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RĪGA STRADIŅŠ UNIVERSITY

Melita Ozola (Vidējā)

# Regulation of Trimethylamine N-oxide in Treatment of Cardiometabolic Diseases

Summary of the 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

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The Doctoral Thesis was developed at Latvian Institute of Organic Synthesis, Laboratory of Pharmaceutical Pharmacology

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## Abbreviations used in the Thesis

| | |
|---|---|
| Ach | acetylcholine |
| ADP | adenosine diphosphate |
| AmA | antimycin A |
| ANOVA | analysis of variance |
| AS | atherosclerosis |
| cDNA | complementary deoxyribonucleic acid |
| CKD | chronic kidney disease |
| CVD | cardiovascular disease |
| F | fatty acid oxidation-dependent pathway |
| FAO | fatty acid oxidation |
| FMD | fasting mimicking diet |
| FMOs | flavin-containing monooxygenases |
| HOMA-IR | Homeostatic Model Assessment for Insulin Resistance |
| K-H | Krebs-Henseleit |
| *K. pneumoniae* | *Klebsiella pneumoniae* |
| LEAK | substrate-dependent state |
| LVDP | left ventricular developed pressure |
| MACE | major adverse cardiovascular events |
| MCT | monocrotaline |
| MetS | metabolic syndrome |
| N | NADH pathway |
| NADH | reduced nicotinamide adenine dinucleotide |
| NYHA | New York Heart Association |
| OXPHOS | oxidative phosphorylation |
| P | pyruvate |
| *P. mirabilis* | *Proteus mirabilis* |

| | |
|---|---|
| PC | palmitoylcarnitine |
| 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 |
| 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), 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. This leads to the production of trimethylamine (TMA), which is further oxidised to TMAO in the host liver.

In preclinical studies, TMAO was 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.

## Tasks 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 almost all studies initially reported a 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 increases 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 the 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 fasting mimicking diet can help reduce cardiovascular risks by improving metabolic parameters and decreasing circulating TMAO levels.

# 1 Methods

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

### 1.1.1 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). Isoflurane was purchased from Chemical Point (Deisenhofen, Germany). All other reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany).

### 1.1.2 Animals and treatment

Thirty male Wistar rats were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). 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 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.

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.

### 1.1.3 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.

### 1.1.4 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 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 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).

Mesenteric artery rings were prepared as described previously (Bridges et al., 2011). 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.

### 1.1.5 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 ADInstruments. 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 %.

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

### 1.2.1 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). 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.

### 1.2.2 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 (MCT)-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 1.1. The experimental animals were randomly separated into four groups: control (n = 10), TMAO (n = 10), MCT (n = 10) and TMAO + MCT (n = 10). The animals in the TMAO group and TMAO + MCT group received TMAO 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 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. 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 RV functionality and invasive direct RV pressure measurement based on our pilot experiments in this model. 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 permeabilised cardiac fibres of the right ventricle.

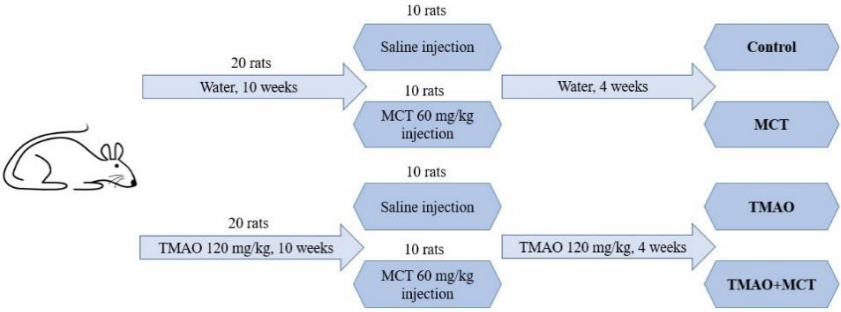


Figure 1.1 **Schematic representation of the study design**

### 1.2.3 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.

After the echocardiography assessment, 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.

### 1.2.4 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).

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

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

### 1.2.5 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), synthesized cDNA, and forward and reverse primers specific for *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 1.1.

Table 1.1

**Primer sequences of qPCR primers**

| Gene symbol | Full name | Primer sequence (5'–> 3') |
|---|---|---|
| *VCP* | Valosin-containing protein | F- AATATTTGACAAGGCACGACAAG |
| | | R- CCGGTTGGTAGCTCCAATGAT |
| *BNP* | Natriuretic peptide type B | F- TAGCCAGTCTCCAGAGCAATTC |
| | | R- TTGGTCCTTCAAGAGCTGTCTC |
| *αMHC* | Myosin heavy chain 6 | F- CTCCATCTCTGACAACGCCTATC |
| | | R- CTCCGGATTCTCCAGTGATGA |
| *βMHC* | Myosin heavy chain 7 | F- GGAGCTGATGCACCTGTAGACA |
| | | R- AGTGCGGACACGGTCTGAA |

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

### 1.3.1 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) were obtained from Envigo (Harlan Laboratories BV), Venray, Netherlands. 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 1.2 A). Plasma samples were collected after 4 and 8 weeks of treatment.

Another 30 db/db male mice and 10 age-matched non-diabetic db/Lean male mice (10 weeks old) were obtained from Envigo for the follow-up experiment based on data obtained from the first study. Here the aim was to test the effects of metformin in case of increased tertiary amine load; choline 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 (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 1.2 B). At the end of the treatment 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. The samples were stored at -80 ºC for future analysis.

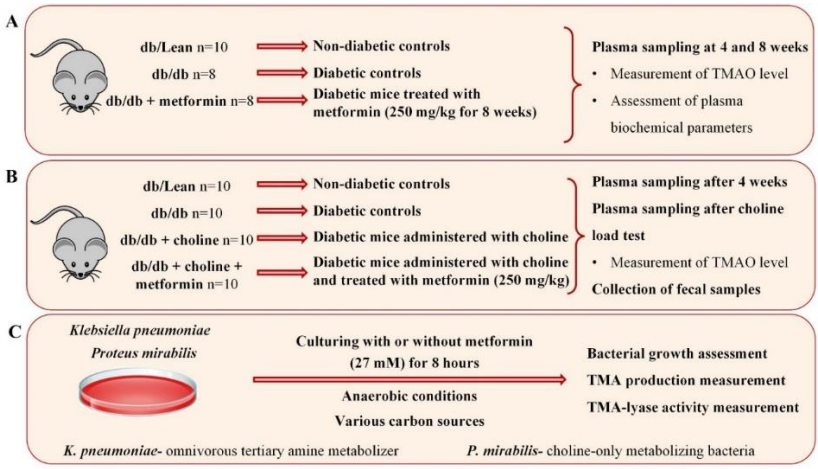


Figure 1.2 **Schematic representation of the study design**

### 1.3.2 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). To test the effect of metformin on choline-dependent TMA production, bacteria were grown under standard conditions with 27 mM choline as the carbon source, with or without 27 mM metformin addition (Figure 1.2 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). 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 (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, liquid chromatography bottles were top-filled with 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.

## 1.4 Fasting mimicking diet as a lifestyle intervention to target TMAO levels

### 1.4.1 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 < 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.

### 1.4.2 Study design

The schematic design of the study is presented in Figure 1.3. 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. 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. Fasting plasma glucose was selected as the main parameter for randomization of the volunteers.

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.

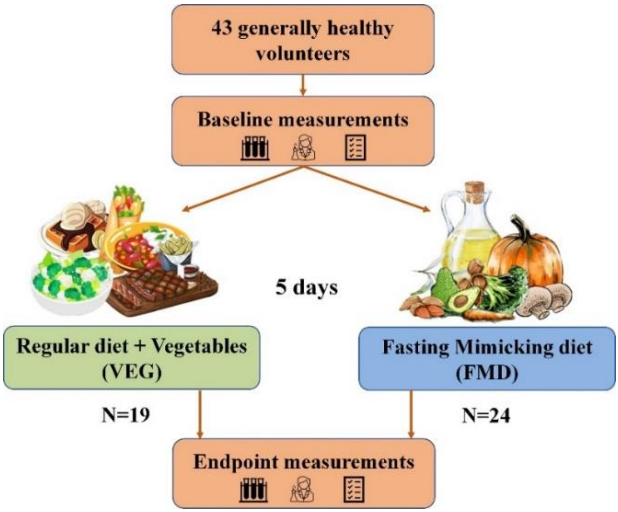


Figure 1.3 **Schematic representation of the study design**

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.

After 5 days of dietary intervention, volunteers were weighed, and blood samples in the fasted state were taken.

### 1.4.3 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.

## 1.5 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 ultra-performance liquid chromatography-tandem mass spectrometry (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).

## 1.6 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. 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. The *ex vivo* experiments described in Publication I were performed in compliance with ethical approval No. 82. For Publication II, experiments were performed according to ethical approval. No. 105. The experimental procedures from Publication III were performed complying with ethical approval No. 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 Riga Stradiņš University, Latvia (No. 6-2/10/51).

# 2 Statistical Analysis

The statistical analysis of the data was performed using GraphPad Prism statistical software (GraphPad, Inc., La Jolla, USA). The 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). For Publication IV, the data are represented as mean value with individual data points for each volunteer. 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.

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).

# 3 Results

## 3.1 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 3.1). 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).

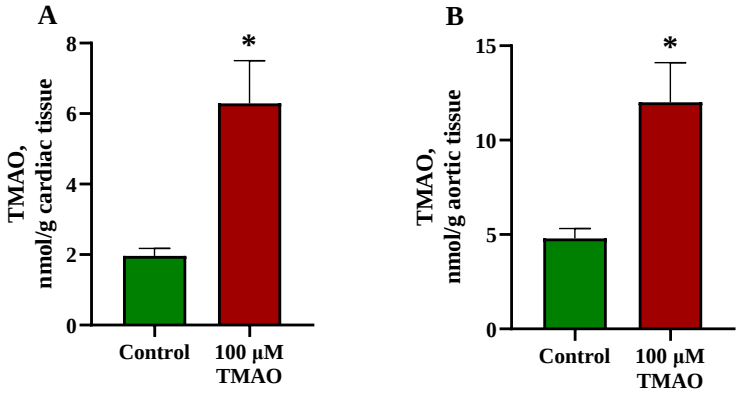


Figure 3.1 **TMAO content in the heart (A) after 1 hour of perfusion and in aortic tissues (B) after 1 hour of incubation in Krebs-Henseleit buffer solution with or without the addition of 100 μM TMAO**

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

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 3.2).

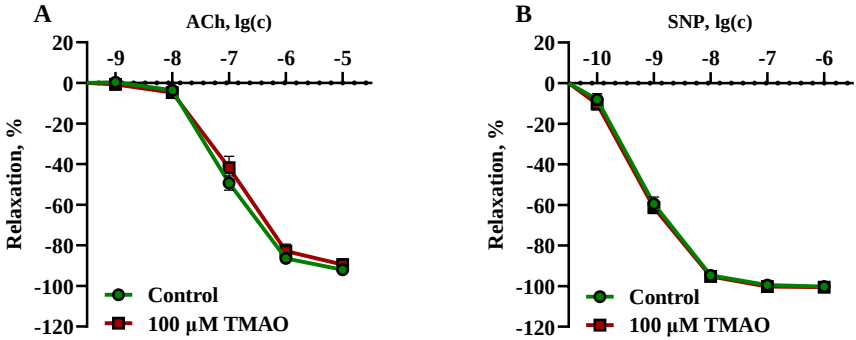


Figure 3.2 **Effects of 100 µM TMAO on endothelium-dependent (A) and endothelium-independent (B) relaxation in aortic rings**

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 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.

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 3.1). 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.

After this, the size of myocardial infarction was compared in both groups. 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 (Table 3.1) was nearly identical (~40 % of the risk zone) in both groups.

Table 3.1

**Effects of 1 mM TMAO on cardiac function and infarct size 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 |
| Infarct size, % of area at risk | 37.5 ± 3.0 | 41.2 ± 1.6 |

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

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

### 3.2.1 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 3.3A). 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 3.3B).

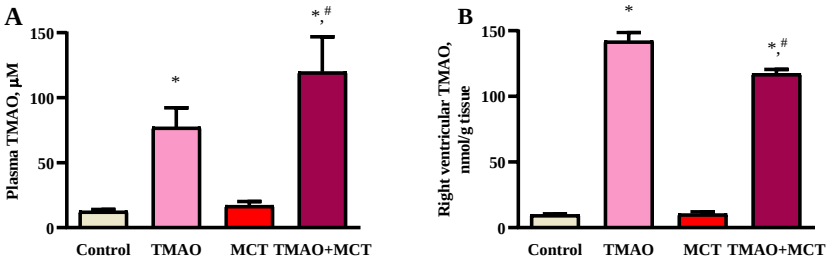


Figure 3.3 **TMAO concentration in plasma (A) and right ventricular tissue (B) after administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks**

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 3.2). Compared with the control, administration of monocrotaline induced a significant increase (approximately 50 %) in direct RV pressure (Table 3.2). 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 3.2

**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 ± 2.0 | 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 | 0.50 ± 0.07 | 0.40 ± 0.04 |
| Right ventricular systolic area, $cm^2$ | 0.20 ± 0.01 | 0.21 ± 0.02 | 0.36 ± 0.07 * | 0.26 ± 0.03 |
| Right ventricular fractional area change, % | 46.6 ± 2.6 | 37.0 ± 2.8 | 29.7 ± 4.8 * | 37.0 ± 5.0 |

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 3.4AB). In the MCT group, a 3-fold decrease in the *α/β-MHC* expression ratio (Figure 3.4A) 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 3.4B). 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 3.4A). The gene expression of BNP was lower in the TMAO + MCT group than in the MCT group (Figure 3.4B).

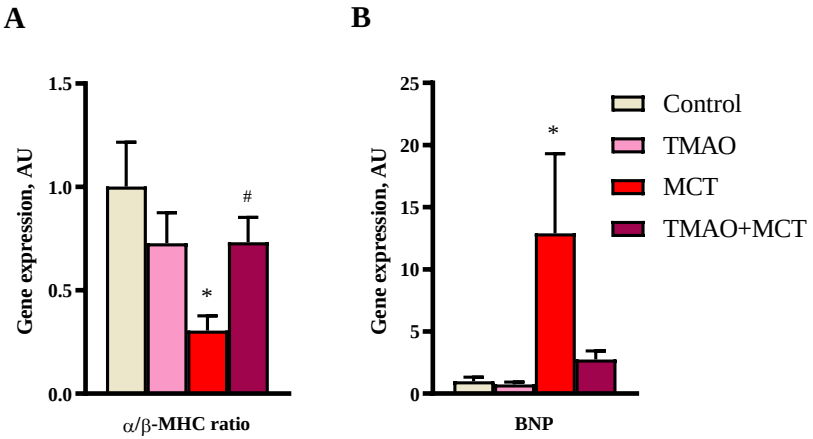


Figure 3.4 **Effect of administration of TMAO at a dose of 120 mg/kg in the drinking water for 14 weeks on heart failure severity-related gene expression in right ventricular tissue**

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, Kruskall-Wallis test followed by Dunn's multiple comparison test for panel B, # indicates a significant difference from the MCT group (unpaired t-test), $p < 0.05$.

### 3.2.2 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 3.5A), resulting in an 11 % decrease in the FAO-dependent OXPHOS coupling efficiency (Figure 3.5B). Although pyruvate metabolism input to overall respiration was increased by approximately 44 % in the TMAO group, as indicated by Flux control factor analysis (Figure 3.5B), it was not sufficient to restore FN and FNS pathway-linked mitochondrial respiration in the OXPHOS state (Figure 3.5A). In the MCT group, there was a 75 % decrease in the FAO-dependent respiration rate in the OXPHOS state

(Figure 3.5A) and a subsequent 13 % decrease in the FAO-dependent OXPHOS coupling efficiency (Figure 3.5B). Similar to the TMAO group, in the MCT group, pyruvate metabolism input to respiration was increased by 50 % (Figure 3.5B), but this increase was not sufficient to restore FN- and FNS-pathway-linked respiration in the OXPHOS state (Figure 3.5A). 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 3.5B). Moreover, in the TMAO + MCT group, mitochondrial energy metabolism was preserved, as shown by normalised respiration rates (Figure 3.5A), preserved FAO-dependent oxidative phosphorylation efficiency and subsequently decreased pyruvate metabolism input (Figure 3.5B).

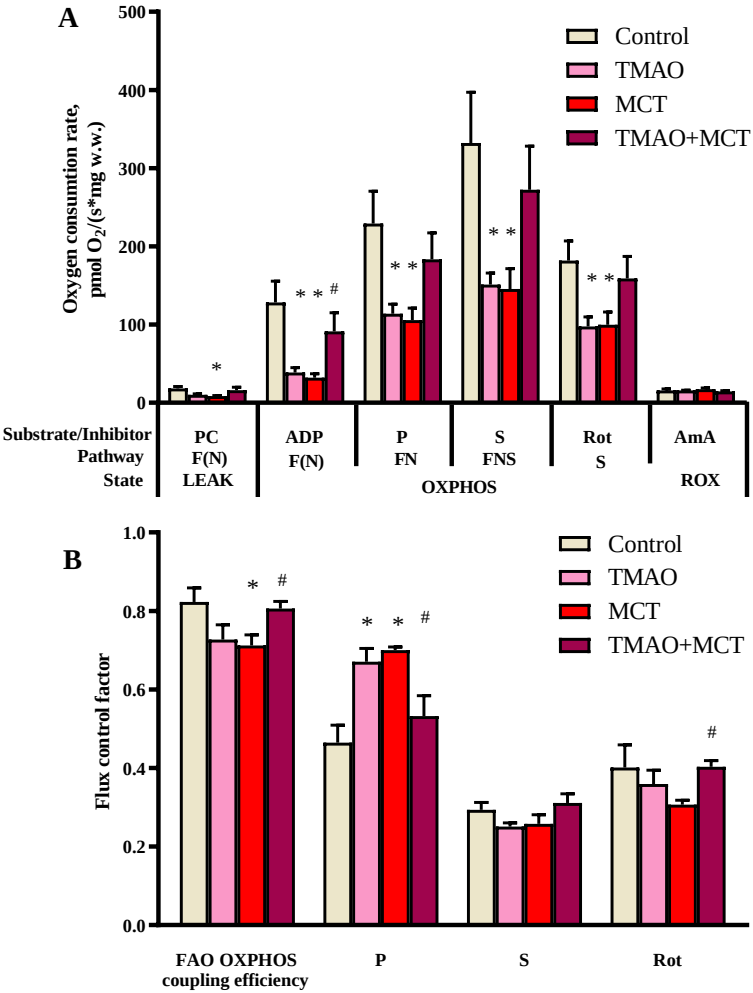


Figure 3.5 **Mitochondrial respiration rate measurements (A) and flux control factors (B) in right ventricular cardiac fibres after administration of TMAO at in a model of monocrotaline-induced right ventricle heart failure**

Results are presented as the mean ± SEM of 6 animals. * significantly different from the control group (one-way ANOVA followed by Dunnett's posttest), # significantly different from the MCT group (unpaired t-test), $p < 0.05$.

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.

## 3.3 Metformin reduces TMAO levels in experimental model of type 2 diabetes

### 3.3.1 TMAO production in db/db mice on normal chow or chow supplemented with choline

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 3.6A).

In db/db animals, glucose and insulin plasma concentrations were significantly increased in both fed and fasted states, and metformin had no effect in any of these states (data not shown). 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.

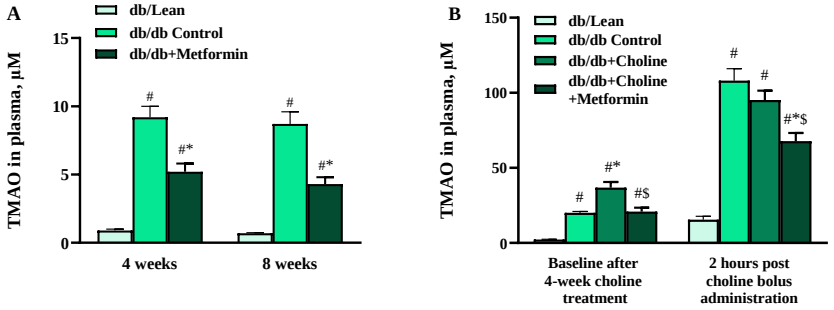


Figure 3.6 **Effects of metformin (250 mg/kg) treatment on plasma TMAO concentrations (A) at 4- and 8-week time points in db/db mice fed a standard laboratory diet. Effects of 4 weeks of metformin treatment on plasma TMAO levels in mice supplemented with choline (0.5 % with drinking water) and after acute choline load (100 mg/kg, bolus) (B).**

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 for panel A. Results are the mean of 10 animals ± SEM for panel B. * 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; $p < 0.05$).

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 3.6B) 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 3.6B).

### 3.3.2 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*. *K. pneumoniae* was chosen because it can produce TMA from all the main precursors – choline (via CutC/D (choline-TMA-lyase complex)) and carnitine/gamma butyrobetaine (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 3.7A), while bacterial growth of *K. pneumoniae* was not affected (Figure 3.7C) 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 3.7B). Unlike with *K. pneumoniae*, metformin significantly decreased *P. mirabilis* bacterial biomass growth when choline was used as the sole carbon source (Figure 3.7D). The effect was present, albeit less pronounced when *P. mirabilis* was grown in media with glucose as the sole carbon source.

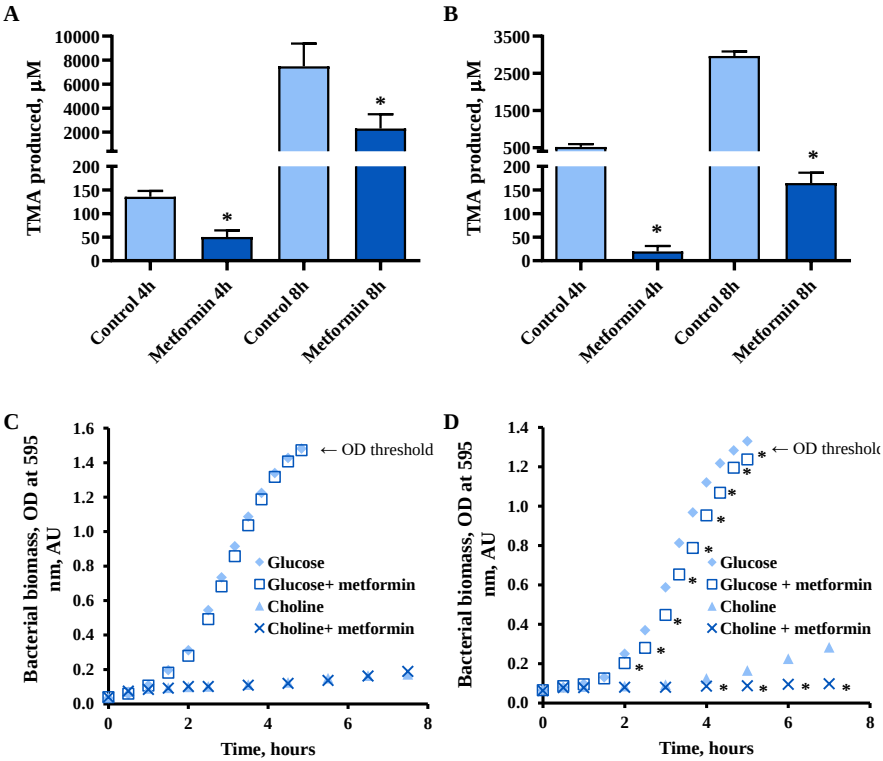


Figure 3.7 **Metformin (27 mM) effects on TMA production from choline (A, B) and bacterial growth (C, D) in *K. pneumoniae* (A, C) and *P. mirabilis* (B, D) under anaerobic conditions**

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).

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.

## 3.4 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. The mean TMAO concentration in plasma was $5.08 \pm 0.74$ μmol/L at baseline. 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 3.8), with a mean increase of $0.43 \pm 0.70$ 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 2$^{nd}$ visit was by $3.01 \pm 1.43$ μmol/L lower than that at the 1$^{st}$ visit.

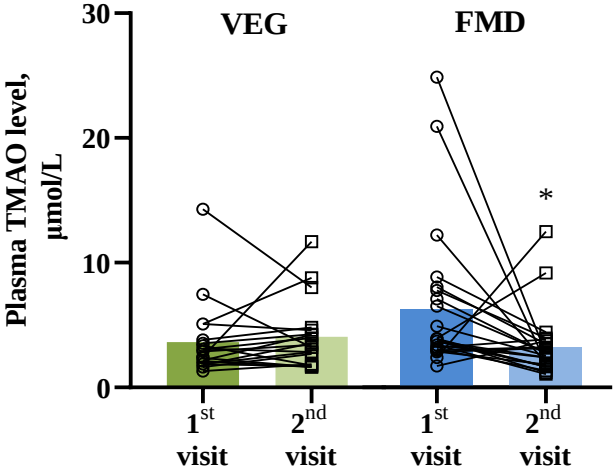


Figure 3.8 **The impact of the 5-day cycle of regular diet supplemented with 4 servings of vegetables (VEG) and fasting mimicking diet (FMD) on the plasma level of TMAO**

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), $p < 0.05$.

Next, we evaluated the effects of a 5-day cycle of the VEG diet and FMD on metabolic parameters. Fasting plasma glucose (Figure 3.9A) 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 3.9B), 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 3.9C). 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 3.9D). In the VEG group, we observed a nonsignificant reduction in HOMA-IR by $0.08 \pm 0.08$ units. In contrast, every volunteer in the FMD group showed a reduced HOMA-IR index, with an average decrease of $0.55 \pm 0.08$ units.

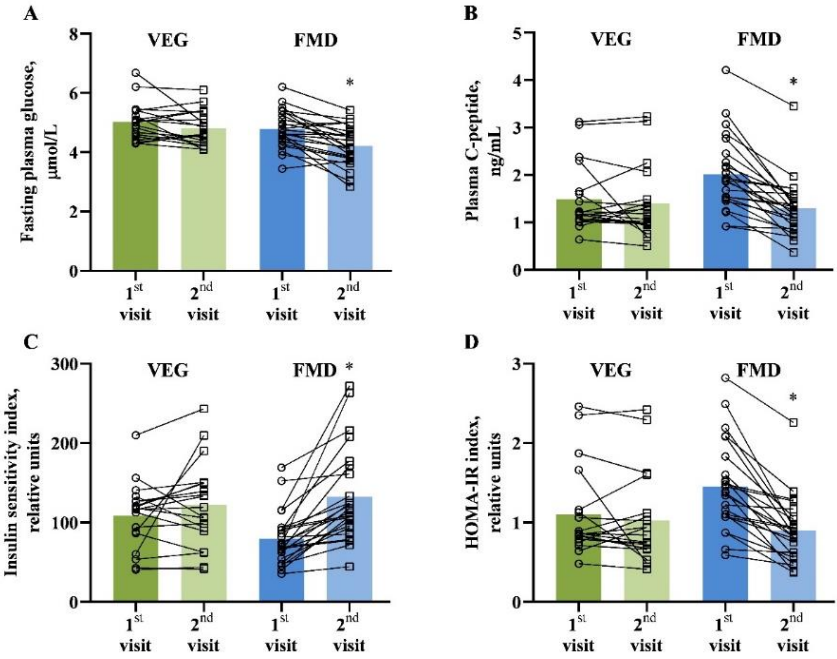


Figure 3.9 **Changes in fasting plasma glucose levels (A), plasma C-peptide levels (B), insulin sensitivity index (C), and HOMA-IR index (D) after the 5-day cycle of regular diet supplemented with additional vegetables (VEG) and fasting mimicking diet (FMD)**

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), $p < 0.05$.

# 4 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, 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 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 dataset 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. In contrast, the second hypothesis was confirmed, as we demonstrated a reduction in circulating TMAO levels by metformin in an animal model of advanced T2D. Moreover, we showed that a 5-day FMD serves as a viable lifestyle strategy to reduce TMAO levels and improve overall metabolic health in generally healthy volunteers.

## 4.1 Increased TMAO bioavailability in experimental models of cardiovascular and metabolic diseases

The data on the role of TMAO in the 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, preclinical studies support the idea of the involvement of TMAO in the 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 a lack of any TMAO-related effects linked to the 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 have 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 a primary cause of cardiac damage, as we did not observe any effects of TMAO on cardiac function either at baseline or during ischaemia–reperfusion in a rat isolated heart model (Table 3.1).

Of course, we cannot assume that the response of all cell types and organ systems to such high TMAO doses would be the same, and the 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 the 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 the development of AS, as deleterious effects of endothelial cells are one of the instigating mechanisms in the 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 3.2), 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 administered to rats for 14 weeks in the drinking water, indicate that the TMAO level in the cardiac tissue can reach up to 140 nmol/g tissue (Figure 3.3). Our results demonstrate that such a 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 3.2). 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 3.5). 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 the case of 14 weeks of TMAO treatment (Figure 3.5). 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 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 an increase in the 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 a metabolic shift was observed. After long-term TMAO administration, FAO was decreased, and pyruvate metabolism was subsequently increased (Figure 3.5) without changes in cardiac functionality (Table 3.2). 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. This ensures preserved energy metabolism and subsequently improves cardiac function recovery after injury. However, such protective effects of TMAO were not observed after a short-term elevation in TMAO levels in isolated rat heart experiments (Table 3.1). 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, utilizing both of the strategies mentioned above.

In conclusion, the results presented here provide evidence that acute elevation of the TMAO level cannot be considered 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.

## 4.2 Targeting the diet-microbiota-TMAO axis using metformin

Knowledge on the mechanism of the antidiabetic 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 the inhibition of mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014), the interactions 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.

Previously, it was shown that 12-week-old db/db mice have a significantly elevated TMAO level up to 9 μM (Dambrova et al., 2016). 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 3.6AB). Treatment with metformin decreased TMAO levels approximately twofold 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 concentrations almost 1.8-fold (Figure 3.6B); however, TMAO plasma levels remained higher than the risk threshold.

Our findings strongly imply that the acute effects of metformin result in overall decrease in TMAO availability through decreased bacterial TMA production (Figure 3.7). The current results prove that metformin inhibits microbial TMA production from choline (Figure 4.1); metformin is unable to decrease TMA production in *K. pneumoniae* when carnitine is used instead of choline as the sole substrate (unpublished observations). Interestingly, metformin had no effect on the anaerobic growth of *K. pneumoniae* (omnivorous tertiary amine metaboliser) but significantly delayed (bacteriostatic effect) the growth of *P. mirabilis* (choline-only metabolizing bacteria) under the same experimental conditions (Figure 3.7), indicating at least two different mechanisms of metformin on gut microbiota. 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, such as co-factor recycling in these microcompartments but not choline-TMA-lyase itself are affected by metformin resulting in overall inhibition of TMA production. Despite this, further studies are required to show how *in vitro* findings and data from the db/db mouse model translate to the 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.

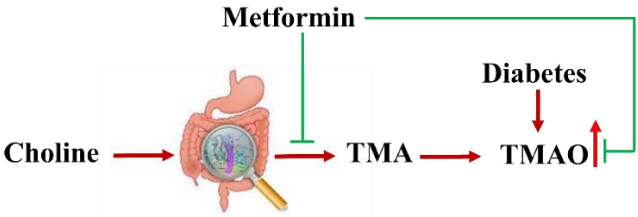


Figure 4.1 **The ability of metformin to reduce the elevated TMAO levels in the T2D model**

To conclude, we present evidence indicating that metformin can induce significant changes in TMAO levels likely due to direct nonlethal inhibition of bacterial TMA production. Moreover, these effects are independent of glucose and insulin plasma concentrations and could be an additional mechanism underlying 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 the patient population is warranted. Moreover, dietary changes would likely be required to reach clinically relevant endpoints.

## 4.3 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 the regulation of TMAO levels 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 characterizing 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 some of the volunteers had extremely high plasma levels of TMAO (Figure 3.8), which were much higher than the CVD risk threshold (up to 24 μmol/L). However, adherence to FMD resulted in a significant decrease in plasma TMAO levels in 75 % of volunteers (Figure 3.8). 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, 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 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 3.8, 3.9). 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. In a recent study, a vegan diet displayed promising results and reduced plasma TMAO levels 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. Another dietary intervention that relies on plant-derived products along with moderate consumption of fish and poultry is the Mediterranean diet. This diet 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 the regulation of TMAO levels is summarised in Figure 4.2. Overall, previous and present observations emphasize 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.

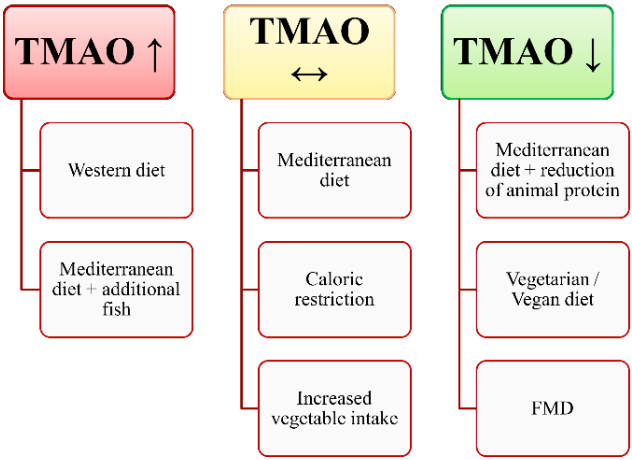


Figure 4.2 **The effects of dietary interventions on circulating TMAO levels in humans**

The main limitation of our dietary intervention study is the short-term nature of the interventions (for only a 5-day period), which imitates 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, 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.

## 4.4 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 contradictory. 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 4.3). The average plasma TMAO level of a healthy person is approximately 3 μM, with some slight deviations because of diet preferences (Wu et al., 2019), age (Li et al., 2018) and sex (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 the Japanese population. In addition, the type of fish or seafood matters, as the increase in TMAO levels differs significantly after consumption of various fish species and products (Wang et al., 2022). 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 approximately 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 emphasizes 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 approximately 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 the 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 approach not only adds crucial insights but also helps to develop a valid dose selection strategy for future investigations.

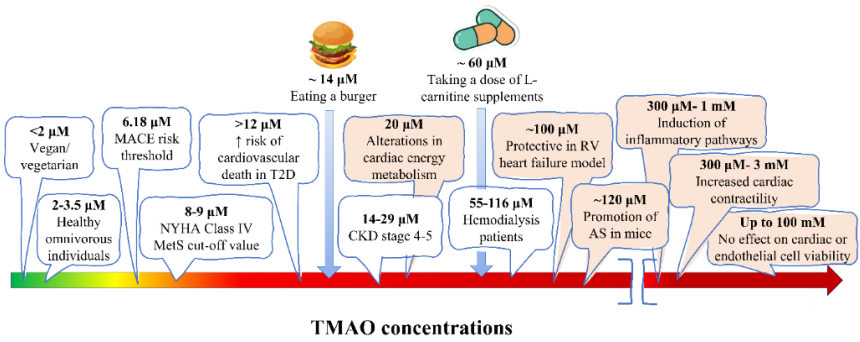


Figure 4.3 **Differential effects observed due to increased TMAO concentrations in preclinical (beige) and clinical (white) settings**

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 with TMAO precursors, which requires mandatory gut microbiota metabolism (Tang et al., 2015; 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; 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 investigations 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 the protective effects of TMAO in some experimental models, many unanswered questions remain: what is the role of TMAO in CVD development and do we truly need to lower our TMAO levels? 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 considered unhealthy due to several crucial factors (Martinez, Leone, and Chang, 2017; Clemente-Suárez et al., 2023). Second, to date, there is no solid evidence that low TMAO values are 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 should be noted that many of the observed protective effects are demonstrated in preclinical animal models, which may not always accurately represent the disease progression 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, a more neutral role can be attributed to TMAO as well. The opinion on the role of the intestinal microbiota in the pathogenesis of noncommunicable diseases has become 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 the case of the kidneys. Moreover, the complex interplay between the 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 with TMAO in an experimental model of right ventricular heart failure exerts preconditioning-like effects by preserving cardiac mitochondrial energy metabolism.
3. Metformin lowers elevated TMAO levels in an 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 on the pathogenesis of cardiovascular and metabolic diseases depend on the disease model or organ system studied, the composition of the 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 in the clinical setting, to ensure the validity of TMAO as a biomarker. 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.

# List of publications, reports and patents on the topic of the Thesis

## Publications

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

2. **Videja M**, Vilskersts R, Korzh S, Cirule H, Sevostjanovs E, Dambrova M, Makrecka-Kuka M. **2021.** Microbiota-Derived Metabolite Trimethylamine N-Oxide Protects Mitochondrial Energy Metabolism and Cardiac Functionality in a Rat Model of Right Ventricle Heart Failure. Frontiers in Cell and Developmental Biology; 8:622741. doi: 10.3389/fcell.2020.622741

3. Kuka J, **Videja M**, Makrecka-Kuka M, Liepins J, Grinberga S, Sevostjanovs E, Vilks K, Liepinsh E, Dambrova M. **2020.** Metformin decreases bacterial trimethylamine production and trimethylamine N-oxide levels in db/db mice. Scientific Reports; 10(1):14555. doi: 10.1038/s41598-020-71470-4

4. **Videja M**, Sevostjanovs E, Upmale-Engela S, Liepinsh E, Konrade I, Dambrova M. **2022.** Fasting-Mimicking Diet Reduces Trimethylamine N-Oxide Levels and Improves Serum Biochemical Parameters in Healthy Volunteers. Nutrients; 14(5):1093. doi: 10.3390/nu14051093

## Reports and theses at international congresses and conferences

1. **Ozola (Videja) M**, Dambrova M. Trimethylamine N-oxide: Cardiometabolic Disease Risk Factor or Just an Osmolyte? Rīga Stradiņš University Research Week (RSU RW 2023). Knowledge for Use in Practice. 29–31 March 2023, Rīga Stradiņš University, Riga, Latvia

2. **Videja M**, Makrecka-Kuka M, Korzh S, Sevostjanovs E, Cirule H, Liepinsh E, Dambrova M. Accumulation of trimethylamine N-oxide in renal tissue induces mitochondrial dysfunction in insulin-resistant mice. 58$^{th}$ EASD Annual Meeting of the European Association for the Study of Diabetes. Stockholm, Sweden, September 19–23, 2022. In: doi: 10.1007/s00125-022-05755-w

3. Petersone G, **Videja M**. Trimethylamine N-oxide vastly accumulates in renal tissue without affecting kidney function in insulin-resistant mice. RSU International student conference 2022, Rīga Stradiņš university, March 24-25, 2022.

4. **Videja M**, Kuka J, Makrecka-Kuka M, Liepins J, Grinberga S, Sevostjanovs E, Vilks K, Liepinsh E, Dambrova M. Metformin decreases the plasma concentration of pro-atherogenic metabolite trimethylamine N-oxide in an experimental model of type 2 diabetes. Research Week (RW2021). Knowledge for Use in Practice. 24–26 March 2021, Rīga Stradiņš university, Riga, Latvia.

5. Ambarova R, **Videja M**. Pro-atherogenic metabolite trimethylamine N-oxide does not affect bone marrow-derived macrophage polarization. Riga Stradiņš University International Student Conference in "Health and Social Sciences" 2021, 22nd–23rd March, 2021, Rīga Stradinš University, Dzirciema Street 16, Riga, Latvia.

6. **Videja M**, Kuka J, Makrecka-Kuka M, Liepins J, Grinberga S, Liepinsh E, Dambrova M. Metformin decreases bacterial trimethylamine production and plasma trimethylamine N-oxide concentration in experimental model of type 2 diabetes. 8th European Virtual Congress of Pharmacology (EPHAR 2021). December 6–8, 2021.

7. Cernihovica A, Supervisors: Dambrova M, Vilskersts R, **Videja M**, Vilks K. Acute effects of TMAO on vascular energy metabolism and reactivity. RSU International Students Conference 2020. Abstract book p.60. Rīga Stradinš University, Riga, Latvia. March 27–28, 2020.

8. **Videja M**, Vilskersts R, Korzh S, Cirule H, Sevostjanovs E, Dambrova M, Makrecka-Kuka M. Trimethylamine N-oxide preserves mitochondrial energy metabolism in rat model of right ventricle heart failure. 4th Annual UCLA Mitochondria Symposium – Los Angeles, CA. Online. November 5–6, 2020.

9. **Videja M**, Sevostjanovs E, Konrade I, Dambrova M. 5-day intermittent fasting reduces proatherogenic metabolite trimethylamine N-oxide level and improves serum biochemical parameters. 3rd International Conference «Nutrition and Health», Riga, Latvia. Online. December 9–11, 2020. Abstract book page 93.

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11. **Videja M**, Kuka J, Liepinsh E, Makrecka-Kuka M, Liepins J, Grinberga S, Dambrova M. Metformin decreases gut microbiota-dependent trimethylamine N-oxide production in diabetic animals. Poster presentation. FEBS3+ conference, Riga, Latvia, 2019, P. 96.

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