy metabolism.
3. Metformin lowers elevated TMAO levels in experimental model of advanced type 2 diabetes by targeting the intestinal microbiota composition and decreasing the rate of TMA production.
4. Fasting mimicking diet is an effective strategy in reducing TMAO levels and improving overall cardiometabolic health in generally healthy volunteers.
5. The effects of TMAO in the pathogenesis of cardiovascular and metabolic diseases depend on the disease model or organ system studied, the composition of intestinal microbiota, the concentrations of TMAO or its precursors used and the duration of exposure.

Proposals

1. There are notable differences between experimental disease models in rodents and the pathogenesis of disease in humans, which must be considered when translating the results from the preclinical to the clinical setting. It is even more important when interpreting the effects caused by TMAO which is derived from the intestinal microbiota, since there are marked differences in the standard diet and the composition of the intestinal microbiota in laboratory rodents and humans.
2. Dietary choices can dramatically affect circulating TMAO levels; therefore, control of food intake for 2 days before measurements is mandatory, to ensure the validity of TMAO as a biomarker in the clinical setting. In addition, differentiation between various types of meat and fish products should be performed in questionnaires, as their impact on TMAO levels varies remarkably.
3. Reduction of animal-derived protein sources, especially red meat, is a reliable approach to regulate TMAO levels. Moreover, beneficial effects can be achieved using this strategy through various popular dietary approaches, for example, vegetarian or vegan diet, FMD or Mediterranean diet.
4. More research is needed to expand our knowledge on the associations between elevated TMAO levels and kidney function, as it can improve its diagnostic and prognostic value in predicting kidney-related outcomes and overall health risks.

Publications and reports on topics of doctoral Thesis

Publications

1. **Videja M**, Vilskersts R, Sevostjanovs E, Liepinsh E, Dambrova M. Data on cardiac and vascular functionality in *ex vivo* and *in vivo* models following acute administration of trimethylamine N-oxide. Data in Brief, **2023**. 108890, ISSN 2352-3409. doi: 10.1016/j.dib.2023.108890
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Annexes

First Publication

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Data in Brief

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Data Article

Data on cardiac and vascular functionality in *ex vivo* and *in vivo* models following acute administration of trimethylamine N-oxide

Melita Videja[a,b,*], Reinis Vilskersts[a,b], Eduards Sevostjanovs[a], Edgars Liepinsh[a], Maija Dambrova[a,b]

[a] Latvian Institute of Organic Synthesis, Aizkraukles street 21, LV-1006, Riga, Latvia
[b] Faculty of Pharmacy, Riga Stradiņš University, Dzirciema street 16, LV-1007, Riga, Latvia

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ABSTRACT

This dataset describes in detail the outcomes of acute trimethylamine N-oxide (TMAO) administration on cardiac, vascular and mitochondrial functionality in *ex vivo* and *in vivo* models.

The accumulation of TMAO in target tissues was assessed after performing heart perfusion or by incubating aortic tissue in a solution containing TMAO. To evaluate the impact of TMAO on mitochondrial function, the aortic rings and heart homogenates of Wistar rats were incubated in a solution containing [9,10-^{3}H] palmitate (5 μCi/ml) or D-[U-^{14}C] glucose (0.625 μCi/ml) in the presence or absence of TMAO with subsequent measurement of substrate oxidation and uptake. The effects of TMAO on the vascular reactivity of isolated conductance and resistance vessels were tested by measuring their response to acetylcholine and sodium nitroprusside. The impact of elevated TMAO levels on cardiac function and infarct size caused by ischemia-reperfusion injury was evaluated in Langendorff perfused heart model. Normal and forced heart functioning was analyzed by echocardiography in CD-1 mouse acute cardiac stress model induced by isoproterenol (10 μg/mouse) upon single and 7 repeated daily administrations of TMAO (120 mg/kg).

* Corresponding author.
E-mail address: melita.videja@farm.osi.lv (M. Videja).
Social media: @Videejaa (M. Videja)

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The data presented in the manuscript provide valuable information on measurements performed under conditions of acutely elevated TMAO levels in experimental models of cardiac and vascular function and energy metabolism. Furthermore, the data have high reuse potential as they could be applied in the planning of future *in vitro*, *ex vivo*, and *in vivo* studies addressing the molecular mechanisms targeted by elevated levels of TMAO.

Specifications Table

Subject	Cardiology and Cardiovascular Medicine
Specific subject area	The outcomes after elevated trimethylamine N-oxide (TMAO) levels in *ex vivo* and *in vivo* models of cardiovascular diseases.
Type of data	Figures and table of analyzed data
How the data were acquired	The data were acquired using following methods: • Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS); • In-vitro radiolabeled substrate oxidation followed by scintillation counting; • Assessment of vascular reactivity using isolated organ bath; • Wire myography; • Ex-vivo Langendorff isolated heart; • Echocardiography.
Data format	Raw and analyzed data
Description of data collection	For acute *ex vivo* experiments, samples were obtained from male Wistar rats immediately after euthanasia and incubated in buffer solution containing 100 µM TMAO. For *ex vivo* isolated heart experiments, the perfusion buffer contained 1 mM TMAO. The effects of TMAO on cardiac function *in vivo* were assessed after single or 7-day administration of TMAO in CD-1 mice at a dose of 120 mg/kg.
Data source location	Institution: Latvian Institute of Organic Synthesis City/Town/Region: Riga Country: Latvia Latitude and longitude: 56.976412171042625, 24.191287962011153
Data accessibility	Repository name: Mendeley Data Data identification number: https://doi.org/10.17632/tv72ryssjg.1 Direct URL to data: https://data.mendeley.com/datasets/tv72ryssjg [1]

Value of the Data

- For more than a decade, TMAO has been studied as a diet- and microbiota-derived metabolite involved in the pathogenesis of cardiometabolic diseases. The data in the manuscript provide useful information on the effects induced by acute TMAO administration in various experimental models for cardiac and vascular functionality.
- These data will be useful for researchers interested in studying the role of TMAO in the development of cardiovascular and metabolic diseases and will help in planning future studies and choosing the experimental conditions for *ex vivo* and *in vivo* experiments.
- The data presented might be reused for comparative studies targeting the acute and chronic effects of increased TMAO concentrations on energy substrate metabolism, cardiac functionality, and vascular reactivity.

1. Objective

Trimethylamine N-oxide (TMAO) has previously been associated with a higher risk of cardiovascular and metabolic diseases. Data from the preclinical and clinical setting suggest that it could be a causal factor for the aforementioned diseases [2], however the evidence background is still conflicting. To better understand the role of acutely elevated TMAO level in the pathogenesis of cardiometabolic conditions, a series of experiments were conducted in *ex vivo* and *in vivo* models of cardiac, vascular, and metabolic function. The dataset described in the manuscript expands the knowledge base and provides novel information on the effects of TMAO on the factors affecting disease development.

2. Data Description

The effects of increased concentrations of trimethylamine N-oxide (TMAO) in incubation buffer on tissue accumulation of TMAO were studied (Fig. 1). For this, samples of male Wistar rat hearts were perfused, and aortic rings were immersed in Krebs-Henseleit (K-H) buffer solution with or without 100 µM TMAO. After 1 hour of perfusion or incubation, the samples were further prepared for UPLC/MS/MS analysis to assess the tissue content of TMAO. After perfusion with a buffer solution containing TMAO, the TMAO content in cardiac tissue increased 3 times (from 2.0±0.2 to 6.3±1.2 nmol/mg tissue) and ~2.5 times in aortic tissue after incubation (from 4.8±0.5 to 12.0±1.2 nmol/mg tissue).

Furthermore, the acute impact of 100 µM TMAO on energy substrate metabolism was assessed by measuring the oxidation of radiolabeled glucose and palmitate and their accumulation in rat aortic rings (Fig. 2). Incubating aortic rings with 100 µM TMAO increased palmitate oxidation nearly two-fold (from 0.26±0.03 to 0.46±0.03 nmol/(h × mg) ^{3}H-palmitate); however, glucose metabolism was not affected. For cardiac tissue, the reaction was performed in tissue homogenates; therefore, only the oxidation of radiolabeled glucose and palmitate was evaluated (Fig. 3). TMAO did not affect energy substrate oxidation in heart homogenates.

Next, the potency of TMAO to affect the reactivity of conductance and resistance vessels was evaluated. Isolated organ bath experiments were conducted in rat aortic rings submerged in a K-H buffer solution with or without 100 µM TMAO for 1 hour to assess the response to acetylcholine (endothelium-dependent relaxation) and sodium nitroprusside (endothelium-independent relaxation) (Fig. 4). A similar procedure was performed on wire myograph with mesenteric arteries (Fig. 5) to assess whether resistance vessels were affected by TMAO in a

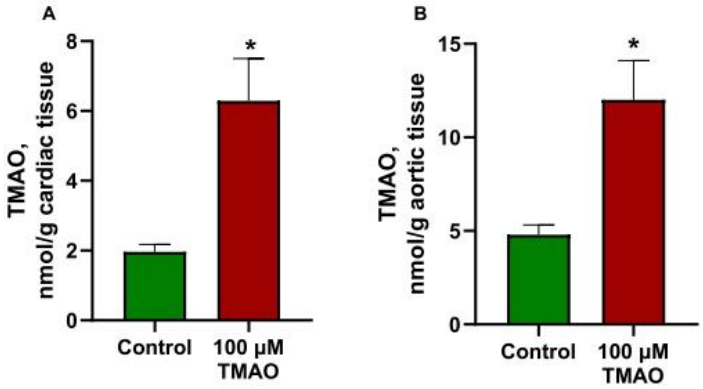

Fig. 1. TMAO content in the heart (A) after 1 hour of perfusion and in aortic tissues (B) after 1 hour of incubation in Krebs-Henseleit buffer solution with or without the addition of 100 µM TMAO. The addition of 100 µM TMAO to the buffer solution increased the content of TMAO in cardiac tissue by three and in the aortic rings by two points five times. Data are shown as the mean±SEM of five experiments. * $p<0.05$ unpaired Student's t test.

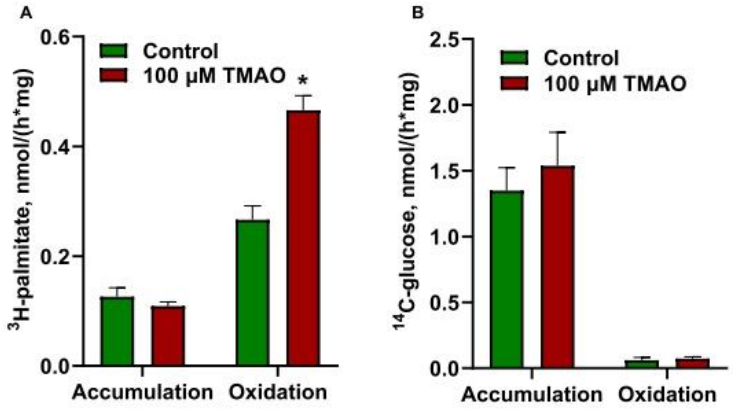

Fig. 2. Effects of 100 µM TMAO on the accumulation and oxidation of ^{3}H-palmitate (A) and ^{14}C-glucose (B) in aortic tissues. Incubation of rat aortic rings with 100 µM TMAO increased ^{3}H-palmitate oxidation but did not influence ^{3}H-palmitate accumulation or ^{14}C-glucose accumulation and oxidation. Data are shown as the mean±SEM of five to six experiments. * $p<0.05$ unpaired Student's t test.

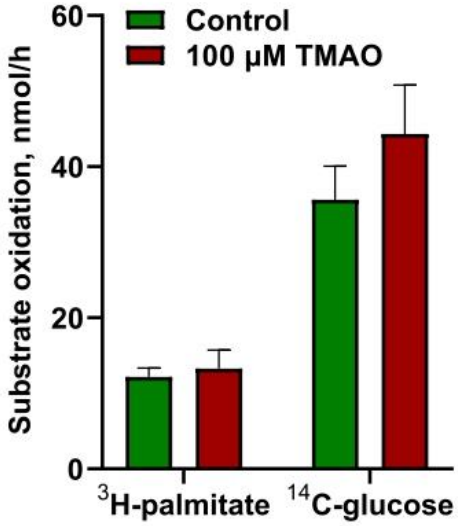

Fig. 3. Effects of 100 µM TMAO on the oxidation of ^{14}C-glucose and ^{3}H-palmitate in heart muscle homogenates. Incubating heart homogenate with 100 µM TMAO did not affect ^{14}C-glucose and ^{3}H-palmitate oxidation. Data are shown as the mean±SEM of five experiments.

similar way as conductance vessels. Incubating the rings of both the aorta and mesenteric artery with 100 µM TMAO did not alter endothelium-dependent or independent relaxation.

In addition, the effects of elevated TMAO concentrations on cardiac function were assessed. First, cardiac functional parameters were tested in a Langendorff isolated rat heart model in the presence of 1 mM TMAO in K-H perfusion buffer (Table 1). Next, the effects of elevated TMAO concentrations in the perfusion buffer (1 mM) on overall cardiac functionality during ischemia–reperfusion were evaluated (Fig. 6). Perfusion of isolated rat hearts with 1 mM TMAO caused no effect on heart function (heart rate, coronary blood flow, contractility, left ventricle developed pressure and cardiac work) at the baseline or during ischemia–reperfusion. After this, the size of myocardial infarction was compared in both groups (Fig. 7). After 30 min of left anterior descending artery occlusion and then 2 h of reperfusion, the necrosis zone was nearly identical (~40% of the risk zone) in both groups.

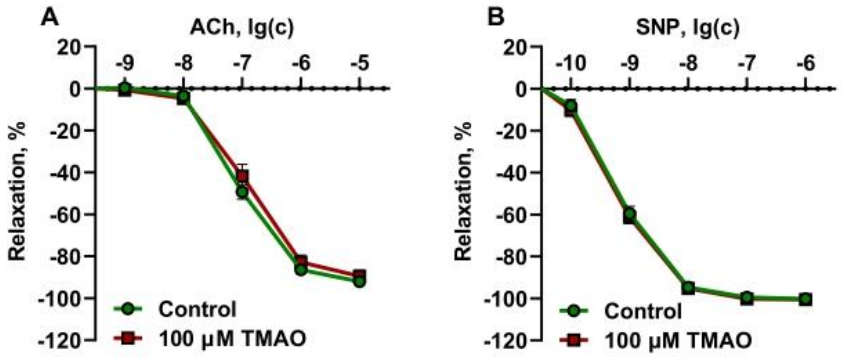

Fig. 4. Effects of 100 µM TMAO on endothelium-dependent (A) and endothelium-independent (B) relaxation in aortic rings. Incubation of the aortic rings for 1 h with 100 µM TMAO did not affect endothelium-dependent and endothelium-independent relaxation. The data are shown as the mean±SEM of twelve aortic rings.

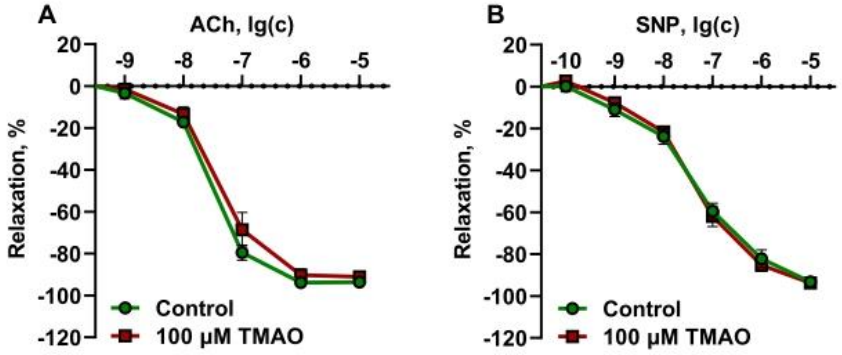

Fig. 5. Effects of 100 µM TMAO on endothelium-dependent (A) and endothelium-independent (B) relaxation in mesenteric artery rings. Incubating the mesenteric artery rings for 1 h with 100 µM TMAO did not affect endothelium-dependent and endothelium-independent relaxation. The data are shown as the mean±SEM of seven to eight mesenteric artery rings.

Table 1
Effects of 1 mM TMAO on heart function. Perfusion of the isolated rat heart with K-H buffer solution containing 1 mM TMAO did not alter functional parameters of the heart.

	Control	1 mM TMAO
Coronary Flow	12.4±1.3	11.5±0.8
LVDP	155±19	153±17
Heart rate	219±12	230±15
Contractility	5458±347	5419±399
Cardiac Work	33±4	34±3

The results are shown as the mean±SEM of six hearts.

Last, the effects of single and 7-day administration of TMAO at a dose of 120 mg/kg in CD-1 mice on the cardiac response to isoproterenol-induced acute cardiac stress in mice were tested (Fig. 8). Administration of TMAO at a 120 mg/kg dose caused no effect on the baseline systolic function of the left ventricle. The ejection fractions in the control and TMAO-treated groups were 77±3% and 79±1%, respectively. Treating animals with isoproterenol (10 µg/mouse) significantly increased left ventricular ejection fraction, fractional shortening, and heart rate; however, neither single administration nor 7-day administration of TMAO at a dose of 120 mg/kg showed any effect on these parameters.

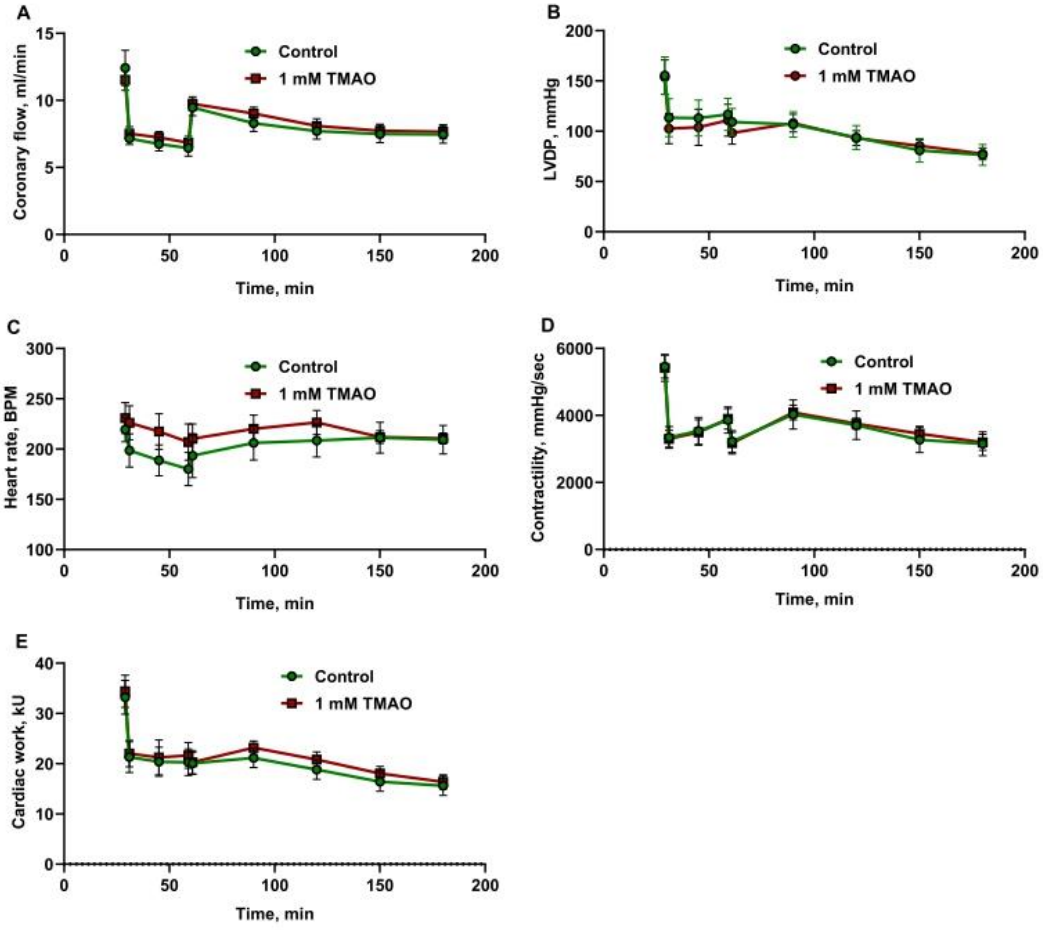

Fig. 6. Effects of 1 mM TMAO in the perfusion buffer solution on functional parameters (coronary flow (A), LVDP (B), heart rate (C), contractility (D), and cardiac work (E)) of the isolated rat heart before and during 30 min of occlusion with 120 min of reperfusion. Perfusion of the isolated rat heart with buffer solution containing 1 mM TMAO did not influence heart function during ischemia–reperfusion. The data are shown as the mean±SEM of six hearts.

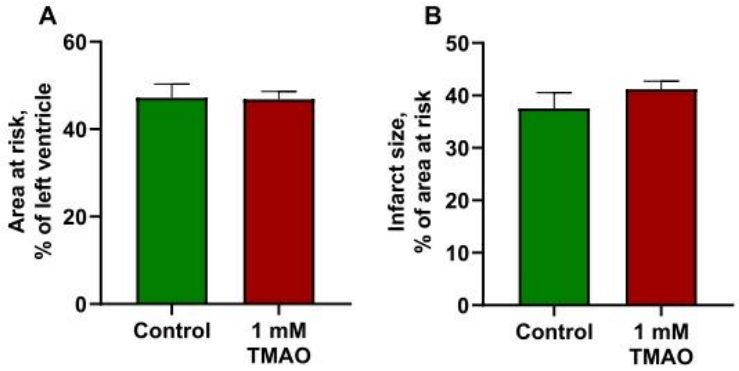

Fig. 7. Effects of 1 mM TMAO on the size of myocardial infarction after 30 min of performing occlusion with the left anterior descending coronary artery and then 120 min of reperfusion. Hearts from both groups showed a similar area at risk in the left ventricle (A). Perfusion of the isolated rat heart with a KH buffer solution containing 1 mM TMAO did not influence the size of the myocardial infarction (B). The data are shown as the mean±SEM of six hearts.

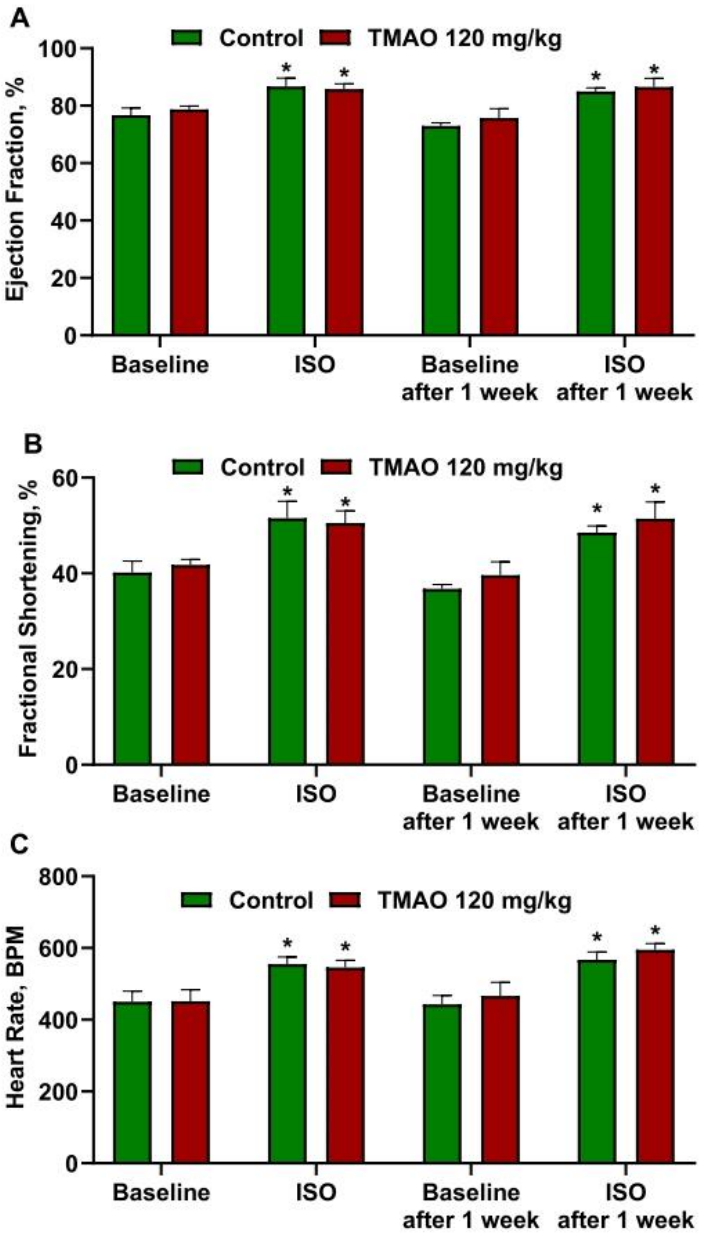

Fig. 8. Effects of single and 7-day administration of TMAO at a dose of 120 mg/kg on left ventricular ejection fraction (A), fractional shortening (B), and heart rate (C). Single and 7-day administration of TMAO did not change the functional parameters of the heart. The results are shown as the mean±SEM of six animals.

3. Experimental Design, Materials and Methods

3.1. Chemicals

TMAO dihydrate was obtained from Alfa Aesar (Kandel, Germany). Sodium pentobarbital (Dorminal) solution was purchased from Alfasan (Woerden, Holland). Heparin sodium was purchased from Panpharma (Fougeres, France). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and 98% formic acid (LC/MS grade) was ob-

tained from Fluka (Buchs, Switzerland). [9,10-^{3}H]-Palmitate (5 µCi/ml) or D-[U-^{14}C]-glucose (0.625 µCi/ml) was purchased from Biotrend (Zürich, Switzerland). ATP was purchased from TCI (Antwerp, Belgium). Isoflurane was purchased from Chemical Point (Deisenhofen, Germany). All other reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany).

3.2. Animals and treatment

Thirty male Wistar rats weighing 200–250 g were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). Twelve male CD-1 mice at the age of 6-8 weeks weighing 25-30 g were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). All experimental animals were housed under standard conditions (21-23°C, 12-hour light/dark cycle, relative humidity 45-65%) with unlimited access to food (R70 diet, Lactamin AB, Kimstad, Sweden) and water. Rats (n=12) were used to determine the levels of TMAO in vascular and myocardial tissue. Six rats were used to assess the effects of TMAO on energy substrate oxidation and vascular reactivity after incubation in TMAO-containing (100 µM) buffer solution, and 12 rats were used in the isolated heart experiments to assess the functionality of the heart and the size of myocardial infarction after perfusion with 1 mM TMAO. Mice (n=12) were used to study the effects of TMAO administration on heart functionality and the response to adrenergic stimulation.

To obtain cardiac and vascular tissue for *ex vivo* experiments, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg·kg−1) and heparin (1000 IU·kg−1). After the onset of anesthesia, the thorax was opened, and the heart and thoracic aorta were removed and placed into ice-cold Krebs-Henseleit (K-H) buffer solution (composition (in mmol/L): NaCl 118, $CaCl_2$ 2.5, $MgCl_2$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 10.0, and EDTA 0.05; pH 7.4 at 37°C) until the tissue was further processed.

3.3. Determination of TMAO concentrations

Rat hearts were perfused, and aortic rings from each experimental animal were immersed in K-H buffer solution with or without the addition of TMAO (100 µM final concentration). After 1 hour of perfusion or incubation, the tissue samples were washed to eliminate the residues of TMAO-containing buffer solution and further homogenized with water in an OMNI Bead Ruptor 24 (Camlab, UK) at a w/v ratio of 1:10. The homogenate was then centrifuged at 20000 × g for 10 min at 4°C. The supernatants were collected and stored frozen (− 80°C) until further analysis.

The TMAO concentrations in the aorta and heart homogenate samples were measured by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) using a positive ion electrospray, as previously described [3,4]. In brief, the sample preparation was performed by deproteinization with an acetonitrile/methanol mixture (3:1, v/v) and centrifugation at 13000 × g for 10 min. The supernatant was then transferred to UPLC vials and analyzed using UPLC/MS/MS. Data acquisition and further processing were carried out in MassLynx 4.1. software with a QuanLynx 4.1. module (Waters, Milford, USA).

3.4. Energy substrate oxidation

To assess the energy substrate oxidation rate, cardiac tissues were minced with scissors and homogenized with a Turrax homogenizer (IKA, Staufen, Germany). Samples were prepared at a ratio of 1:10 w/v in Isolation Buffer A (composition (in mmol/L): KCl 180, Tris-base 10, EDTA 0.5). Heart homogenates were centrifuged at 1000 × g for 5 min, and the supernatant was used to assess fatty acid and glucose oxidation. The aortic rings remained intact. The reaction mix for fatty acid oxidation contained 1 mM NAD, 5 mM ATP, 100 µM CoA, 1 mM malate, 700 µM

L-carnitine, and [9,10-^{3}H] palmitate (specific activity, 5 μCi/ml) in K-H buffer solution with or without 100 μM TMAO. The glucose oxidation reaction mix consisted of 1 mM NAD, 5 mM ATP, 100 μM CoA and D-[U-^{14}C] glucose (specific activity, 0.625 μCi/ml) in K-H buffer solution with or without 100 μM TMAO.

Glucose and palmitate oxidation rates were determined as described previously [5]. Briefly, glucose oxidation was assessed by measuring the $^{14}CO_2$ released from the metabolism of D-[U-^{14}C] glucose. Palmitate oxidation was determined by measuring the $^{3}H_2O$ released from [9,10-^{3}H] palmitate. Glucose and palmitate uptake in the aorta was calculated from the amount of radiolabeled substrates oxidized and the amount found in aortic tissues when the reaction ended.

3.5. Effects of TMAO on vascular reactivity of conductance and resistance vessels

Vascular reactivity of aortic rings was assessed as described previously [6]. In brief, the excised thoracic aorta was immersed in ice-cold K-H buffer solution and the surrounding tissue were removed. The vessels were cut into 3- to 4-mm-long rings that were suspended between two stainless steel hooks in a 10 mL organ bath filled with K-H buffer solution saturated with 95% O_2 and 5% CO_2, and four parallel samples were prepared from the same animal. The aortic rings were stretched to a resting tension of 2 g and equilibrated to the new conditions for 60 min. During this adaptation, the incubation buffer solution was changed every 15 min. The maximal contraction force of each ring was determined by adding 60 mM potassium chloride. After washing, TMAO at a concentration of 100 μM was added to half of the aortic rings and incubated for 1 h. Aortic rings were then washed once more with buffer solution and precontracted with phenylephrine to 70%-80% of maximal contraction. Endothelium-dependent relaxation was assessed by adding cumulative concentrations of acetylcholine (10^{-9} to 10^{-5} mol/L). Endothelium-independent relaxation was assessed by adding cumulative concentrations of SNP (10^{-10} to 10^{-5} mol/L). The relaxation of the aortic rings in response to acetylcholine or SNP was expressed as a percentage of the phenylephrine-induced constriction.

Vascular reactivity in mesenteric artery rings was assessed as described previously [7] with modifications. The intestine with the mesenteric arcade attached was excised and transferred to ice-cold physiological salt solution (PSS) with the following composition (mmol/L): NaCl 130, KCl 4.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 14.9, glucose 5.5, and EDTA 0.026. Second-order mesenteric arteries were cleaned and dissected of adjoining fat and connective tissues. The arteries were cut into ring segments 2 mm in length, and four parallel samples were prepared from the same animal. Each segment was mounted in a Multi-Myograph System (Danish Myograph Technology, Aarhus, Denmark) in PSS saturated with a gas mixture of 95% O_2 and 5% CO_2. Further assessment of endothelium-dependent and endothelium-independent function after incubation with 100 μM TMAO was performed in a similar manner as for the aorta.

3.6. Experimental heart infarction ex vivo

The infarction was performed according to the Langendorff technique as described previously [8], with some modifications. For the infarction studies, the hearts were perfused with K-H buffer solution with or without 1 mM TMAO at a constant perfusion pressure of 60 mmHg. Heart rate, left ventricular end-diastolic pressure, and left ventricular developed pressure were recorded continuously. Coronary flow was measured using an ultrasound flow detector (HSE) and PowerLab systems from ADInstruments. The isolated rat hearts were left to adapt for 30 min, and then the left anterior descending coronary artery (LAD) was occluded for 30 min, followed by 120 min of reperfusion. Further analysis was performed as described by Liepinsh et al. [9]. In brief, LAD was reoccluded and perfused with 0.1% methylene blue. Afterward, the heart was transversely cut into 2-mm-thick slices, treated with triphenyltetrazolium chloride (TTC) and photographed. Computerized planimetric analysis was carried out using Image-Pro Plus v6.3

software (Media Cybernetics Inc., Rockville, MD, USA) to determine the area at risk (AR) and the area of necrosis (AN). Each area was then expressed as a percentage of the total left ventricle area. The infarct size (IS) was then calculated as a percentage of the risk area according to the formula IS = AN/AR × 100%.

3.7. Isoproterenol-induced cardiac stress model

The isoproterenol-induced acute cardiac stress model was established as previously described [10] with some modifications. Before the study, experimental animals were randomly divided into two experimental groups (n=6) and weighed. Mice from both groups were anesthetized using 5% isoflurane dissolved in 100% oxygen. After the onset of anesthesia, the concentration of isoflurane was decreased to 2.5%, the experimental animals were placed in a decubitus position, and the chest was shaved. M-mode tracings of the left ventricle were recorded at the papillary muscle level using an iE33 ultrasonograph equipped with a linear L15-7io transducer (Philips Health care, Andover, USA). Afterward, the mice from the first experimental group received isoproterenol at a dose of 10 µg/mouse, but the animals from the second group received isoproterenol and TMAO at doses of 10 µg/mouse and 120 mg/kg, respectively. After 30 min, the experimental animals were anesthetized with isoflurane once more to record the cardiac response to acute cardiac stress and the impact of TMAO on the inotropic and chronotropic effects. For the next seven days, the mice in the second group received TMAO together with drinking water at a dose of 120 mg/kg, while the animals from the first group received pure drinking water. After one week of treatment, the experimental animals were anesthetized, and echocardiography was performed before and after administration of isoproterenol or a combination of isoproterenol and TMAO as described before.

3.8. Statistical methods

All data are represented as the mean ± standard error of the mean (SEM). For statistical analysis, Student's t test or one-way ANOVA with Tukey's post-test were used. A two-sided p value less than 0.05 was considered statistically significant. Statistical calculations were performed using Prism software (GraphPad, San Diego, California).

Ethics Statements

The experimental procedures described here were performed in accordance with the EU Directive 2010/63/EU for animal experiments and local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia. *Ex vivo* experiments were performed in compliance with ethical approval Nr. 82; isoproterenol-induced cardiac stress model was performed in compliance with ethical approval Nr. 84. All studies involving animals are reported in accordance with the ARRIVE guidelines [11].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data on cardiac and vascular functionality in ex vivo and in vivo models following acute administration of trimethylamine N-oxide (Original data) (Mendeley Data).

CRediT Author Statement

Melita Videja: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft, Funding acquisition; **Reinis Vilskersts:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing; **Eduards Sevostjanovs:** Data curation; **Edgars Liepinsh:** Conceptualization, Supervision; **Maija Dambrova:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

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Second Publication

frontiers in Cell and Developmental Biology

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Microbiota-Derived Metabolite Trimethylamine N-Oxide Protects Mitochondrial Energy Metabolism and Cardiac Functionality in a Rat Model of Right Ventricle Heart Failure

Melita Videja[1,2]*, *Reinis Vilskersts*[1,2], *Stanislava Korzh*[1], *Helena Cirule*[1], *Eduards Sevostjanovs*[1], *Maija Dambrova*[1,2] *and Marina Makrecka-Kuka*[1]

[1] *Latvian Institute of Organic Synthesis, Riga, Latvia,* [2] *Faculty of Pharmacy, Riga Stradiņš University, Riga, Latvia*

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***Correspondence:**
Melita Videja
melita.videja@farm.osi.lv

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Aim: Trimethylamine N-oxide (TMAO) is a gut microbiota-derived metabolite synthesized in host organisms from specific food constituents, such as choline, carnitine and betaine. During the last decade, elevated TMAO levels have been proposed as biomarkers to estimate the risk of cardiometabolic diseases. However, there is still no consensus about the role of TMAO in the pathogenesis of cardiovascular disease since regular consumption of TMAO-rich seafood (i.e., a Mediterranean diet) is considered to be beneficial for the primary prevention of cardiovascular events. Therefore, the aim of this study was to investigate the effects of long-term TMAO administration on mitochondrial energy metabolism in an experimental model of right ventricle heart failure.

Methods: TMAO was administered to rats at a dose of 120 mg/kg in their drinking water for 10 weeks. Then, a single subcutaneous injection of monocrotaline (MCT) (60 mg/kg) was administered to induce right ventricular dysfunction, and treatment with TMAO was continued (experimental groups: Control; TMAO; MCT; TMAO+MCT). After 4 weeks, right ventricle functionality was assessed by echocardiography, mitochondrial function and heart failure-related gene and protein expression was determined.

Results: Compared to the control treatment, the administration of TMAO (120 mg/kg) for 14 weeks increased the TMAO concentration in cardiac tissues up to 14 times. MCT treatment led to impaired mitochondrial function and decreased right ventricular functional parameters. Although TMAO treatment itself decreased mitochondrial fatty acid oxidation-dependent respiration, no effect on cardiac functionality was observed. Long-term TMAO administration prevented MCT-impaired mitochondrial energy metabolism by preserving fatty acid oxidation and subsequently decreasing pyruvate metabolism. In the experimental model of right ventricle heart failure, the impact of TMAO on energy metabolism resulted in a tendency to restore right ventricular function, as indicated by echocardiographic parameters and normalized organ-to-body weight indexes. Similarly, the expression of a marker of heart failure severity, brain natriuretic peptide, was substantially increased in the MCT group but tended to be restored to control levels in the TMAO+MCT group.

Conclusion: Elevated TMAO levels preserve mitochondrial energy metabolism and cardiac functionality in an experimental model of right ventricular heart failure, suggesting that under specific conditions TMAO promotes metabolic preconditioning-like effects.

Keywords: trimethylamine N-oxide, right ventricular dysfunction, monocrotaline, mitochondrial function, cardiovascular diseases

INTRODUCTION

Impaired energy metabolism is one of the cornerstones of heart failure pathophysiology (Rosca and Hoppel, 2013). Normally, 60–90% of energy is generated through fatty acid oxidation (FAO) (Lopaschuk et al., 2010; Liepinsh et al., 2014); however, in the early stages of heart failure, a shift from fatty acid oxidation toward glucose utilization is observed and is a mechanism of metabolic adaptation (Ventura-Clapier et al., 2011). Compared to healthy patients, heart failure patients exhibit decreased FAO (Dávila-Román et al., 2002), which correlates with cardiac hypertrophy and reduced ejection fraction (Neglia et al., 2007; Byrne et al., 2016). During the progression of heart failure, overall cardiac oxidative metabolism decreases, resulting in energy starvation (Sabbah, 2020). One of the risk factors that lead to disturbances in energy metabolism and further progression of cardiovascular diseases is unhealthy dietary patterns. For instance, high intake of fat, red meat and processed food, as in Western diet, is shown to damage myocardial oxidative capacity, leading to impaired mitochondrial energy metabolism (Neves et al., 2014). Moreover, dietary choices determine the composition of intestinal microbiota, which further unambiguously affects the host metabolism (Lindsay et al., 2020). It has been shown that chronic heart failure is characterized by substantial alterations in gut microbiome composition and reduced microbial variety (Kummen et al., 2018; Mayerhofer et al., 2020). Previous studies suggest a link between the human gut microbiome and the homeostasis of energy metabolism, however, a clear causal relationship between them remains elusive.

In 2011, in a targeted metabolomics study, trimethylamine N-oxide (TMAO) was identified as a metabolite, which is both microbiota- and diet-derived, and associated with the incidence of adverse cardiovascular outcomes (Wang et al., 2011). TMAO is produced in organisms by gut microbiota during the metabolism of common food constituents, such as carnitine, choline and betaine; this process first leads to the production of trimethylamine (TMA), which is subsequently oxidized by the host liver enzyme group called flavin-containing monooxygenases (Janeiro et al., 2018). High intake of TMAO and its precursor has been shown to promote atherosclerosis and exacerbate cardiovascular risks (Wang et al., 2011; Koeth et al., 2013; Ding et al., 2018). A positive correlation between the circulating levels of TMAO and the severity of metabolic syndrome has been observed (Barrea et al., 2018), and elevated TMAO plasma levels were observed in patients with diabetes (Lever et al., 2014; Dambrova et al., 2016). Moreover, in heart failure patients, increased TMAO concentrations correlate with heart failure severity, as shown by NYHA class (Tang et al., 2014, 2015; Trøseid et al., 2015) and heart failure-associated mortality (Suzuki et al., 2016). Although extensive studies of TMAO in various patient populations clearly demonstrate that TMAO can serve as a biomarker, it remains uncertain whether TMAO is directly involved in the pathogenesis of cardiometabolic diseases (Nowiński and Ufnal, 2018).

Diet supplementation with TMAO or its precursors has been shown to exacerbate cardiac dilation, leading to reduced ejection fraction and increased cardiac fibrosis, in an experimental model of heart failure (Organ et al., 2016). In addition, a reduction of circulating TMAO levels by 3,3-dimethyl-1-butanol or iodomethylcholine resulted in attenuated cardiac remodeling after aortic banding (Organ et al., 2020; Wang et al., 2020). On the other hand, the Mediterranean diet, which is focused on regular consumption of TMAO-rich fish and seafoods (Cho et al., 2017), is inversely correlated with fatal coronary heart disease (He et al., 2004) and is proposed to be a strategy for preventing and reducing the risk of cardiovascular and metabolic diseases (Tørris et al., 2014; Widmer et al., 2015; Estruch et al., 2018). Moreover, it was recently shown that chronic treatment with low-dose TMAO was associated with preserved cardiac hemodynamic parameters in Spontaneously Hypertensive rats and Spontaneously Hypertensive Heart Failure rats (Huc et al., 2018; Gawrys-Kopczynska et al., 2020). Such controversial results raise the question of whether increased availability of TMAO plays detrimental or protective roles in the progression of cardiovascular diseases.

It has been shown that both acute and chronic TMAO treatment can cause disruptions in energy metabolism in the heart by impairing pyruvate and fatty acid metabolism (Makrecka-Kuka et al., 2017). However, there is no evidence that TMAO-induced metabolic alterations result in impaired cardiac functionality. It could be hypothesized that long-term TMAO administration could exert preconditioning-like effects, thus improving cardiovascular outcomes after stress conditions, such as hypoxia, pressure overload and altered energy substrate availability. Thus, the aim of the present study was to investigate the effects of long-term TMAO administration in an experimental rat model of monocrotaline-induced right ventricle heart failure. To mimic the chronic increase in TMAO in plasma and tissues, as observed in cases of regular consumption of seafood, TMAO pretreatment for 10 weeks prior to monocrotaline injection was chosen. The effects of the administration of TMAO on indicators of heart failure severity (cardiac functional parameters, heart failure and hypertrophy-related gene and protein expression) and cardiac mitochondrial energy metabolism were studied.

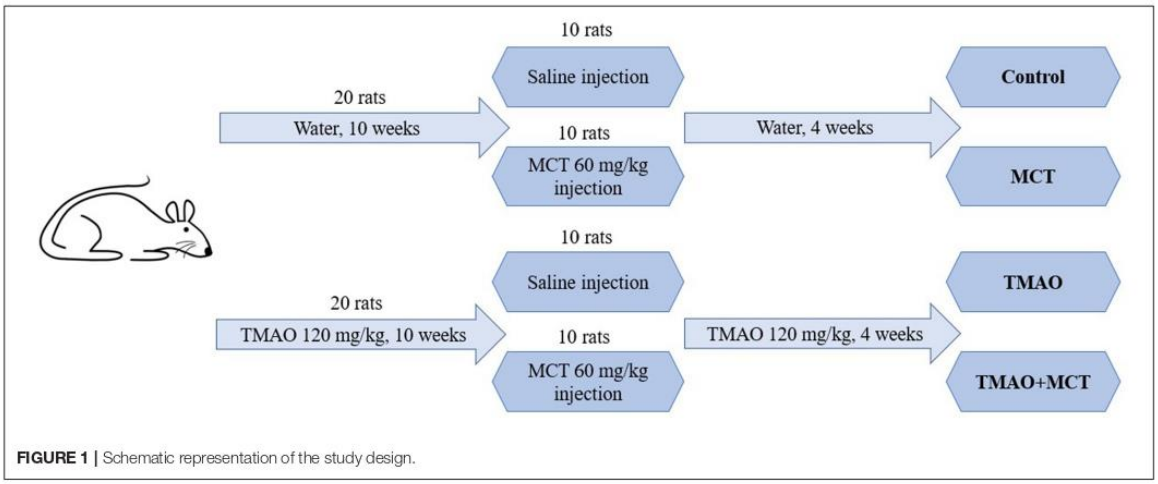


FIGURE 1 | Schematic representation of the study design.

MATERIALS AND METHODS

Experimental Animals

Wistar rats ($n = 40$) weighing 280–380 grams (6–8 weeks old) were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia) and housed under standard conditions (21–23°C, 12-h light/dark cycle, relative humidity 45–65%) for 2 weeks prior to the start of the experiment. The animals were fed a standard R70 diet (Lantmännen, Stockholm, Sweden) with unlimited access to food and drinking water. All the experimental procedures were performed in accordance with the guidelines reported in the EU Directive 2010/63/EU and in accordance with local laws and policies, and all of the procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia (Food and Veterinary Service Ethical approval Nr. 105). These studies are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010). Our previous experiments, in which right ventricular functionality was assessed, indicated that due to interindividual variability, 8–10 animals per group are necessary to obtain significant results; therefore, $n = 10$ per group was chosen. The data from previous experiments in which mitochondrial energy metabolism was studied were subjected to statistical power analysis, and the calculations indicated that the mitochondrial respiration assay requires at least $n = 5$ or 6 per group to produce significant results with a power >0.95.

Experimental Design

The schematic representation of the study design is shown in **Figure 1**. The experimental animals were randomly separated into four groups: control ($n = 10$), TMAO ($n = 10$), MCT (monocrotaline) ($n = 10$) and TMAO+MCT ($n = 10$). One animal in the control group and 2 animals in the other groups died during the experiment due to reasons not related to the experimental protocol and treatment. The samples from these rats were excluded from further analysis; therefore, the final animal count was nine rats in the control group and eight rats in the other groups. The animals in the TMAO group and TMAO+MCT group received TMAO (Alfa Aeser, Kandel, Germany) at a dose of 120 mg/kg in their drinking water daily for 10 weeks. To induce pulmonary hypertension and right ventricular remodeling and dysfunction, a single subcutaneous injection of monocrotaline (MCT) (Sigma-Aldrich, Schnelldorf, Germany) at a dose of 60 mg/kg was administered to the animals in the MCT and TMAO+MCT groups. TMAO treatment was continued in both groups that previously received TMAO until the end of the experiment. The rats were weighed twice a week to monitor their general health condition. Since the time from MCT injection to right ventricular failure onset differs markedly (Hardziyenka et al., 2006), a 4-week time point after the administration of MCT was chosen for the echocardiographic assessment of right ventricle functionality based on our pilot experiments in this model. In addition, invasive direct right ventricular pressure measurement was performed. After the assessment of cardiac functionality, the animals were sacrificed, and cardiac tissue and plasma samples were immediately frozen and stored at −80°C for further analysis. In addition, a mitochondrial functionality study was performed using permeabilized cardiac fibers of the right ventricle.

Echocardiography Assessment and Direct Right Ventricle Blood Pressure Measurement

The rats were anesthetized using 5% isoflurane dissolved in 100% oxygen. After the onset of anesthesia, the concentration of isoflurane was decreased to 2.5%, the experimental animals were placed in a decubitus position, and the chest and upper part of the abdomen were shaved. The animals were connected to a Philips iE33 ultrasonograph (Philips Healthcare, Andover, USA) to record ECG from the II lead. Then, the rat was placed on the left side, and a four-chamber view was recorded from the apical point of view using a Philips (Philips Healthcare, Andover, USA) S12-4 sector array transducer. Right ventricular

(RV) end-diastolic area (RV-EDA) and RV end-systolic area (RV-ESA) were recorded. ECG was used to determine the exact time of RV systole and diastole. Furthermore, RV-EDA and RV-ESA were used to calculate RV fractional area change (RVFAC). The rat was again placed in a decubitus position, and functional parameters of the left ventricle were recorded at the papillary muscle level using a Philips (Philips Healthcare, Andover, USA) linear L15-7io transducer.

After the echocardiographic assessment of ventricular anatomy and functioning, invasive direct right ventricular pressure measurement was performed. The anesthetized rat was intubated using a 16-G intravenous catheter and mechanically ventilated with 2% isoflurane dissolved in 100% oxygen at a tidal volume of 1.5 ml/100 g. The abdominal cavity was opened, and the diaphragm was incised to expose the pleural cavity. The ribs on both sides of the chest were cut to access the heart. An 18-G needle was connected to a pressure transducer (AD Instruments, Sidney, Australia) and inserted into the cavity of the right ventricle through the apex of the heart. The right ventricular pressure was measured until a stable pressure reading was obtained.

Measurement of Organ Mass

To calculate the organ-to-body weight indexes, the heart and lungs were excised and weighed. Then, the right ventricle (excluding the septum) was separated from the heart and weighed.

Measurements of Plasma Biochemical Parameters

To obtain plasma, the blood samples were centrifuged at 1,000 g and 4° for 10 min and then stored at −80°C until further analysis. The levels of triglycerides and total cholesterol in plasma were measured using commercially available kits from Instrumentation Laboratory (Milan, Italy). The level of free fatty acids (NEFA) was measured using a commercially available kit from Wako Chemicals (Neuss, Germany). All measurements were carried out according to the manufacturer's instructions.

Quantification of TMAO in Plasma and Tissue Samples

Determination of the TMAO concentrations in the plasma and heart homogenate samples was performed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) using the positive ion electrospray mode as previously described (Dambrova et al., 2013; Grinberga et al., 2015). Briefly, obtained tissues were homogenized with water in OMNI Bead Ruptor 24 (Camlab, Cambridge, United Kingdom) at a w/v ratio of 1:10. The obtained homogenates were centrifuged at 20,000 g for 10 min at 4°C. The supernatants were collected and stored at −80°C until further analysis.

Sample preparation was performed by deproteinization with an acetonitrile–methanol mixture (3:1, v/v). The samples were then vortexed and centrifuged at 15,000 g for 20 min. The supernatant was transferred to UPLC vials and used for UPLC/MS/MS analysis. MassLynx 4.1. software with a QuanLynx 4.1. module (Waters, Milford, USA) was used for data acquisition and processing.

Measurements of Tissue Brain Natriuretic Peptide (BNP)

A Rat BNP 45 ELISA Kit (Abcam, Cambridge, United Kingdom, ab108816) was used to test the levels of makers of congestive heart failure in right ventricular tissue extracts. Extract preparation and analysis were carried out according to the manufacturer's instructions.

Isolation of RNA and qPCR Analysis

Total RNA was isolated from right ventricular tissues using TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's recommended protocol. First-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, Foster City, CA, USA) following the manufacturer's instructions. The qPCR mix consisted of SYBR® Green Master Mix (Applied BiosystemsTM, Foster City, CA, USA), synthesized cDNA, and forward and reverse primers specific for VCP, BNP, αMHC, and βMHC. These genes were chosen to characterize heart failure severity and cardiac hypertrophy. The reaction was carried out in an Applied Biosystems Prism 7500 instrument according to the protocol provided by the manufacturer. The relative expression levels of each of the genes of interest were calculated with the ΔΔCt method and were normalized to the expression level of the VCP gene. The primer sequences used for the qPCR analysis are available in **Supplementary Table 1**.

Measurements of Mitochondrial Respiration in Permeabilized Cardiac Fibers

Mitochondrial function was assessed in permeabilized cardiac fibers from the right ventricle that were prepared as previously described (Kuka et al., 2012). The mitochondrial respiration measurements were performed in MiR05 media (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, 0.1% BSA essentially free of fatty acids) at 37°C using an Oxygraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria). Mitochondrial functionality measurements were performed using a previously described respirometry protocol (Makrecka-Kuka et al., 2020). Briefly, palmitoylcarnitine (PC) and malate (10 μM and 0.5 mM, respectively) were used to measure FAO-dependent mitochondrial respiration (F(N)-pathway) in a substrate-dependent LEAK (L) state. Then, ADP was added to a concentration of 5 mM to initiate oxidative phosphorylation-dependent respiration (OXPHOS state). Next, pyruvate (5 mM, complex I substrate, N-pathway) was added to reestablish FN-pathway-linked respiration. Succinate (10 mM, complex II substrate, S-pathway) was added to reconstitute convergent FNS-linked respiration. Then, rotenone (0.5 μM, complex I inhibitor) and antimycin A (2.5 μM, complex III inhibitor) were added to determine the S-linked respiration and residual oxygen consumption (ROX), respectively.

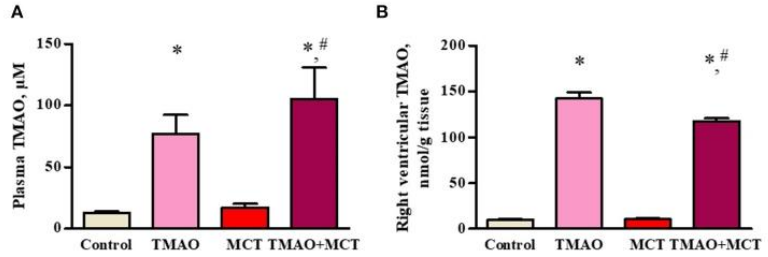


FIGURE 2 | TMAO concentration in plasma **(A)** and right ventricular tissue **(B)** after administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks. The results are presented as the mean ± SEM of 8–9 animals. * Indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's post-test), # indicates a significant difference from the MCT group (unpaired *t*-test), $p < 0.05$.

TABLE 1 | Echocardiographic assessment of right ventricle functionality after administration of TMAO at a dose of 120 mg/kg for 14 weeks in a monocrotaline-induced model of right ventricle heart failure.

	Control	TMAO	MCT	TMAO+MCT
Right ventricular pressure, mmHg	21.9 ± 2	22.7 ± 1.3	33.5 ± 5.3*	26.1 ± 1.8
Right ventricular diastolic area, cm^2	0.37 ± 0.02	0.33 ± 0.02	0.5 ± 0.07	0.4 ± 0.04
Right ventricular systolic area, cm^2	0.2 ± 0.01	0.21 ± 0.02	0.36 ± 0.07*	0.26 ± 0.03
Right ventricular fractional area change, %	46.6 ± 2.6	37 ± 2.8	29.7 ± 4.8*	37 ± 5

*The results are presented as the mean ± SEM of 8–9 animals. * Indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's post-test), $p < 0.05$.*

To determine the contribution of each substrate to the respiration rate, the flux control factor was calculated as follows:

$$1 - \frac{Resp.rate\ before\ the\ addition\ of\ substrate}{Resp.rate\ after\ the\ addition\ of\ substrate}.$$

To determine the mitochondrial mass in the heart, the citrate synthase activity in tissue homogenates was measured spectrophotometrically as previously described (Srere, 1969).

Statistical Analysis

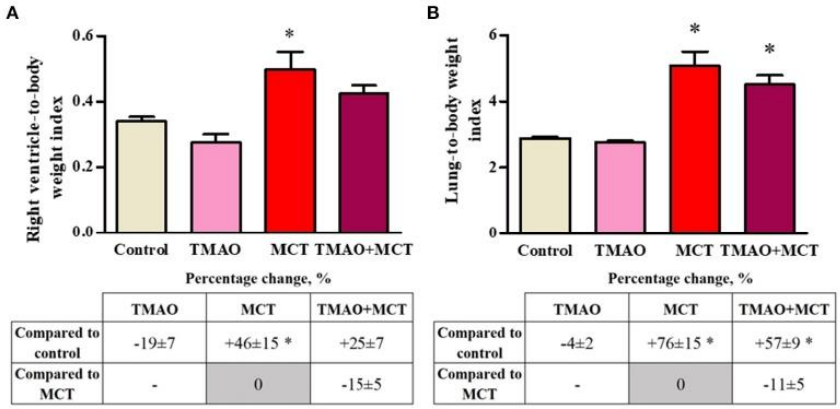
The statistical analysis of the data was performed using GraphPad Prism (GraphPad, Inc., La Jolla, USA) software. All the data are represented as the mean ± standard error of the mean (SEM). Data distribution was determined using Shapiro-Wilk's normality test. The statistical significance of the experimental results was verified by one-way ANOVA followed by Dunnett's multiple comparison test (to compare each experimental group to the control group) following an unpaired *t*-test (to compare the MCT group to the TMAO+MCT group). If the data were not normally distributed, the Kruskal-Wallis test followed by Dunn's multiple comparison test was used. The results were considered statistically significant if the *p*-value was <0.05.

RESULTS

Effects of Long-Term TMAO Administration on Heart Failure Severity

Administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks resulted in a 6-fold increase in the TMAO plasma concentrations (up to 100 µM) in both the TMAO and TMAO+MCT groups (**Figure 2A**). The analysis of the TMAO content in the tissues of the right ventricle revealed that treatment with TMAO resulted in a 14-fold increase in the TMAO tissue content (up to 140 nmol/g tissue) in both groups that received TMAO (**Figure 2B**).

The echocardiographic assessment did not reveal any significant differences in cardiac function between the control and TMAO groups. Administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks did not affect direct right ventricular (RV) pressure, RV systolic and diastolic area or RV fractional area change (**Table 1**) as well as ejection fraction and fractional shortening of the left ventricle (**Supplementary Table 2**). Compared with the control, administration of monocrotaline induced a significant increase (~50%) in direct right ventricular pressure (**Table 1**). In addition, dilatation of the right ventricle was observed in the hearts of the animals in the MCT group, as indicated by 34 and 83% increases in the RV diastolic and systolic areas, respectively. Subsequently, the right ventricular fractional area change was significantly decreased in the MCT group compared to the control group. Compared to those in the MCT control group, the direct RV pressure measurement was decreased by 22%, the RV diastolic and systolic areas were decreased by up to 27%, and therefore, the RV fractional area change was increased by 25% in the TMAO+MCT group. None of the measured parameters in the TMAO+MCT group were significantly different from those in the control group. Overall, these results indicate that chronically elevated TMAO levels in plasma and cardiac tissue do not affect



Percentage change, %

	TMAO	MCT	TMAO+MCT
Compared to control	-19±7	+46±15 *	+25±7
Compared to MCT	-	0	-15±5

Percentage change, %

	TMAO	MCT	TMAO+MCT
Compared to control	-4±2	+76±15 *	+57±9 *
Compared to MCT	-	0	-11±5

FIGURE 3 | Changes in the right ventricle-to-body weight **(A)** and lung-to-body weight **(B)** indexes after monocrotaline injection. The results are presented as the mean ± SEM of 8–9 animals. * Indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's post-test), $p < 0.05$.

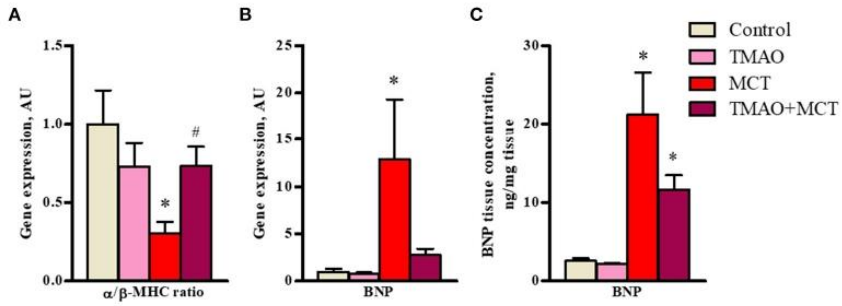


FIGURE 4 | Effect of administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks on heart failure severity-related gene expression **(A,B)** and BNP protein expression **(C)** in right ventricular tissue of a rat model of monocrotaline-induced heart failure. The results are presented as the mean ± SEM of 6–8 animals. * Indicates a significant difference from the control group [one-way ANOVA followed by Dunnett's posttest for **(A)**, Kruskall-Wallis test followed by Dunn's multiple comparison test for **(B)** and **(C)**], # indicates a significant difference from the MCT group (unpaired *t*-test), $p < 0.05$.

cardiac functionality, while long-term TMAO administration preserves myocardial mechanical function in monocrotaline-induced heart failure.

To evaluate MCT-induced cardiac and pulmonary remodeling, the organ mass indexes were calculated. There was no difference in the whole heart-to-body weight index or in the left ventricle-to-body weight index between the experimental groups (**Supplementary Figure 1**). Long-term TMAO administration did not impact either the right ventricle (**Figure 3A**) or lung-to-body weight (**Figure 3B**) indexes. Compared to the control group, the MCT group exhibited increased right ventricle hypertrophy and pulmonary remodeling, as indicated by significant increases in the organ-to-body weight indexes by 46 and 76%, respectively (**Figures 3A,B**). In the TMAO+MCT group, the right ventricle and lung-to-body weight indexes were decreased by 15 and 11%, respectively, compared to those in the MCT group (**Figures 3A,B**) suggesting that long-term TMAO administration can partially prevent monocrotaline-induced organ remodeling and hypertrophy.

In addition, long-term TMAO administration did not cause any significant changes in the expression of genes related to heart failure and hypertrophy (**Figures 4A,B**) or in the protein expression of BNP45 (**Figure 4C**). In the MCT group, a 3-fold decrease in the *α/β-MHC* expression ratio (**Figure 4A**) was observed, indicating a shift in favor of the *β* isoform caused by right ventricle hypertrophy. In addition, the expression of a maker of heart failure severity, *BNP*, was upregulated by 12-fold in the MCT group (**Figure 4B**). Consistent with the gene expression results, BNP45 protein expression in cardiac tissue was significantly increased by 10-fold in the MCT group compared to the control group (**Figure 4C**). In the TMAO+MCT group, the *α/β-MHC* expression ratio was 2-fold higher, suggesting less pronounced cardiac hypertrophy compared to that in the MCT group (**Figure 4A**). The gene and protein expression of BNP was lower in the TMAO+MCT group than in the MCT group (**Figures 4B,C**). Moreover, measurements of total cholesterol, triglycerides and free fatty acids in plasma did not reveal any significant changes between experimental

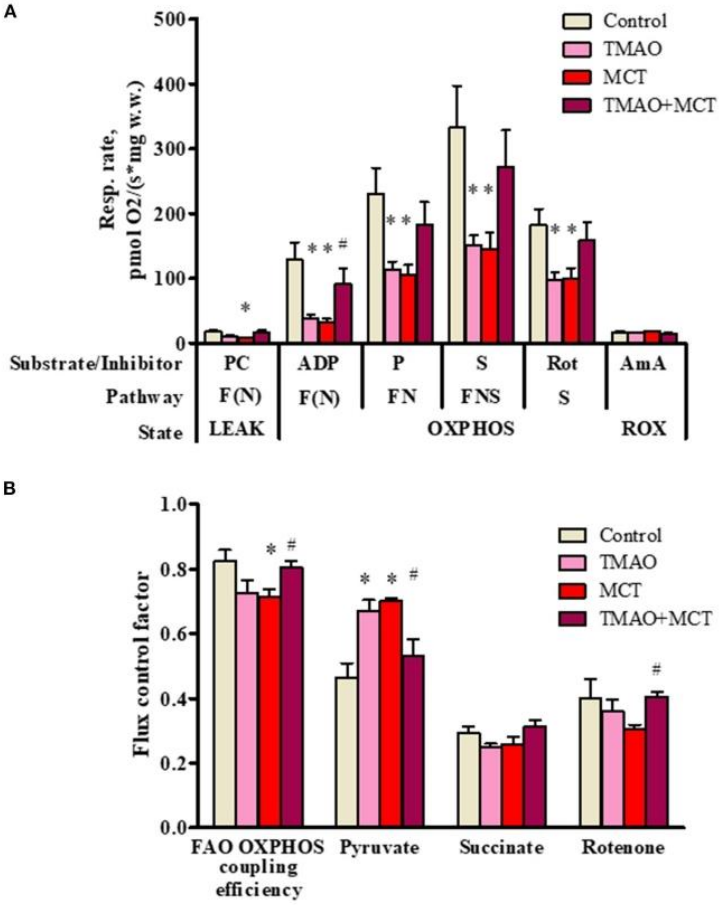


FIGURE 5 | Mitochondrial respiration rate measurements **(A)** and flux control factors **(B)** in right ventricular cardiac fibers using different energy substrates after administration of TMAO at a dose of 120 mg/kg for 14 weeks in a model of monocrotaline-induced right ventricle heart failure. The results are presented as the mean ± SEM of 6 animals. * Indicates a significant difference from the control group (one-way ANOVA followed by Dunnett's post-test), # indicates a significant difference from the MCT group (unpaired *t*-test), $p < 0.05$. Flux control factor, the contribution of each substrate/pathway to the respiration rate; PC, palmitoylcarnitine; ADP, adenosine diphosphate; P, pyruvate; S, succinate; Rot, rotenone; AmA, antimycin A; F, fatty acid oxidation-dependent pathway; N, NADH pathway; LEAK, substrate-dependent state; OXPHOS, oxidative phosphorylation-dependent state; ROX, residual oxygen consumption.

groups (**Supplementary Table 3**), therefore our observed effects of TMAO on cardiac function are independent of plasma lipid profile. Overall, these findings indicate that long-term TMAO administration partially reduced the severity of heart failure induced by monocrotaline administration.

Effects of Long-Term TMAO Administration on Cardiac Mitochondrial Energy Metabolism

To further investigate the effects of long-term TMAO administration on energy metabolism, mitochondrial respiration measurements were performed using permeabilized cardiac fibers prepared from right ventricular tissue samples. Long-term TMAO administration decreased the FAO-dependent respiration rate by 69% in the OXPHOS state (**Figure 5A**), resulting in an 11% decrease in the FAO-dependent OXPHOS coupling efficiency (**Figure 5B**). Although pyruvate metabolism input to overall respiration was increased by ∼44% in the TMAO group, as indicated by Flux control factor analysis (**Figure 5B**), it was not sufficient to restore FN and FNS pathway-linked mitochondrial respiration in the OXPHOS state (**Figure 5A**). In the MCT group, there was a 75% decrease in the FAO-dependent respiration rate in the OXPHOS state (**Figure 5A**) and a subsequent 13% decrease in the FAO-dependent OXPHOS coupling efficiency (**Figure 5B**). Similar to the TMAO group, in the MCT group, pyruvate metabolism input to respiration was increased by 50% (**Figure 5B**), but this increase was not sufficient to restore FN- and FNS-pathway-linked respiration in the OXPHOS state (**Figure 5A**). In contrast to

the TMAO group, in the MCT group, the flux control factor for rotenone was reduced ($p = 0.06$), indicating partial complex I dysfunction (**Figure 5B**). Moreover, in the TMAO+MCT group, mitochondrial energy metabolism was preserved, as shown by normalized respiration rates (**Figure 5A**), preserved FAO-dependent oxidative phosphorylation efficiency and subsequently decreased pyruvate metabolism input (**Figure 5B**). Moreover, measurements of the citrate synthase activity (**Supplementary Figure 2**) in right ventricular tissue showed that there were no differences in mitochondrial mass between the experimental groups. Taken together, the obtained results show that long-term TMAO administration itself induces mitochondrial metabolic preconditioning by causing a switch from fatty acid utilization to pyruvate utilization without affecting electron transfer functionality; moreover, in right ventricle heart failure, TMAO treatment can preserve cardiac mitochondrial energy metabolism.

DISCUSSION

In the present study, we demonstrate the effects of long-term TMAO administration on cardiac mitochondrial energy metabolism and on right ventricular heart failure progression. Although long-term TMAO administration decreases mitochondrial fatty acid oxidation, it has no adverse effects on cardiac mechanical function. However, unexpectedly, long-term TMAO administration results in preserved mitochondrial energy metabolism, leading to reduced heart failure severity and maintained cardiac functionality. Taken together, these results suggest that elevated TMAO concentrations can exert preconditioning-like effects and exhibit cardioprotective properties.

The role of TMAO as a risk factor in the development of cardiovascular diseases is widely debated around the world. It has been shown that even a 100-fold increase in circulating TMAO levels (up to 60 μM) in rats did not affect cardiac functionality (Ufnal et al., 2014). Similarly, no effects on cardiac parameters in mice were observed after 3 weeks of administration of 0.12% TMAO in the chow (Organ et al., 2016). Although the TMAO concentration in target tissues was not determined in previously mentioned studies, it has recently been shown that TMAO at concentrations up to 10 mM does not affect cell viability, mitochondrial membrane potential or ROS production in rat cardiomyocytes (Querio et al., 2019). Our results complement previous findings, reporting the TMAO level reached in cardiac tissue (up to 140 nmol/g tissue) after 14-week administration of TMAO in drinking water and, thus, provide a rationale behind further dose selection strategies in *in vitro* experiments. In our experimental design, TMAO was administered directly (120 mg/kg in their drinking water) and no further metabolization was needed by gut bacteria. However, the possible interindividual variability caused by alterations in gut microbiota composition should be considered if TMAO precursors (i.e., choline or carnitine) were administered during the experiment, since various bacterial genera are able to metabolize different precursors to synthesize TMA (Jameson et al., 2018). It has also been shown that a retroconversion of TMAO to TMA is possible (Hoyles et al., 2017); however, in our study a low variability of TMAO levels in right ventricular tissue was observed, suggesting that gut microbiota composition and TMA/TMAO production capacity was similar in our experimental animals. Moreover, our results demonstrate that a long-term increase in plasma TMAO levels up to 100 μM and a subsequent increase in TMAO levels in cardiac tissue (up to 140 nmol/g tissue) do not affect cardiac function. In addition, recent studies indicated that TMAO administration does not exacerbate the condition of already present stressors (Querio et al., 2019), such as H_2O_2, which is a major contributor to oxidative stress (Nita and Grzybowski, 2016), and doxorubicin, which is known to cause disturbances in cardiac energy substrate metabolism similar to those caused by heart failure (Wu et al., 2016). In our experimental setup, long-term TMAO administration shifted mitochondrial energy substrate utilization from FAO to glucose metabolism, but in contrast to the heart failure group, the TMAO treatment group did not exhibit altered mitochondrial electron transfer system functionality. Since the shift from compensated cardiac hypertrophy to heart failure is preceded by respiratory complex I and II dysfunction (Griffiths et al., 2010), unaltered complex I and complex II function could explain our observations of maintained cardiac functionality even after 14 weeks of TMAO administration, notwithstanding altered energy metabolism. Overall, our findings suggest that despite this metabolic shift, long-term elevations in TMAO levels in plasma and cardiac tissue do not exert detrimental effects on cardiac function.

Previously, increased plasma TMAO levels in experimental models of heart failure led to worsening of cardiac parameters, suggesting that TMAO is a detrimental factor in cardiovascular disease pathophysiology. It has been shown that administration of TMAO and its precursor, choline, exacerbates left ventricle remodeling and cardiac function loss (Organ et al., 2016). Moreover, withdrawal of dietary TMAO even 6 weeks after aortic constriction reversed those changes, indicating an ability of the heart to recover from detrimental changes caused by TMAO (Organ et al., 2020). In addition, a reduction in circulating TMAO levels by 3,3-dimethyl-1-butanol or iodomethylcholine alleviated cardiac hypertrophy and remodeling after aortic banding (Organ et al., 2020; Wang et al., 2020). TMAO-induced impairment in cardiomyocyte contractility and calcium handling (Savi et al., 2018) as well as T-tubule formation (Jin et al., 2020) were suggested as possible mechanisms that may link TMAO to heart failure. In contrast, our results demonstrate that an increase in TMAO levels in plasma and tissues partially prevents the remodeling of the right ventricle and the development of right-sided heart failure. Previously, it has been shown that TMAO treatment reduces cardiac fibrosis and improves cardiac functionality in a model of Spontaneously Hypertensive rats (Huc et al., 2018). Consistent with these findings, our study shows that TMAO administration can partially prevent right ventricular hypertrophy as shown by normalized organ-to-body weight indexes and hypertrophy-related gene expression. Moreover, consistent with our results, protective effects of TMAO were also observed in Spontaneously Hypertensive Heart Failure rats, in which long-term TMAO treatment improved survival and cardiac parameters and lowered plasma NT-proBNP levels (Gawrys-Kopczynska et al., 2020). Similarly, in our study, TMAO

administration preserved right ventricular function, as indicated by normalized direct RV pressure and RV fractional area change; moreover, TMAO decreased the expression of a marker of heart failure severity, BNP, in right ventricular tissue. In addition, a recent study showed that administration of betaine, a common TMAO precursor, attenuated pulmonary artery hypertension (Yang et al., 2018). Interestingly, monocrotaline injection was used to induce pulmonary artery hypertension in the previous study; this is the same method we used in our study to induce right ventricle heart failure. Although ∼100-fold less TMAO is produced from betaine than from choline (Wang et al., 2014), at least to some extent, the observed protective effects of betaine might be explained by increased bioavailability of TMAO. Overall, previous and present observations suggest that long-term TMAO administration can reduce right ventricular remodeling and improve cardiac function in right-sided heart failure. Moreover, our findings also support the hypothesis that a TMAO-rich Mediterranean diet may prevent and reduce the risk of cardiovascular diseases (Estruch et al., 2018).

The protective role of TMAO was previously explained by its ability to reduce endoplasmic reticulum stress (Makhija et al., 2014), oxidative-nitrative stress and the subsequent vascular and diabetic complications (Lupachyk et al., 2013; Fukami et al., 2015). More recently, some protective effects were attributed to the ability of TMAO to increase diuresis and natriuresis (Gawrys-Kopczynska et al., 2020). In addition, our study proposes preserved cardiac energy metabolism as a possible mechanism underlying the observed protective effects of TMAO. The heart is capable of adapting to both physiological and pathological stressors by shifting from FAO as a dominant energy source to more pronounced utilization of glucose (Brown et al., 2017). In physiological states, this shift could be considered a preconditioning strategy, since the heart is thus better prepared for future stress conditions, such as hypoxia, due to higher reliance on more energy-efficient substrates in the case of oxygen deficiency (Karwi et al., 2018). In the present study previously described metabolic shift was observed, when after long-term TMAO administration FAO was decreased, and pyruvate metabolism was subsequently increased without changes in cardiac functionality. It has been previously shown that TMAO decreases FAO with a mechanism not related to inhibition of carnitine palmitoyl transferase 1 (CPT1) (Makrecka-Kuka et al., 2017). Since we did not observe hindered pyruvate metabolism by accumulation of acylcarnitines, which might occur if carnitine/acylcarnitine translocase (CACT) or CPT2 was inhibited (Chegary et al., 2008; Makrecka et al., 2014; Makrecka-Kuka et al., 2020), most likely the decrease in FAO induced by TMAO administration is not related to direct inhibition of CACT or CPT2. Another possible explanation of our observation that TMAO reduces FAO could be that TMAO indirectly inhibits CACT by a nitric oxide (NO)-dependent mechanism (Tonazzi et al., 2017). However, TMAO is not reported to act as NO donor, moreover, it is shown to downregulate NO production *in vitro* (Sun et al., 2016; Chou et al., 2019) and *in vivo* (Li et al., 2017). Thus, it is unlikely that TMAO could cause indirect inhibition of CACT via NO pathway. Overall, an increase in TMAO concentration appears to induce a metabolic shift, possibly through direct inhibition of β-oxidation, toward more efficient substrate metabolism under stress conditions, such as hypoxia, thus ensuring preserved energy metabolism and subsequently improving cardiac function recovery after injury. Taken together, our results suggest that TMAO administration exhibits cardioprotective properties during heart failure by maintaining metabolic flexibility and preserving fatty acid oxidation, both of which are vital strategies to restore cardiac bioenergetic balance (Kolwicz et al., 2012; Karwi et al., 2018). Moreover, our findings demonstrate that long-term consumption of TMAO-rich foods (i.e., Mediterranean diet) might induce metabolic preconditioning-like effects.

In conclusion, our study presents evidence that chronic TMAO administration protects cardiac functionality by preserving mitochondrial energy metabolism in an experimental model of monocrotaline-induced right ventricle heart failure, where TMAO acts as a preconditioning factor. In addition, our results provide a novel insight on the theory, that the role of TMAO in the pathogenesis of cardiometabolic diseases is not limited to either detrimental or protective effects, suggesting that it might actually be dual and depend on specific conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia; Food and Veterinary Service Ethical approval Nr. 105.

AUTHOR CONTRIBUTIONS

MV, RV, MD, and MM-K performed planning of the study. MV, RV, SK, HC, and MM-K conducted experiments. MV, RV, SK, and MM-K performed data analysis. ES performed bio-analytical assays and data analysis. MV wrote the manuscript with input from RV, MD, and MM-K. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.622741/full#supplementary-material

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Metformin decreases bacterial trimethylamine production and trimethylamine N-oxide levels in db/db mice

Janis Kuka[1]✉, Melita Videja[1,2], Marina Makrecka-Kuka[1], Janis Liepins[3], Solveiga Grinberga[1], Eduards Sevostjanovs[1], Karlis Vilks[1], Edgars Liepinsh[1] & Maija Dambrova[1,2]

The current study aimed to explore whether metformin, the most widely prescribed oral medication for the treatment of type 2 diabetes, alters plasma levels of cardiometabolic disease-related metabolite trimethylamine N-oxide (TMAO) in db/db mice with type 2 diabetes. TMAO plasma concentration was up to 13.2-fold higher in db/db mice when compared to control mice, while in db/db mice fed choline-enriched diet, that mimics meat and dairy product intake, TMAO plasma level was increased 16.8-times. Metformin (250 mg/kg/day) significantly decreased TMAO concentration by up to twofold in both standard and choline-supplemented diet-fed db/db mice plasma. In vitro, metformin significantly decreased the bacterial production rate of trimethylamine (TMA), the precursor of TMAO, from choline up to 3.25-fold in *K. pneumoniae* and up to 26-fold in *P. Mirabilis*, while significantly slowing the growth of *P. Mirabilis* only. Metformin did not affect the expression of genes encoding subunits of bacterial choline-TMA-lyase microcompartment, the activity of the enzyme itself and choline uptake, suggesting that more complex regulation beyond the choline-TMA-lyase is present. To conclude, the TMAO decreasing effect of metformin could be an additional mechanism behind the clinically observed cardiovascular benefits of the drug.

Type 2 diabetes is characterized by alterations in the composition of intestinal microbiota, notably by a decrease in butyrate-producing bacteria and an increase in opportunistic pathogens, bacteria that can exploit weakened immune system to cause an infection[1,2]. While gut bacteria have been known to play fundamental roles in the pathogenesis of many diseases, such as obesity, diabetes and cardiovascular disease, a clear link underlying these roles was missing. A breakthrough in predicting cardiometabolic risks and potential outcomes based on intestinal microbiota composition came when trimethylamine N-oxide (TMAO) was linked to the development and progression of cardiovascular diseases[3,4]. Thus, it was shown that intestinal microbiota produces trimethylamine (TMA) from dietary tertiary amines like choline, carnitine and butyrobetaine. Upon entering circulation TMA is further metabolized to TMAO by host liver enzyme flavin-containing monooxygenase 3 (FMO3). Recently it was shown that type 2 diabetes is also associated with higher plasma level of TMAO[5,6].

In recent years, an interesting relationship between TMAO, insulin and insulin resistance-induced cardiovascular diseases has been elucidated. Thus, it was shown that the expression of TMAO producing enzyme FMO3 is suppressed by insulin[7]; subsequently, it was shown that FMO3 knockout mice are protected from the development of hyperglycaemia, hyperlipidaemia and atherosclerosis due to the suppression of FOXO1, a transcription factor that is the main target of insulin signalling. Very recently, it was shown that the endoplasmic reticulum stress kinase PERK acts as a receptor for TMAO; thus, TMAO at physiologically relevant concentrations selectively activates PERK, which in turn induces the transcription factor FOXO1[8] and promotes metabolic dysfunction.

The most widely prescribed medication for the treatment of type 2 diabetes is metformin. However, approximately one fourth of patients receiving metformin report side effects directly attributed to the therapy, and almost 62% of side effects reported are gastrointestinal ones such as nausea and diarrhoea[9] suggesting of changes in microbiota composition. Indeed, recent findings indicate that both therapeutic effects and side effects of

[1]Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, Riga 1006, Latvia. [2]Faculty of Pharmacy, Rīga Stradiņš University, Dzirciema Str. 16, Riga 1007, Latvia. [3]Institute of Microbiology and Biotechnology, University of Latvia, Jelgavas Str. 1, Riga 1004, Latvia. ✉email: janis.kuka@farm.osi.lv

metformin at least to some extent depend on drug use-induced changes in intestinal microbiota[10,11]. It has been shown that metformin treatment can partially normalize the aberrant Firmicutes/Bacteroidetes ratio in high-fat diet-fed mice by decreasing *Firmicutes* and restoring *Bacteroidetes* and *Verrucomicrobia* levels[12] and improve the function of a high-fat diet impaired glucose-SGLT1-sensing glucoregulatory pathway in rats[13]. Metformin-induced intestinal effects might be related to relatively high mM concentrations than can be achieved in the lumen of intestine following oral administration of the drug at higher doses[14]. Although anti-diabetic effects and more recently[15,16] beneficial cardiovascular effects in diabetes and non-diabetes patients of metformin are well characterized, molecular mechanisms and particularly biochemical mechanisms involving the effects on intestinal microbiota remain to be elucidated. First, the experimental setup of the present study aimed to explore the effects of metformin on TMAO levels under conditions of type 2 diabetes in db/db mice. Second, since TMA and subsequently TMAO are mostly formed from dietary tertiary amines, mainly from choline and carnitine/butyrobetaine[4,17,18], additional experiments were performed to evaluate the efficacy of metformin in db/db mice under increased choline load to mimic high meat and dairy product (rich in choline, carnitine and other tertiary amines/their derivatives) intake as in typical Western diet. Third, the direct effects of metformin on opportunistic pathogens present in human intestines that differ significantly in their ability to produce TMA from different sources were evaluated in vitro. Thus, *Klebsiella pneumoniae* was chosen because it can produce TMA from all the main precursors—choline (via *CutC/D* (choline-TMA-lyase complex)) and carnitine/butyrobetaine (via *CntA/B* (Carnitine monooxygenase complex)), while *Proteus mirabilis* was chosen because it has just *CutC/D* and can produce TMA only from choline[19,20].

Results

Effects on TMAO production in db/db mice fed standard chow and diet supplemented with choline. The TMAO concentration in plasma of db/db control mice fed standard chow (R70) was significantly, up to 13.2-fold, higher than that in db/Lean mice plasma. Metformin administration to db/db mice at a dose of 250 mg/kg for 8 weeks significantly decreased TMAO levels up to 2.0-fold when compared to those of db/db control mice (Fig. 1A).

Because TMA and subsequent TMAO production depend on diet, we next evaluated whether metformin treatment can affect chronic and acute dietary choline load-induced increases in TMAO levels. Based on findings with standard chow, where the efficacy of metformin was similar after 4 and 8 weeks of treatment, administration of metformin for 4 weeks was chosen for follow-up studies using a choline-supplemented diet. In this experiment, choline pretreatment for 4 weeks resulted in a further 1.8-fold increase in basal TMAO levels (36.9 µM) when compared to db/db mice on a standard diet (Fig. 1B) and 16.8-fold increase when compared to control db/Lean mice. Two h after acute choline load, TMAO plasma levels increased in all experimental groups, and highest concentrations up to 95–108 µM were observed in the db/db control and choline pre-treated groups. In db/db mice on a diet supplemented with choline, metformin treatment significantly decreased both basal and 2 h post-choline-load TMAO levels (Fig. 1B). The choline plasma concentration in db/db control mice plasma was 45 µM before and 58 µM after acute choline load, and no significant changes in choline plasma concentrations due to treatment with metformin were observed among experimental groups.

To assess the population of selected bacteria in the faecal samples (from mice treated with choline or choline in combination with metformin for 4 weeks) we evaluated metformin-induced changes in abundance of *P. mirabilis* and *K. pneumoniae.* While levels of *K. pneumoniae* were too low to be detected, *P. mirabilis* bacteria were detected and we observed tendency for relatively higher *P. mirabilis* bacteria presence in db/db mice that received choline and also tendency for metformin to prevent this increase (Fig. 1C). In addition, we investigated the effect of metformin on bacterial genera *Olsenella* and *Desulfovibrio,* members of which are known to be able to produce TMA from choline[21]. Metformin treatment resulted in a significant relative population increase of *Desulfovibrio* species (Supplementary Fig. 1). Flavin-containing monooxygenase 3 (FMO3) activity in the liver microsomal fraction in the presence of metformin was measured and metformin did not change FMO3 activity at concentrations up to 2 mM (Supplementary Fig. 2).

Glucose and insulin plasma concentrations were significantly increased in both fed and fasted states, and metformin had no effect in any of these states (Fig. 2A,B). Lactate plasma concentration was significantly increased only in the fasted state in db/db control mice plasma but not in metformin-treated db/db mice plasma (Fig. 2C). Taken together, metformin treatment had no glucose-lowering and insulin sensitivity-improving effect in db/db mice with type 2 diabetes; thus, the effects observed in this study were independent of glucose and insulin plasma concentrations.

Metformin effects on bacterial TMA production. Since treatment with metformin significantly decreased TMAO production in mice, we tested whether this effect could be related to changes in ability of gastrointestinal tract bacteria to produce TMA, which is required for host liver to produce TMAO. For this we chose human gastrointestinal tract bacteria *K. pneumoniae* and *P. mirabilis* both known to be able to produce TMA[19,20]. Metformin significantly decreased TMA production 3.25-fold in *K. pneumoniae* (Fig. 3A), while bacterial growth of *K. pneumoniae* was not affected (Fig. 3C) when choline or glucose was used as the sole carbon source for up to 8 h. Metformin significantly decreased TMA production (up to 26-fold) in *P. mirabilis* (Fig. 3B). Unlike with *K. pneumoniae*, metformin significantly decreased *P. mirabilis* bacterial biomass growth when choline was used as the sole carbon source. The effect was present, albeit less pronounced when *P. mirabilis* was grown in media with glucose as the sole carbon source (Fig. 3D). Decrease in choline concentration in incubation media indicated that choline is converted to TMA by bacteria (Supplementary Fig. 3).

To determine if the administration of metformin changes the expression of bacterial genes encoding key proteins of choline metabolism, choline lyase enzyme complex subunits cutC and cutD and enzyme enclosure

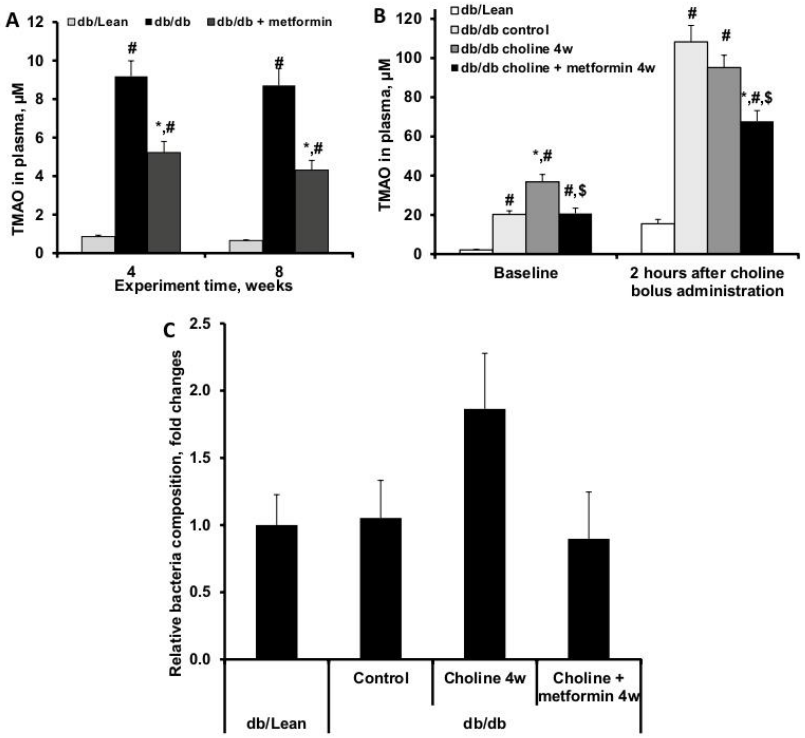

Figure 1. Effects of metformin (250 mg/kg) treatment on TMAO plasma concentrations and relative bacteria composition in db/db mice. Panel A, effects of 4 and 8 weeks of treatment with metformin in mice fed a standard laboratory diet; Panel B, effects of 4 weeks of metformin treatment in mice supplemented with choline. Panel C, effects of 4 weeks of metformin treatment in mice supplemented with choline on relative presence of *P. mirabilis* in gut microbiota. The results are the mean of 8 animals ± SEM in the db/db and db/db + metformin groups and 10 animals in the db/Lean group for panel A. The results are the mean of 10 animals ± SEM for panel B. The results are mean of 9–10 animals for panel C, one sample was excluded in choline group and one in choline + metformin group after identified as outliers by ROUT analysis. * Significantly different from the respective db/db control group, # significantly different from the respective db/Lean group, $ significantly different from the respective db/db + choline 4w group (ANOVA followed by Tukey's test; $P < 0.05$).

microcompartment structural protein cmcA[22,23], *K. pneumoniae* were cultivated with choline (0.5% in broth) as the sole carbon source, and metformin was added at the final concentration of 27 mM (0.5% in broth). Transcription samples were extracted after 0, 4 and 8 h of anaerobic cultivation with choline and with or without metformin. The expression of the choline-TMA-lyase complex subunits (cutC and cutD) and the microcompartment structural gene (cmcA) increased over time; however, metformin had no significant effect on the expression of any of the tested genes (Fig. 4A,B,C). Moreover, choline-TMA-lyase activity was determined in *P. mirabilis* bacterial lysate, and we found no metformin effect on the TMA production rate (Fig. 4D).

Because metformin did not affect the expression of genes coding choline-TMA-lyase and enzymatic activity, we next evaluated its effects on choline uptake. To confirm bacterial viability and functional capacity, carnitine uptake was tested as a positive control as described previously[19]; [^{3}H]-carnitine uptake was time-dependent with a maximal velocity of 0.77 ± 0.07 fmol/min/mg bacterial protein, indicating that the bacteria were viable and functional. [^{3}H]-choline was used for uptake measurements in *K. pneumoniae,* and metformin was added at a final concentration of 27 mM. The choline concentration in bacteria after 10 min of incubation was 1.49 fmol/mg bacterial protein and was unchanged by metformin treatment (1.50 fmol/mg bacterial protein).

Discussion

Knowledge on the mechanism of the anti-diabetes action of metformin has become increasingly complex over the years from complex I inhibition to suppression of gluconeogenesis by inhibition of mitochondrial glycerophosphate dehydrogenase[24] to interaction with intestinal microbiota[10,25] to the suggested potential iron chelating activity of metformin[26]. We now show that metformin is able to directly inhibit bacterial TMA production and decrease TMAO availability in mice. In this study db/db mice had pronounced hyperglycaemia and obesity;

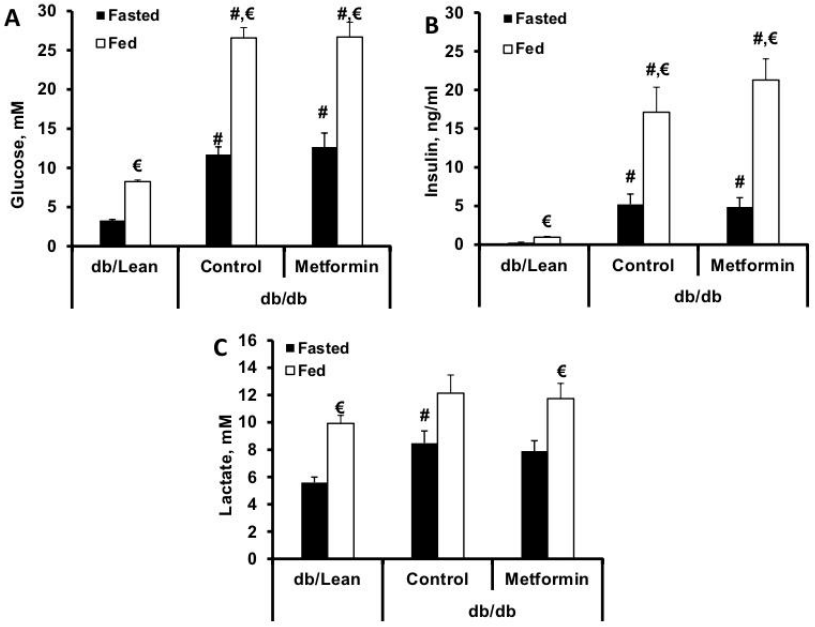

Figure 2. Effects of an 8-week metformin treatment at a dose of 250 mg/kg on blood glucose levels in fasted and fed states (**A**), plasma insulin in fasted and fed states (**B**) and plasma lactate levels in fasted and fed states (**C**). The results are the mean ± SEM of 8 animals in the db/db and db/db + metformin groups and 10 animals in the db/Lean mice group. $^{\#}P < 0.05$ vs. db/Lean group (ANOVA, followed by Tukey's test for A, Kruskal–Wallis test followed by Dunn's multiple comparison test for B and C). $^{€}$ significantly different from the respective group at fasted state (paired t-test).

under our setup, treatment with metformin did not affect blood glucose and insulin levels, indicating that metformin-induced changes in TMAO levels are at least partially independent of glucose and insulin plasma concentrations.

TMAO has recently been shown to cause endothelial dysfunction through cellular inflammation, elevation of oxidative stress and suppression of endothelial progenitor cell functions[27–29]. In turn, in the clinical setting, metformin treatment is beneficial in attenuating endothelial dysfunction in patients with prediabetes, metabolic syndrome and type 1 and 2 diabetes[30,31]. While decreasing glucose levels and improving insulin sensitivity are largely responsible for the beneficial effects of metformin, in light of the present findings, there is a basis for attributing part of the protective effects of metformin on the cardiovascular system to the decrease in circulating pro-atherogenic TMAO levels. Indeed, in recent years, metformin treatment has been shown to improve the endothelial glycocalyx barrier in db/db mice without changing blood glucose levels[32] and to improve endothelial function in spontaneously hypertensive rats with type 1 diabetes independent of glycaemia control[33].

Previously, we have shown that 12-week-old db/db mice have a significantly elevated TMAO level of 9 μM[5], which, if translated to human patients, indicates a significantly increased risk of major adverse cardiovascular events as the TMAO plasma level is above the risk threshold of 6.18 μM[3]. It has been recently reported that TMAO levels in db/db mice are higher during the dark cycle[34], and plasma samples in the current study were also collected during the dark cycle. Thus, in db/db control mice fed a standard laboratory R70 diet, TMAO plasma levels were well above 6.18 μM, while treatment with metformin decreased TMAO levels approximately two-fold and, importantly, below the threshold of TMAO plasma levels associated with increased risk of major cardiovascular complications. In animals on a diet supplemented with choline metformin treatment significantly decreased TMAO plasma concentration almost 1.8-times; however, TMAO plasma levels remained higher than the risk threshold. It should be noted that our experimental results differ from the previously published effects of metformin treatment on TMAO levels in patients. Thus, in metabonomics study metformin administration (dose not stated) was associated with increased TMAO availability[35], while interventional cross-over study where metformin was used at a dose of 2 g per day[36] showed no effect on TMAO levels. However, in both studies there were no indications that the patients were controlled for seafood intake. Seafood is very rich in TMAO and consumption of fish or fish products should be clear exclusion criteria when looking at TMAO plasma availability[5,37]. Given our present data and previously reported metformin concentrations that can be reached in the intestines after the use of high doses[14,38], dose of 2 g per day as in the study by Velebova and colleagues[36] should have been sufficient to induce decrease in bacterial TMA production. It must be stressed that we did determine effects of metformin on abundance of only selected intestinal bacteria and their ability to produce TMA in mice. Further

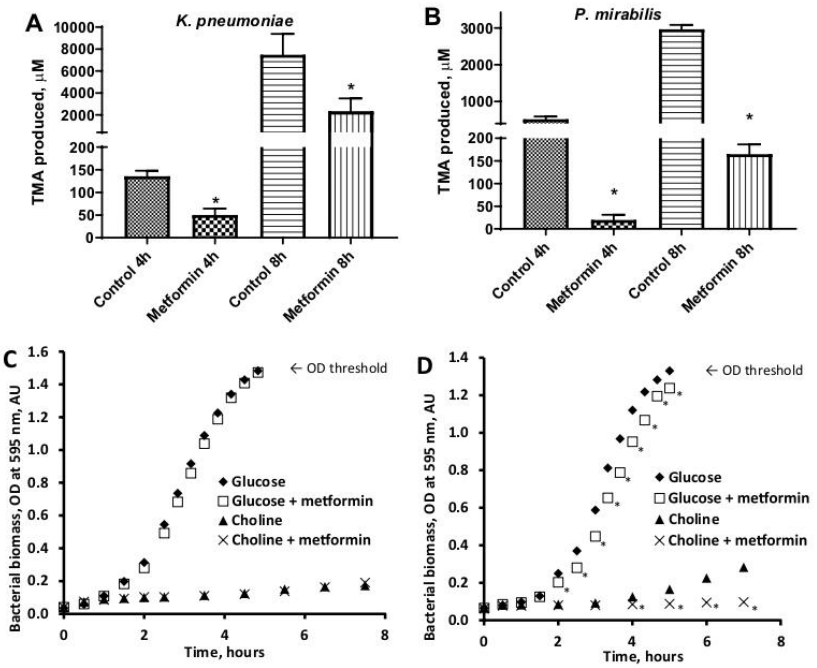

Figure 3. Metformin (27 mM) effects on TMA production from choline (**A**, **B**) and bacterial growth (**C**, **D**) in *K. pneumoniae* (**A** and **C**) and *P. mirabilis* (**B** and **D**) under anaerobic conditions. The results are the mean ± SD of 3 independent replicates for A and 4 for B, C and D. * $P < 0.05$ vs. respective time point control (t-test). The maximum absorbance value for turbid suspensions in the colorimeter used in the experiments was 1.4.

studies would be required to show how in vitro findings and data from db/db mice model translate to clinical setting under diet controlled conditions, and how metformin changes the abundance of selected bacteria that produce TMA from tertiary dietary amines in patients. Taken together, although metformin can decrease plasma TMAO availability in mice below the risk threshold, dietary changes would likely be required to reach clinically relevant endpoints and follow-up study in patient population is warranted.

Our findings strongly imply that acute effects of metformin result in overall decrease in TMAO availability through decreased bacterial TMA production. We found that metformin treatment does not result in uniform alterations in TMA-producing bacteria (both decrease and increase in bacterial population can be observed) and does not inhibit FMO3 activity. The effect of metformin on TMA synthesis also appears to be rather complex. Choline degradation to TMA in bacteria occurs in specialized microcompartments that contain choline-TMA-lyase[39]. Our data imply that other components or processes like co-factor recycling in these microcompartments but not choline-TMA-lyase itself are affected by metformin resulting in overall inhibition of TMA production. The current results prove that metformin inhibits microbial TMA production from choline; metformin is unable to decrease TMA production in *K. pneumoniae* when carnitine instead of choline is used as the sole substrate (unpublished observations). Metformin has no effect on anaerobic growth of *K. pneumoniae* (omnivorous tertiary amine metabolizer) but significantly delays (bacteriostatic effect) growth of *P. mirabilis* (choline-only metabolizing bacteria) under the same experimental conditions. To conclude, we present proof that metformin can induce significant changes in TMAO levels likely due to direct non-lethal inhibition of bacterial TMA production. Moreover, these effects are independent of glucose and insulin plasma concentrations and could be an additional mechanism behind the known cardiovascular benefits of metformin therapy.

Materials and methods

Animals and treatment. Sixteen male db/db (BKS.Cg-+Leprdb/+Leprdb/OlaHsd) mice and 10 age-matched non-diabetic db/Lean (db/+(BKS.Cg-+Leprdb/+/OlaHsd)) male mice (10 weeks old, Envigo, Venray, Netherlands) were housed for two weeks prior to treatment under standard conditions (21–23 °C, reverse 12-h light/dark cycle, relative humidity 45–65%) with unlimited access to water and food (R70 diet from Lantmännen, (Stockholm, Sweden)). The experimental procedures involving animals were performed in accordance with the guidelines of the European Community and local laws and policies, and all of the procedures were approved by Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. Studies involving animals are reported in accordance with the ARRIVE guidelines[40,41]. Db/db mice were randomly divided into two experimental groups and given daily oral doses of water (db/db control group, n = 8) or 250 mg/kg met-

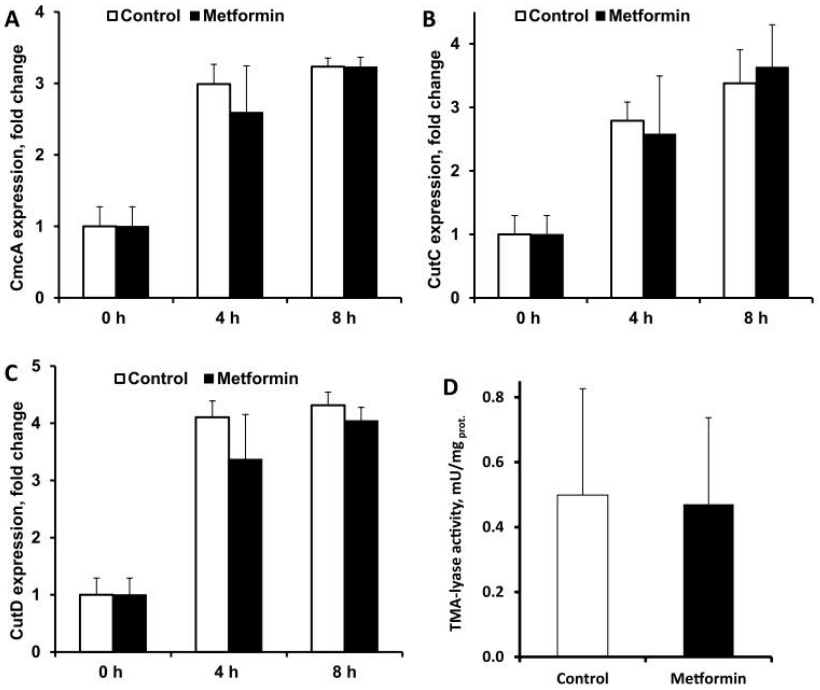

Figure 4. Effects of metformin (27 mM) under anaerobic conditions on the expression of genes that encode enclosure microcompartment structural proteins (**A**) and choline-TMA-lyase subunit proteins (**B** and **C**) in *K. pneumoniae* and on the choline-TMA-lyase activity in *P. mirabilis* bacterial lysate (**D**). The results are the mean ± SD of 3 independent replicates for A, B and C and the mean of 5 independent replicates for D.

formin (db/db metformin group, n = 8) for 8 weeks. Db/Lean mice (n = 10) were used as a control. Plasma samples were collected after 4 and 8 weeks of treatment. To test metformin (TCI Europe N.V., Zwijndrecht, Belgium) effects in case of increased tertiary amine load, choline (TCI EUROPE N.V., Zwijndrecht, Belgium) was chosen as the most common food tertiary amine.

Another 30 db/db male mice (BKS.Cg- + Leprdb/ + Leprdb/OlaHsd) and 10 age-matched non-diabetic db/Lean (db/ + (BKS.Cg- + Leprdb/ + /OlaHsd)) male mice (10 weeks old, Envigo, Venray, Netherlands) were obtained for the follow-up experiment based on data obtained from the first study. Db/db mice were divided into three experimental groups and given daily oral doses of water (db/db control group, n = 10), 0.5% choline in drinking water for 4 weeks to facilitate bacterial TMA and subsequently host TMAO production (db/db choline 4w group, n = 10) or 0.5% choline in drinking water and 250 mg/kg metformin (db/db choline + metformin 4w group, n = 10). Db/Lean mice (n = 10) were used as a control. Faecal samples were collected after 4 weeks of choline administration. At the end of the treatment these 40 mice received bolus dose of choline (100 mg/kg) to evaluate the overall capacity to produce TMAO and the ability of metformin to decrease TMAO production after acute substrate load. For this, plasma samples were collected immediately before and 2 h after choline load (100 mg/kg). Plasma samples were collected from tail veins during the dark cycle; samples were centrifuged, and the plasma was stored at -80 °C for future analysis. All experiments were performed in a blinded manner.

Glucose, lactate and insulin concentrations. The plasma insulin concentration was determined using a Sensitive Insulin RIA Kit (EMD Millipore, Billerica, MA, USA). Blood glucose was measured using a MediSense Optium glucometer from Abbott Diabetes Care (Maidenhead, UK). Lactate in plasma was determined using a kit from Instrumentation Laboratory (Lexington, MA, USA).

Determination of choline, TMAO and TMA concentrations. Determination of TMAO concentration in blood plasma was performed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) in positive ion electrospray mode, as described previously[42]. Choline quantification and trimethylamine concentration determination were performed as described previously[19].

Microbial cultures, TMA production, assay of choline uptake and FMO3 activity. To test microbial TMA production, two bacterial species were used: *Klebsiella pneumoniae* (obtained from the Microbial

Strain Collection of Latvia (MSCL), strain number 535) and *Proteus mirabilis* (MSCL, strain number 590). Bacteria were maintained on LB agar plates. A single colony was used as inoculum for each experiment. To test the effect of metformin on choline-dependent TMA production, bacteria were grown in M9 mineral broth supplemented with 0.2% casamino acids (Merck, Darmstadt, Germany)[43] with 27 mM choline as the carbon source, with or without 27 mM metformin addition. Metformin concentration of 27 mM was chosen as it represents levels of a drug that could be achieved in the intestines after administration of high (≥850 mg) metformin doses to patients[14,38].

Micro-anaerobic cultivations were performed essentially as described previously[19]. Briefly, to ensure anaerobic conditions 2 ml test tubes were filled with broth and covered with airtight caps; the test tubes were kept still in an incubator at +37 °C. Samples were harvested after 4 and 8 h of cultivation, fixed with formic acid to 5% final concentration and centrifuged. Supernatants were frozen (−80 °C) and stored until further analyses.

The effect of metformin on bacterial growth was assessed by spectrophotometric recording of *K. pneumoniae* and *P. mirabilis* growth dynamics in anaerobic tubes, as previously described[44]. Briefly, microbial cells were grown in M9 broth with glucose as the sole carbon source overnight. Cells were harvested by centrifugation, washed and resuspended in fresh M9 broth with different carbon sources, glucose or choline (final concentration of 27 mM) with or without metformin (final concentration 27 mM). Airtight, clear glass HPLC bottles (vol. 4.5 mL, diameter 1 cm) were top-filled with different microbial suspensions and left to incubate at +37 °C. The absorbance of the microbial suspensions was recorded using a WPA colorimeter (Biochrom Ltd, UK) set to 590 nm. Choline uptake in *K. pneumoniae* cells was measured using [^{3}H]-choline as a substrate as described previously[19].

FMO3 activity was determined in C57Bl6 lean mice liver microsomal fraction based on methimazole/DTNB assay as described previously[45]. Choline-TMA-lyase enzymatic activity was determined based on a method previously described by Roberts and colleagues[46] with modifications as indicated below. *P. mirabilis* bacterial cells were lysed, and the reaction was carried out in an inflatable glove bag filled with nitrogen gas to ensure anaerobic conditions. An enzymatic activity assay was performed using non-labelled choline, and the conversion of choline to TMA was determined with UPLC/MS/MS. The reaction mixture contained clarified bacterial lysate in lysis buffer and the necessary co-factors (1 mM SAM, 10 mM NaDT, and 2 mM NADH (Sigma-Aldrich, Schnelldorf, Germany)). Metformin was added to the reaction mixture (27 mM final concentration) to evaluate its effect on choline-TMA-lyase activity. The mixture was then allowed to incubate for 15 min before the initiation of the reaction by adding choline (1 mM final concentration). Baseline samples were collected, and the reaction was carried out for 2 h at room temperature in the dark. Afterwards, samples were harvested, fixed with formic acid (1.5% final concentration) and stored at -80 °C until further analysis.

Choline-TMA-lyase gene expression. To determine if a metformin–induced decrease in TMA production was associated with changes in the transcription of choline metabolism-related genes in gut bacteria, we tested the transcription of three *K. pneumoniae* choline-TMA-lyase complex coding genes (cmcA, CutC, CutD)[23]. Gene expression was evaluated in *K. pneumoniae* after 4 and 8 h of cultivation with and without 27 mM metformin. Total RNA was isolated and purified from *K. pneumoniae* cell pellets (stored at -80 °C) using a PureLink RNA extraction kit (ThermoFisher Scientific, USA) as recommended by the manufacturer's protocol. First-strand cDNA synthesis and quantitative RT-PCR analysis for genes were performed as described previously[47]. Relative expression levels for each gene were calculated with the ΔΔCt method and normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) gene. The primer sequences used for the quantitative RT-PCR analysis were as follows: 5′- TGTTGATGTCGTTGTGCGGA-3′ (Fw cmcA), 5′- CGTCGAGCTTATCGGCTATGA-3′ (Rv cmcA), 5′-TCGGTAACCAGACCCGTAAA-3′ (Fw cutC), 5′-GGCGCGAGTTTTCTCTTCTA-′3 (Rv cutC), 5′-GATTAACACCGCCGTCGAAA-3′ (Fw cutD), 5′-TCCACCAGCCATTCGAGATT-3′ (Rv cutD), 5′- ACCGTTCGTCTGGAAAAAGC-3′ (Fw GAPDH) and 5′- ACGAAGTTGTCGTTCAGTGC-3′ (Rv GAPDH).

DNA isolation from faecal samples and qPCR analysis. Total DNA from mice faeces was isolated[48] using FastDNA SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions. qPCR analysis and relative bacteria quantification was performed by using KAPA SYBR FAST master mix (Sigma-Aldrich) and MIC qPCR Cycler (Bio-Molecular Systems) and using the following conditions: Polymerase activation 95 °C for 3 min; Touchdown 10 cycles [95 °C for 15 s, 65 °C for 15 s-0.5 C per cycle decrease, 68 °C for 10 s]; Cycling 60 cycles [95 °C for 5 s, 60 °C for 15 s, 72 °C for 15 s]. Primers specific for selected bacterial species and genera were selected from literature and checked using the Primer-BLAST tool[49] and are listed in Supplementary Table 1. The relative bacteria composition was determined for each genera or species by using ΔΔCt method and normalized to the Ct of universal bacteria primer (TotBact-176).

Statistical methods. Data are presented as the mean ± SD (standard deviation) for bacterial data and as the mean ± SEM (standard error of the mean) for animal data. Statistically significant differences in the mean values were evaluated based on data normality analysis using a one-way ANOVA or Kruskal–Wallis test. A t-test was used when only two groups were compared, repeated measures t-test was used when changes over time were compared within one group. If ANOVA or Kruskal–Wallis test provided $P < 0.05$, Tukey's or Dunn's test was performed, respectively, and the differences were considered significant when $P < 0.05$. None of the animal samples were excluded from the analysis. After performing ROUT analysis to identify outliers several samples were excluded from analysis for bacterial composition data in faecal samples and exclusions are indicated in the respective figure legends. The data were analysed using GraphPad Prism statistical software (GraphPad Inc., San Diego, CA, USA).

Ethics approval. The experimental procedures involving animals were performed in accordance with the guidelines of the European Community (Directive 2010/63/EU) and local laws and policies, and all of the procedures were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. Studies involving animals are reported in accordance with the ARRIVE guidelines.

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Author contributions
J.K., M.M.K., E.L., M.D. performed planning of the study. J.K., J.L., M.V. performed in vitro bacterial assays and data analysis. J.K., M.M.K., M.V., E.L. performed in vivo assays and data analysis. S.G., E.S. performed bio-analytical assays and data analysis. M.V and K.V. performed faecal sample assays and data analysis. M.D., J.L., S.G. contributed animals/cells/reagents/materials/analysis tools. J.K. wrote the manuscript with input from M.M.K., J.L., E.L., M.D. All authors have read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to J.K.

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Article

Fasting-Mimicking Diet Reduces Trimethylamine N-Oxide Levels and Improves Serum Biochemical Parameters in Healthy Volunteers

Melita Videja [1,2,*], Eduards Sevostjanovs [1], Sabine Upmale-Engela [1,3], Edgars Liepinsh [1], Ilze Konrade [3,4] and Maija Dambrova [1,2]

[1] Latvian Institute of Organic Synthesis, LV-1006 Riga, Latvia; eduards@osi.lv (E.S.); upsabine@farm.osi.lv (S.U.-E.); ledgars@farm.osi.lv (E.L.); maija.dambrova@farm.osi.lv (M.D.)
[2] Faculty of Pharmacy, Riga Stradiņš University, LV-1007 Riga, Latvia
[3] Department of Endocrinology, Riga East University Hospital, LV-1038 Riga, Latvia; ilze.konrade@rsu.lv
[4] Department of Internal Diseases, Riga Stradiņš University, LV-1007 Riga, Latvia
* Correspondence: melita.videja@farm.osi.lv

Abstract: Elevated plasma levels of trimethylamine N-oxide (TMAO) have been proposed as a diet-derived biomarker of cardiometabolic disease risk. Caloric restriction is the most common dietary intervention used to improve cardiometabolic health; however, novel trends suggest a fasting-mimicking diet (FMD) as a more feasible alternative. FMD is a variation of intermittent fasting, based on caloric restriction and limitation of protein sources of animal origin, applied in daily cycles during a 5-day period. As TMAO is intensively produced by gut microbiota after the consumption of animal-derived products, we aim to investigate whether a 5-day FMD affects plasma TMAO levels and markers of metabolic health. To investigate whether an increase in vegetable intake possesses similar effects on TMAO levels and metabolic parameters, healthy volunteers (n = 24) were subjected to a 5-day FMD and 19 volunteers served as a reference group (VEG). This group of volunteers consumed an additional four servings of vegetables per day, but otherwise stayed on their usual diet. FMD resulted in a twofold decrease in plasma TMAO levels, which was not evident in the volunteers from the VEG group. Moreover, FMD led to a weight loss of 2.8 ± 0.2 kg and a subsequent reduction in BMI compared to baseline. The FMD group exhibited a significant elevation in plasma ketone bodies (14-fold compared to baseline) and a decrease in IGF-1 levels by 37 ± 8 ng/mL. Since fasting glucose and C-peptide levels decreased, all volunteers in the FMD group showed improved insulin sensitivity and a decreased HOMA-IR index. In contrast, in the VEG group, only a slight reduction in plasma levels of fasting glucose and triglycerides was noted. In conclusion, we show that FMD is a viable strategy to reduce plasma levels of TMAO by limiting caloric intake and animal-derived protein consumption. The reduction in the level of TMAO could be an additional benefit of FMD, leading to a reduced risk of cardiometabolic diseases.

Keywords: trimethylamine N-oxide; fasting-mimicking diet; weight loss; insulin sensitivity; cardiometabolic risk

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1. Introduction

Despite the well-known relationship between unhealthy dietary patterns and an increased risk of cardiovascular and metabolic diseases, research continuously tries to identify novel diet-derived culprits that are responsible for the molecular mechanisms that cause the detrimental health effects. One study that attempted to link dietary choices and cardiometabolic health identified that the gut microbiota metabolite trimethylamine N-oxide (TMAO) is associated with a higher incidence of adverse cardiovascular outcomes [1]. In recent years, TMAO concentrations have been extensively studied in various patient populations. Since its initial discovery, strong associations have been reported between systemic TMAO levels and coronary artery atherosclerosis [2–5], which is known to be the leading cause of CVDs. Other possible mechanisms linking TMAO to the pathogenesis of

CVDs include platelet activation, increased probability of thrombosis [6], aggravation of vascular inflammation [7,8] and prolongation of the hypertensive effect of angiotensin II [9], as indicated by preclinical research. Clinical studies, in turn, have added to this knowledge by identifying a TMAO plasma concentration of 6.18 µmol/L as a major adverse cardiovascular event risk threshold [10]. An increase in TMAO plasma levels also correlates with heart failure severity according to the New York Heart Association (NYHA) classification (NYHA II-3.5 ± 0.9; NYHA III-6.0 ± 0.8; NYHA IV-8.1 ± 1.0 µmol/L, respectively) [11] and correlates with the advancement of T2D [12]. Subsequently, T2D patients with elevated plasma levels of TMAO are also more susceptible to major adverse cardiovascular events, such as myocardial infarction, hospitalization for heart failure and unstable angina [13]. In addition, a dose–response meta-analysis revealed that the relative risk for all-cause mortality increased by 7.6% per 10 µmol/L increase in TMAO levels [14].

The initial step of TMAO formation occurs in the intestines, where a variety of gut microbial enzymes metabolize dietary precursors, such as L-carnitine, betaine and choline, to form trimethylamine (TMA). These precursors of TMA are highly abundant in protein sources of animal origin, such as red meat, liver, high-fat dairy products and eggs as well as some legumes [2,10]. Furthermore, TMA is oxidized to TMAO in the liver by the enzyme group flavin-containing monooxygenases (FMOs) [2,15]. Moreover, fish and other seafood contain high concentrations of TMAO; therefore, the consumption of marine products can also increase plasma levels of TMAO [16]. To date, it has been shown that a diet rich in saturated fat modifies the gut microbiota and leads to increased TMAO levels in rodents [17] and humans [18]. Moreover, the adherence to a Western-style diet also results in impaired cardiac function, which can be prevented if TMA formation is inhibited pharmacologically [8]. Thus far, such pharmacological means as antibiotics, metformin, meldonium and structural analogues of choline have been considered as possible TMAO-lowering strategies [19,20]. However, the pharmacological inhibition of TMAO production cannot prevent disturbances in the lipid profile and obesity [8], indicating that, in addition to pharmacological intervention, further lifestyle changes would still be necessary. Therefore, studying dietary approaches targeting the level of TMAO together with other metabolic parameters would be of great significance.

To date, caloric restriction has been the most well-known and widely applied dietary strategy; it has been used to achieve a healthy weight, improve metabolic health and promote longevity in humans [21,22]. However, problems with long-term compliance with caloric restriction have been identified in clinical studies [23–25]. Recently, intermittent fasting has gained scientific interest as a novel dietary regimen with the aim of improving metabolic health [26]. The main strategies of intermittent fasting rely either on restriction of food intake for periods ranging from 16 to 48 h (time-restricted fasting, alternate-day fasting, or 5:2 days cycle) or the reduction in total caloric intake and abstaining from specific macronutrients (fasting-mimicking diet (FMD)) [27]. FMD is a hypocaloric, vegetable-based diet with strictly limited animal protein intake, and it is applied in cycles of 5 subsequent days a month [28]. Recent studies have shown the beneficial effects of FMD on cardiovascular disease (CVD) risk markers, such as BMI, total and trunk body fat, systolic and diastolic blood pressure and insulin resistance [29]. This type of periodic energy restriction imitates the metabolic patterns of prolonged fasting; however, it is easier to comply with and safer than the complete cutback of calories [28].

Although there is some evidence that TMAO levels could be targeted by some types of caloric restriction [30–32], reduced protein intake [33] or diets supplemented with sources of dietary fibers and unsaturated fatty acids [34,35], it has not been thoroughly investigated. As FMD has shown potential in reducing CVD risks, our objective is to investigate whether this short-term reduction in caloric intake by decreasing the consumption of animal-derived proteins, as in the case of FMD, could also serve as an effective dietary strategy to reduce plasma TMAO levels in healthy omnivorous volunteers. As FMD is based on a high vegetable intake, we compared the clinical biochemistry measurements of the volunteers undergoing the cycle of FMD to those who incorporated additional amounts of vegetables

in their usual diet. This was to specify that the effects of FMD are attributed to intermittent energy restriction and the reduction in protein, not the high abundance of vegetables in the diet. As a measure of volunteer compliance, we assessed the levels of plasma ketone bodies and insulin-like growth factor-1 (IGF-1), parameters that are usually affected by prolonged fasting and reduced protein intake.

2. Materials and Methods

2.1. Volunteers

A total of 44 omnivorous volunteers were subjected to an interventional study approved by the local Ethics Committee of Riga Stradiņš University, Latvia (No. 6-2/10/51). Routine biochemistry tests and blood counts were performed to assess the general health of all volunteers prior to joining the study. The exclusion criteria were as follows: BMI < 18.5 kg/m^2; abnormal levels in any of the blood biochemistry measurements that indicate severe health problems; and taking antibiotics, probiotics or dietary supplements containing TMAO precursors within 2 months before the start of dietary interventions. All volunteers were informed about the aim and nature of this study. The recruitment of the volunteers and study procedures were carried out between December 2019 and June 2021.

2.2. Study Design

The schematic design of the study is presented in Figure 1. Baseline anthropometric measurements and biochemical tests were performed in a fasted state before the planned dietary intervention. All participants were instructed to fast ≥ 10 h prior the blood sampling; drinking pure water was allowed during the fasting time. As fish consumption could interfere with the measurement of the TMAO level, volunteers were asked to abstain from sea food consumption for two days prior to sampling. Participants were asked to maintain their usual levels of physical activity throughout the intervention. The research was carried out as a parallel arm study, and the volunteers were assigned to either the reference group (VEG) or fasting-mimicking diet (FMD) group for 5 days. The baseline characteristics of the participants are presented in Table 1. Fasting plasma glucose was chosen as the main parameter for the randomization of the volunteers.

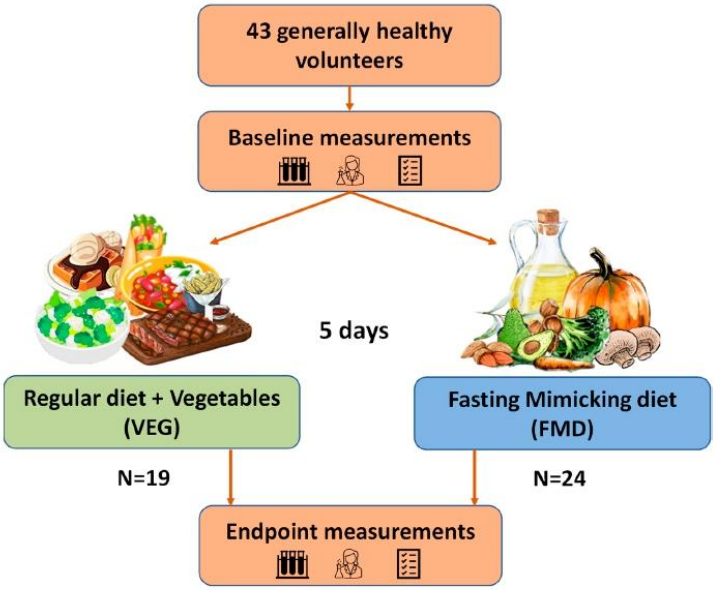

Figure 1. Schematic representation of the study design.

Table 1. Baseline data characterizing bio-anthropometric and biochemical parameters of the study participants.

Baseline Characteristics	VEG (n = 19)	FMD (n = 24)	p-Value
Age, years	37 ± 3	39 ± 2	0.660
Sex, n (%)			
Men	6 (31.6)	9 (37.5)	
Women	13 (68.4)	15 (62.5)	
BMI, kg/m^2	25.2 ± 0.9	28.8 ± 0.9	0.004
Body type (regional fat distribution), n (%)			
Abdominal	8 (42.1)	10 (41.7)	
Gluteofemoral	11 (57.9)	14 (58.3)	
Plasma biochemistry			
Hemoglobin, g/L	144.0 ± 3.5	150.3 ± 6.2	0.350
Glucose, mmol/L	4.99 ± 0.13	4.87 ± 0.11	0.470
HDL cholesterol, mmol/L	1.51 ± 0.07	1.49 ± 0.08	0.841
LDL cholesterol, mmol/L	3.33 ± 0.16	3.37 ± 0.19	0.857
Triglycerides, mmol/L	1.44 ± 0.22	1.30 ± 0.09	0.440
Creatinine, µmol/L	75.7 ± 3.8	75.2 ± 6.6	0.941
eGFR, mL/min/1.73 m^2	86.6 ± 5.9	92.7 ± 6.9	0.527
ALT, U/L	21.5 ± 2.7	24.7 ± 3.4	0.478
Total bilirubin, µmol/L	9.7 ± 1.1	10.3 ± 1.5	0.788
Lipase, U/L	41.6 ± 1.9	37.3 ± 1.8	0.149
ESR, mm/h	2.9 ± 0.9	2.3 ± 0.2	0.641
CRP, mg/L	1.26 ± 0.33	1.24 ± 0.22	0.964
TMAO, µmol/L	3.65 ± 0.68	6.22 ± 1.16	0.083
Physical activity, n (%)			
Low	11 (57.9)	15 (62.5)	
Moderate	6 (31.6)	8 (33.3)	
High	2 (10.5)	1 (4.2)	
Meat consumption, n (%)			
>5 servings per week	10 (52.6)	14 (58.3)	
3–5 servings per week	9 (47.4)	9 (37.5)	
<3 servings per week	0 (0.0)	1 (4.2)	

Data are presented as the mean ± SEM, unless indicated otherwise. ALT, Alanine aminotransferase/Glutamate pyruvate transaminase; BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, Erythrocyte Sedimentation Rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TMAO, trimethylamine N-oxide.

FMD as a dietary regimen was based on the plan developed by the team of Prof. Valter D. Longo [28]. Briefly, participants in the FMD group were subjected to a 5-day hypocaloric diet that provides 34–54% of regular caloric intake (approximately 1100 kcal on the first day and approximately 800 kcal on the four subsequent days). The volunteers in the FMD group were asked to consume primarily complex carbohydrates and unsaturated fat, but to limit protein intake (the caloric intake of these macronutrients was distributed as follows: 40–45%; 45–50%; 10–15%, respectively). The meals in the FMD group mainly

consisted of vegetables, seeds, nuts and vegetable oils. Legumes were allowed only on the first day as they are considered a protein source.

Volunteers in the VEG group were expected to continue their usual dietary regimen, with the exception that they were asked to incorporate 4 servings (each approximately 100–125 g) of vegetables into their diet per day. The sizes of the meals, the caloric intake and the macronutrient content of the diet were otherwise unrestricted.

The volunteers subjected to this interventional study were under careful supervision throughout the study. Detailed information leaflets were prepared and distributed to volunteers, containing all the important information about the dietary intervention to which they were assigned and the list of allowed products together with their nutritional value. Volunteers usually ate two identical meals together (breakfast and lunch), For the evaluation of dinner, a special WhatsApp Messenger group was created, where volunteers shared photos of their meals prepared at home, which was also used as a measure of volunteer compliance.

After 5 days of dietary intervention, volunteers were weighed and blood samples in the fasted state (fasting at least 10 h prior the blood sampling) were taken. One volunteer from the FMD group withdrew from the study due to difficulties adhering to the dietary regimen. Samples previously taken from this volunteer were excluded from further analysis.

2.3. Determination of Biochemical Measurements

Blood sampling was carried out in the fasted state immediately before the start of the dietary intervention and the morning after the 5-day dietary intervention. The samples obtained were stored on ice and delivered to the Limited Liability Company "E. GULBJA LABORATORIJA" (accredited by the Latvian National Accreditation Bureau, accreditation No. M-365) within two hours. The samples were subjected to clinical chemistry analyses. β-Hydroxybutyrate (plasma ketone bodies) was measured using a commercially available enzymatic kit (Biosystems S. A, Barcelona, Spain; Lot 39099) according to the manufacturer's instructions. Briefly, the obtained plasma was 5-fold diluted. The standard curve was generated from 3-Sodium hydroxybutyrate (Alfa Aeser, Ward Hill, MA, USA) and assayed in duplicate. All samples were assayed in duplicate. The enzyme assay is based on oxidation of β-Hydroxybutyrate in the presence of NAD+, to form acetoacetate and NADH. The NADH produced is further involved in an indicator reaction that results in the formation of formazan that can be detected spectrophotometrically [36].

2.4. Measurement of TMAO Levels by UPLC/MS/MS

The concentration of TMAO in plasma samples was determined by ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) using the positive ion electrospray mode described previously [37,38]. In brief, the obtained blood samples were centrifuged at 3000× *g* for 5 min at 4 °C to separate plasma. Plasma samples were collected and stored at −80 °C, until further analysis. The samples were prepared for further analyses by deproteinization with an acetonitrile–methanol mixture (3:1, *v*/*v*), followed by vortexing and centrifugation at 13,000× *g* for 10 min. The supernatant was transferred to UPLC vials and used for UPLC/MS/MS analysis. MassLynx 4.1. software with the QuanLynx 4.1. module (Waters, Milford, PA, USA) was used for data acquisition and processing. A sample of an original data file of TMAO detection using UPLC/MS/MS analysis is available in Supplementary Table S1.

2.5. Data Analysis

The calculation of insulin sensitivity and insulin resistance indices was performed using homeostatic model assessment and HOMA2 Calculator (version 2.2.3, available online, developed by Diabetes Trial Unit, University of Oxford, Oxford, U.K.) [39].

Statistical analysis of the data was performed using GraphPad Prism computer software (GraphPad, Inc., San Diego, CA, USA). The results are reported as the mean ± SEM. Statistical significance between two groups was evaluated using paired Student's *t*-test or

Wilcoxon matched-pairs test, depending on the data distribution, which was determined using Shapiro–Wilk test. Differences were considered significant when the two-sided p value was below 0.05.

3. Results

All recruited volunteers were generally healthy, as baseline biochemistry measurements did not indicate any severe health-related conditions of any of the organ systems. Anthropometric measurements, on the other hand, suggested that the volunteers were slightly overweight with a mean BMI of 27.2 ± 0.7 units. The mean TMAO concentration in plasma was 5.08 ± 0.74 µmol/L at baseline.

The measurement of plasma TMAO levels (Figure 2) revealed that 5 days of the regular diet supplemented with four servings of vegetables per day (VEG) did not result in significant changes in plasma TMAO levels, with a mean increase of 0.43 ± 0.70 µmol/L. In 8 out of 19 volunteers, we observed a reduction in plasma TMAO levels after the dietary intervention; however, 11 volunteers experienced an increase in plasma TMAO levels. In contrast, 75% (18 out of 24) of the volunteers who followed the FMD experienced a notable reduction in plasma TMAO levels. Despite the fact that the interindividual variability of the baseline TMAO levels was high, we observed a strong correlation between the plasma TMAO levels at baseline and the decrease in plasma TMAO levels for those who underwent 5 days of FMD. Moreover, the average plasma level of TMAO in the FMD group at the second visit was 3.01 ± 1.43 µmol/L lower than that at the first visit.

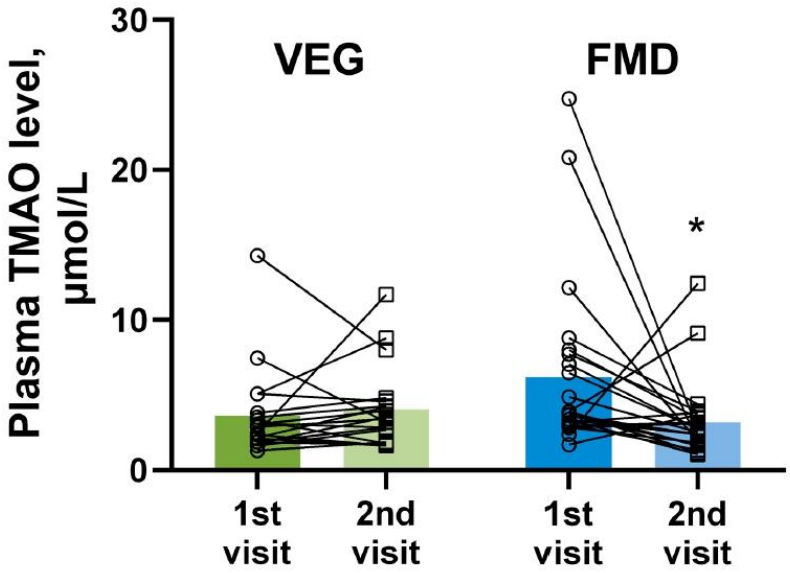

Figure 2. The impact of the 5-day cycle of regular diet supplemented with 4 servings of vegetables (VEG) and fasting-mimicking diet (FMD) on the plasma level of trimethylamine N-oxide (TMAO) in healthy volunteers. The results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

As FMD as a dietary regimen is based on imitating the molecular effects of prolonged fasting, we next evaluated the effects of both diets on plasma ketone body concentrations. As shown in Figure 3A, 5 days of the VEG diet resulted in only a slight increase in plasma ketone body levels from 0.11 ± 0.02 mmol/L to 0.16 ± 0.04 mmol/L. In contrast, the FMD group exhibited a significantly higher increase in plasma ketone body levels by 1.87 ± 0.32 mmol/L

(14-fold elevation compared to baseline measurement). We also observed a significant reduction in plasma insulin-like growth factor-1 (IGF-1) concentrations (Figure 3B) in the FMD group by 37 ± 8 ng/mL, which was not present in the VEG diet group.

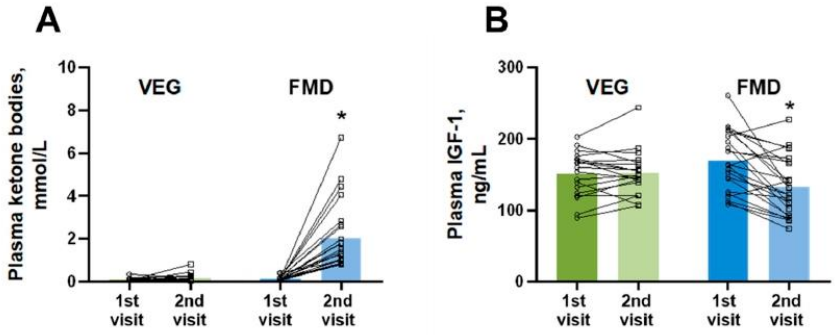

Figure 3. Changes in the levels of plasma ketone bodies (**A**) and insulin-like growth factor-1 (IGF-1) (**B**) induced by 5 days of the regular diet supplemented with additional vegetables (VEG) and fasting-mimicking diet (FMD). The results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

To investigate the contribution of applied dietary strategies to weight loss, the volunteers were weighed before the study and after 5 days of the applicable diet. At baseline, volunteers in the VEG group weighed 78 ± 4 kg. The baseline weight of the volunteers in the FMD group was slightly higher (88 ± 3 kg). Only five volunteers subjected to the VEG diet experienced a slight weight reduction of an average of 0.28 ± 0.15 kg of body weight (Figure 4A). However, each of the volunteers who followed FMD experienced significant weight loss. The average weight loss in the FMD group after 5 days of the dietary intervention was 2.8 ± 0.2 kg of body weight. These changes in body weight resulted in a more pronounced reduction in the body mass index (Figure 4B) in the FMD group (0.90 ± 0.06 units in the FMD group compared to 0.09 ± 0.05 units in the VEG group).

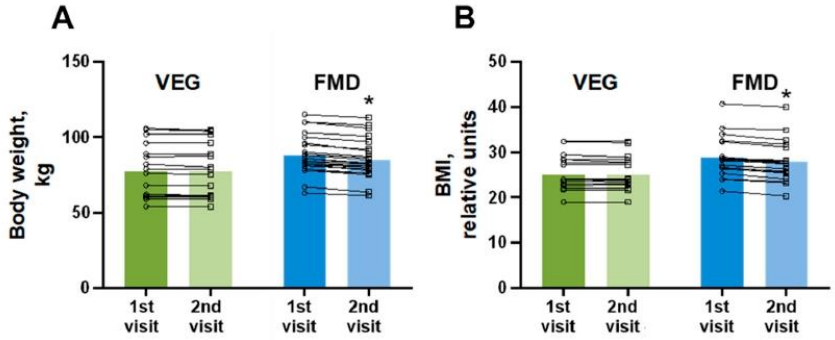

Figure 4. Effects of a 5-day regular diet with additional intake of vegetables (VEG) and fasting-mimicking diet (FMD) on weight (**A**) and BMI (**B**) in healthy volunteers. The results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

Next, we evaluated the effects of a 5-day cycle of the VEG diet and FMD on metabolic parameters. Fasting plasma glucose (Figure 5A) in the VEG group was reduced by 0.22 ± 0.12 mmol/L. Meanwhile, in the FMD group, the lowering of fasting plasma glucose was 2.7 times more pronounced (a decrease of 0.57 ± 0.11 mmol/L). A similar pattern was observed in plasma C-peptide levels (Figure 5B), where the FMD group exhibited a significant reduction in plasma C-peptide compared to the VEG group (a decrease of 0.72 ± 0.11 ng/mL and 0.09 ± 0.11 ng/mL, respectively).

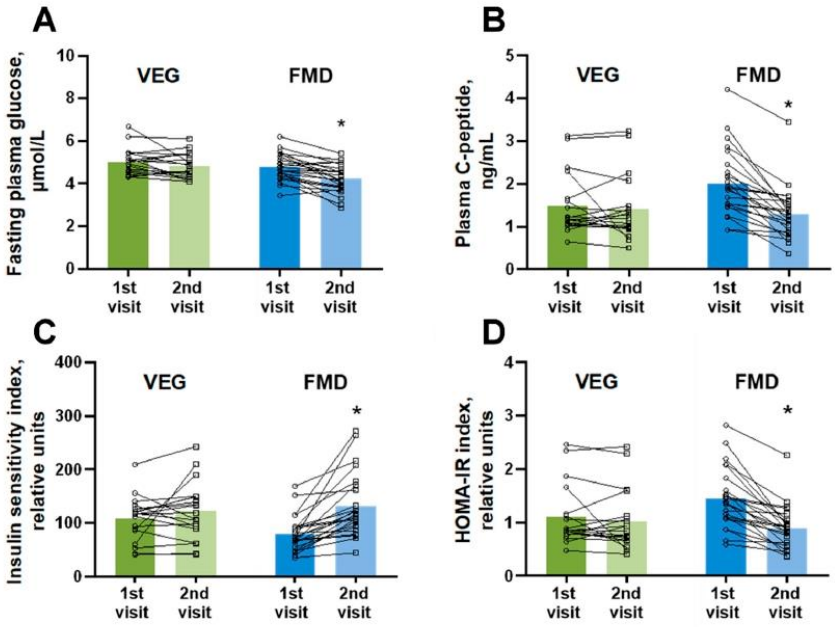

Figure 5. Changes in fasting plasma glucose levels (**A**), plasma C-peptide levels (**B**), insulin sensitivity index (**C**), and HOMA-IR index (**D**) after the 5-day cycle of regular diet supplemented with additional vegetables (VEG) and fasting-mimicking diet (FMD). The results are presented as the mean and independent values of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

Subsequently, volunteers in the FMD group also had an improved insulin sensitivity index (Figure 5C). The increase in insulin sensitivity was 3.8 times greater than that in the VEG group and exceeded the baseline measurement by more than 60%. The benefits of FMD were even more pronounced when we calculated the HOMA-IR index, which defines the extent of insulin resistance (Figure 5D). In the VEG group, we observed a nonsignificant reduction in HOMA-IR by 0.08 ± 0.08 units. In contrast, every volunteer in the FMD group showed a reduced HOMA-IR index, with an average decrease of 0.55 ± 0.08 units.

In the FMD group, we also observed a slight reduction in plasma high-density lipoprotein (HDL) levels after the 5-day dietary intervention. Both diets showed similar effects on plasma triglycerides (a reduction of up to 15%). However, no other significant changes in the plasma lipid profile were evident in any of the experimental groups (Table 2).

Table 2. The effects of a 5-day regular diet supplemented with 4 servings of vegetables (VEG) and fasting-mimicking diet (FMD) on the plasma lipid profile.

	VEG		FMD	
	1st Visit	**2nd Visit**	**1st Visit**	**2nd Visit**
High-density lipoprotein, µmol/L	1.51 ± 0.07	1.51 ± 0.07	1.49 ± 0.08	1.30 ± 0.07
Low-density lipoprotein, µmol/L	3.33 ± 0.16	3.32 ± 0.15	3.38 ± 0.19	3.41 ± 0.20
Triglycerides, µmol/L	1.44 ± 0.22	1.22 ± 0.20 *	1.30 ± 0.09	1.10 ± 0.07 *

The results are presented as the mean ± SEM of 19 volunteers in the VEG group and 24 volunteers in the FMD group. * Indicates a significant difference from the respective group at the 1st visit (Wilcoxon matched-pairs test), $p < 0.05$.

4. Discussion

In the present study, we demonstrate that 5 days of FMD is a viable dietary strategy to reduce plasma levels of TMAO, which is a diet-derived cardiovascular and metabolic disease risk biomarker. Moreover, our data suggest that the reduction in TMAO and improvement in the parameters that characterize glucose metabolism and the general metabolic state in healthy volunteers are attributed to intermittent energy restriction and the limitation of animal-derived protein consumption rather than increased vegetable intake.

The baseline characteristics of the volunteers in our study showed that they were slightly overweight and had plasma levels of TMAO that ranged from low values to extremely high values that are way above the CVD risk threshold (up to 24 µmol/L), indicating the high individual variability of TMAO [40]. Because of this, fasting plasma glucose, as one of the main parameters characterizing metabolic health, was chosen as the key criterion for randomization in our study. Nevertheless, the adherence to FMD resulted in a significant decrease in plasma TMAO levels in 75% of the volunteers. At the endpoint, 22 out of 24 volunteers had plasma TMAO levels below the CVD risk threshold in the FMD group. Moreover, a recent study reported that the benefits of FMD are more pronounced in individuals at risk than in those whose metabolic markers are within the normal range [41], which is in line with our findings. The same applied to TMAO levels, as the most noticeable reduction in TMAO concentrations was also observed in volunteers of the FMD group with higher baseline TMAO plasma concentrations.

In addition to the beneficial effects attributed to FMD, such as a decrease in fasting plasma glucose, reduction in C-peptide concentrations and overall improvement of metabolic health, the volunteers in the FMD group also presented a slight reduction in HDL levels, which may raise concerns about the development of atherosclerosis [42]. However, FMD can be defined as a very low-calorie diet (VLCD), as the caloric intake is ~800 kCal [43] for 4 subsequent days. Although previously reported studies applying VLCD are very heterogeneous, there is some evidence that adherence to VLCD can result in a decrease in HDL levels; however, after the completion of VLCD, HDL levels tend to regain previous levels or even surpass them [44], which was also evident in the pilot data of our study (data not shown). Therefore, based on the cyclic and short-term regimen of FMD, we believe it should not be considered detrimental in terms of the reduction in HDL levels and development of atherosclerosis.

Although the data from observational studies suggest that increased vegetable intake is also inversely associated with biomarkers of metabolic diseases [45,46], these findings are poorly supported by the evidence from interventional studies [47]. Our results also indicate that a short-term increase in vegetable intake, as in the VEG group, may not be sufficient to reduce plasma TMAO levels and provide noticeable benefits with respect to metabolic health, as we only observed a significant reduction in plasma triglyceride levels in the VEG group. Moreover, volunteers in the VEG group were expected to proceed with their usual caloric intake and dietary habits in terms of meat consumption, which has been associated with an increased risk of metabolic syndrome [48,49] and T2D [50]. An alternative to FMD, in terms of limiting the consumption of products of animal origin, would be a vegan diet, which in a recent study displayed promising results and reduced plasma TMAO

levels already a week after switching to a plant-based diet [51]. However, the TMAO concentration returned to the previous level after the reintroduction of the usual diet [51], indicating that a vegan diet should be used as a permanent dietary regimen to sustain TMAO levels within the normal range. This in turn could lead to lowered compliance with the diet [52], a problem previously reported with continuous caloric restriction as well [23–25]. FMD, on the other hand, due to its cyclic nature, is associated with high compliance [53], which we also observed in our study. Overall, our previous and present observations emphasize the importance of reduced animal-derived protein consumption and limited calorie intake to achieve beneficial results, as in the case of FMD.

The main limitation of our study is the short-term nature of the designated dietary interventions (for only a 5-day period) imitating an acute change in diet. However, it has already been reported that such 5-day cycles of FMD could also serve as a long-term strategy if repeated each month [28]. Since our pilot study indicates that, to some extent, the reduction in TMAO levels in plasma can also be observed a week after the completion of the FMD cycle (data not shown), further research should be conducted to assess the durability of the beneficial effects of FMD on TMAO levels in the plasma after returning to the usual diet. As the production of TMA is strictly microbiota-dependent, another limitation is that we were not able to collect samples to assess the impact of FMD on gut microbiota. Some studies state that alterations in microbiota composition that favour TMA-producing bacteria are a possible mechanism by which plasma TMAO levels increase in T2D patients [54,55]. However, recent research shows that some of the typical deviations observed in gut microbiota composition in patients with T2D [56,57] or atherosclerosis [58] can be restored by FMD [59,60], thus possibly lowering TMA production and reducing CVD risks. Overall, these data suggest that the benefits of FMD are not limited to only the exclusion of dietary sources of TMAO [61–63], but could also be explained through the impact on gut microbiota composition. Moreover, it would also be of great interest to investigate the changes in the abundance of specific TMA-producing bacterial genera after following the FMD cycle and upon reintroduction of the usual diet, as it has been shown that some of the beneficial effects on gut microbiota composition occur only after continuation of the usual diet [60].

To conclude, our results show that FMD, a vegetable-based, low-calorie variation of intermittent fasting with a strict exclusion of animal-derived protein sources, is an efficient strategy to reduce plasma TMAO levels. Our results add a novel component to the interaction of FMD and the metabolic state of a person, suggesting that TMAO reduction should be considered one of the noteworthy benefits of FMD with respect to improving metabolic health. However, further research is needed to assess the potential of compliance to FMD and the effects on TMAO levels after the completion of several cycles of the diet, as well as upon the reintroduction of the regular diet.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14051093/s1, Table S1: The original data sample of TMAO detection using UPLC/MS/MS analysis.

Author Contributions: Conceptualization, M.V., I.K. and M.D.; methodology, M.V. and I.K.; formal analysis, M.V. and E.S.; investigation, M.V.; resources, I.K. and M.D.; data curation, M.V. and S.U.-E.; writing—original draft preparation, M.V.; writing—review and editing, E.L., I.K. and M.D.; visualization, M.V.; supervision, E.L., I.K. and M.D.; project administration, M.D.; funding acquisition, M.V. and M.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the local Ethics Committee of Riga Stradiņš University, Latvia (No. 6-2/10/51, 28 November 2019.).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
3.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
4.	Profesore Regīna Kleina	Dr.med.	patalogs
5.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
6.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
7.	Docente Iveta Jankovska	Dr.med.	ortodonts
8.	Docents Kristaps Circenis	Dr.med.	docētājs

Pieteikuma iesniedzējs/i: **Edžus Beinārs**
Medicīnas fakultāte

Pētījuma / pētnieciskā darba nosaukums: "Piecas dienas badošanos imitējošās diētas ("fasting mimicking diet,FMD") protokola ietekme uz indivīdu ar virssvaru metabolajiem rādītājiem"

Iesniegšanas datums: 27.11.2019.

Pētījuma protokols:
Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot klīnisku pētījumu (asins paraugu ņemšanu un izdarot analīzes), veicot ķermeņa mērījumu un pielietojot īpašu diētu, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (dalībnieku) informēta brīvprātīga piekrišana piedalīties, personu iegūto datu apstrāde, to pielietošana, glabāšana, aizsardzība, anonimitāte un konfidencialitāte ir nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. theol., prof.

Paraksts

RĪGAS STRADIŅA UNIVERSITĀTE ĒTIKAS KOMITEJA

I.Bēniņa
67061596