

**The Effects of TMA Lyase Inhibition on Organ Preservation in
Prediabetic Mice**

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The Effects Of TMA Lyase Inhibition On Organ Preservation In Prediabetic Mice

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Submitted in fulfilment of the requirements of the degree of Master of Medical Research

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I. Abstract

Introduction: For patients suffering from end-stage heart failure (HF), heart transplant (HTx) can often be the only viable option for treatment. However, the availability of hearts and success of surgery is a requisite for decreasing overall mortality. It has long been known that diet has an influence on overall cardiovascular health and wellbeing. Hence, this study observes two factors effected by diet; (1) diabetes, (2) TMA lyase inhibition, a therapeutic acting within the gut to reduce trimethylamine N-oxide (TMAO) – a potential dietary marker of disease. As donor hearts aren't always in ideal health, it is important to understand the effects of these factors on the outcomes of cold static storage (CSS) for HTx.

Design/Methods: A prediabetic (Pre-Db) mouse model was generated using IP streptozotocin (STZ; 75mg/kg) administration, followed by a 14-week high sugar and high fat diet. TMAO production was inhibited with TMA lyase inhibitor (3,3-dimethyl-1-butanol (DMB); 1% v/v) in a subset of animals in their drinking water. Cardiac function and response to cold ischemia was measured using a Langendorff perfusion apparatus in which hearts underwent 3 hours of CSS at 4 °C, followed by 45 minutes of normothermic reperfusion. Cell death was then identified through completion of a lactate dehydrogenase (LDH) assay on coronary effluent at two separate timepoints during reperfusion. Additionally, cardiac mitochondrial respiration was measured post-reperfusion via an Oroboros Oxygraph-2k device as a measurement of mitochondrial function. Finally, specific cardiac protein expression of glycogen synthase kinase-3 β (GSK-3 β), extracellular signal-regulated kinases $\frac{1}{2}$ (ERK1/2), protein kinase B (AKT), signal transducer and activator transcription 3 (STAT3) and proteins involved in mitochondrial oxidative phosphorylation were assessed using a standard western blot protocol. Hepatic flavin monooxygenase 3 (FMO3) protein expression was also analysed.

Results: The Pre-Db and Pre-Db + DMB animals displayed increased body weight gain and hyperglycaemia compared to control (CTRL) mice while fasting insulin levels were comparable between groups. Ischemic contracture severity was reduced and contractile function recovery after CSS was improved in CTRL mice treated with DMB. However, these cardioprotective effects were lost in Pre-Db mice. Pre-Db mice also had reduced expression of key mitochondrial oxidative phosphorylation proteins after CSS, while limited changes in cardiac mitochondrial respiration were observed. Interestingly, phosphorylated ERK1/2 expression was significantly reduced in DMB treated CTRL mice after CSS. However, no major changes were seen in other RISK and SAFE protein pathways.

Conclusions: Collectively, data suggest DMB treatment improves ischaemic tolerance in hearts exposed to CSS storage for HTx. However, these effects were limited in Pre-Db mice. Since tissue mitochondrial function and protein expression was unchanged between groups, further study is required to identify the exact mechanisms associated with these changes in ischaemic tolerance.

II. Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

III. Table of Contents

<i>I. Abstract</i>	2
<i>II. Statement of Originality</i>	4
<i>III. Table of Contents</i>	5
<i>IV. List of Tables and Figures</i>	9
<i>V. List of Abbreviations & Terms</i>	11
<i>VI. Acknowledgments</i>	13
<i>Chapter 1. Literature Review</i>	14
1.1 Introduction to Cardiovascular Disease	16
1.2 Diet and Cardiovascular Disease	17
1.2.1 Western Diets.....	17
1.2.2 Other Diets.....	19
1.3 Type 2 Diabetes	19
1.3.1 Type 2 Diabetes and Cardiovascular Disease.....	21
1.3.2 A Western Diet and Type 2 Diabetes.....	23
1.3.3 Prediabetes.....	24
1.4 Gut Microbiota and Disease	25
1.4.1 Trimethylamine N-Oxide and Gut Microbiota.....	25
1.4.2 Trimethylamine N-Oxide and Cardiovascular Disease.....	27
1.4.2.1 Atherosclerosis and Vascular Dysfunction.....	27
1.4.2.2 Heart Failure.....	29
1.4.3 Trimethylamine N-Oxide and Type 2 Diabetes.....	30
1.4.3.1 Trimethylamine N-Oxide and Prediabetes.....	32

1.5 Pre-transplant Organ Preservation	32
1.5.1 Heart Transplantation	34
1.5.2 Myocardial Ischemia-Reperfusion Injury.....	36
1.5.2.1 Oxidative Phosphorylation	38
1.5.2.2 Reperfusion Injury Survival Kinase Pathway.....	41
1.5.2.3 Survival Activating Factor Enhancement Pathway	43
1.5.3 Variability of the Donor Pool	44
1.5.3.1 Type 2 Diabetes and Donor Heart Viability	44
1.5.3.2 Trimethylamine N-Oxide.....	46
 Chapter 2. Aims & Hypotheses	 47
2.1 Aims	48
2.2 Overall Hypothesis	49
 Chapter 3. Research Methods	 50
3.1 Animal Model	51
3.1.1 Prediabetic Murine Model & Composition of Diet	52
3.1.2 3,3-Dimethyl-1-Butanol Water Supplement.....	53
3.2 Phenotypic Characteristics.....	53
3.2.1 Glucose Tolerance Tests.....	53
3.2.2 Biochemical Assessment	54
3.3 Cardiac Outcomes	54
3.3.1 Heart Perfusion and Post I-R Assessments.....	54
3.3.2 Cell Death via Lactate Dehydrogenase Efflux	55
3.3.3 Heart Tissue Dissection	56
3.3.4 Other Tissue Dissection.....	56
3.3.5 Mitochondrial Respirometry.....	56
3.4 Protein Detection & Analysis	57
3.4.1 Subcellular Fractionation.....	57

3.4.2 Western Immunoblotting.....	58
3.5 Statistical Analysis	59
Chapter 4. Results.....	60
4.1 Phenotype Characteristics.....	61
4.1.1 Body Weight.....	61
4.1.2 Prediabetic Phenotype	62
4.1.2.1 Blood Glucose.....	62
4.1.2.2 Insulin	64
4.1.3 Water Consumption.....	65
4.2 Cardiac Ischemic and Post-ischemic Function.....	66
4.2.1 Cardiac Perfusion and I-R Response	66
4.2.2 Lactate Dehydrogenase Release	69
4.2.3 Mitochondrial Function	70
4.2.3.1 Baseline Mitochondrial Respiration	70
4.2.3.2 Mitochondrial Respiration After Cold Static Storage and Reperfusion	74
4.3 Protein Expression	77
4.3.1 Protein Expression in Hearts at Baseline.....	77
4.3.2 Protein Expression in Hearts After Cold Static Storage and Reperfusion.....	79
4.3.3 Liver FMO3 Expression	82
Chapter 5. Discussion.....	84
5.1 Phenotypic Effects of Western Diet, STZ and TMA Lyase Inhibition.....	85
5.1.1 Body Weight.....	85
5.1.2 Glucose Homeostasis.....	87
5.1.3 Implications of Changes in Water Consumption in Groups.....	89
5.2 Effects of Prediabetes and TMA Lyase Inhibition on Ischemic Tolerance to CSS.....	90
5.2.1 Ischemic Contracture	90
5.2.2 Myocardial Tolerance to CSS and Myocardial Function During Reperfusion	92

5.2.3 LDH Release and Cell Death.....	94
5.2.4 Mitochondrial Function	95
5.3 Effects of Prediabetes and TMA Lyase Inhibition on Myocardial Protein Expression	97
5.3.1 Oxidative Phosphorylation Protein Expression	97
5.3.2 RISK Pathway Protein Expression	99
5.3.3 SAFE Pathway Protein Expression	100
5.3.4 Liver FMO3 Expression	101
5.4 Limitations and Future Research Directions.....	102
5.5 Conclusion.....	103
<i>References.....</i>	<i>105</i>
<i>Appendices.....</i>	<i>137</i>

IV. List of Tables and Figures

Ordered as they appear in thesis:

Figure 1.1. TMAO formation.	25
Figure 1. 2. Metabolic and biochemical changes during I-R.	36
Figure 1. 3. Overview of cell survival pathways activated during reperfusion highlighting RISK (blue) and SAFE (orange) pathways.	42
Figure 3.1. Animal model experimental groups.	52
Figure 4.1. Final Body Weights (post-14-week feeding protocol).....	61
Figure 4.2. Combined Fasting Glucose and GTT at week 6.	62
Figure 4.3. Combined Fasting Glucose and GTT at week 12.	63
Figure 4.4. Combined Fasting Insulin at week 12.....	64
Figure 4.5. Daily Bottle Weight Changes per Mouse.	65
Figure 4.6. Extent and rate of cardiac ischemic contracture.	66
Figure 4.7. Post-ischemic outcomes following 3 hours of cold ischemia/ 45 minutes of reperfusion.....	67
Figure 4.8. Change in pressure over change in time as a percentage of baseline.....	68
Figure 4.9. LDH release from coronary effluent after CSS.....	69
Figure 4.10. Left ventricular oxygen consumption in murine hearts at baseline.	70
Figure 4.11. Left ventricular oxygen consumption in murine hearts at baseline.	71
Figure 4.12. Left ventricular oxygen consumption in murine hearts at baseline.	72
Figure 4.13. Left ventricular oxygen consumption in murine hearts at baseline.	73

Figure 4.14. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion.	74
Figure 4.15. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion.	75
Figure 4.16. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion.	76
Figure 4.17. Whole Homogenate OXPHOS complex subunit expression in left ventricles of murine heart at baseline.....	77
Figure 4.18. Cytosolic protein expression at baseline.	78
Figure 4.19. Mitochondrial OXPHOS complex subunit expression in left ventricles of murine heart after CSS and reperfusion.....	79
Figure 4.20. Cytosolic AKT expression in left ventricles of murine hearts after CSS and reperfusion.	80
Figure 4.21. Cytosolic ERK1/2 expression in left ventricles of murine hearts after CSS and reperfusion.....	81
Figure 4.22. Cytosolic GSK- 3 β expression in left ventricles of murine hearts after CSS and reperfusion.	81
Figure 4.23. Cytosolic STAT3 expression in left ventricles of murine hearts after CSS and reperfusion.....	82
Figure 4.24. Whole homogenate FMO3 expression in murine livers.	82

V. List of Abbreviations & Terms

AKT/PKB	Protein Kinase B
AUC	Area Under Curve
ATP	Adenosine Triphosphate
BNP	Brain Natriuretic Peptide
CHD	Coronary Heart Disease
CSS	Cold Static Storage
CTRL	Control
CVD	Cardiovascular Disease
DALYs	Disability Affected Life Years
DMB	3,3-Dimethyl-1-Butanol
EDP	End Diastolic Pressure
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Signal-Regulated Kinases $\frac{1}{2}$
ETC	Electron Transport Chain
FMO	Flavin Monooxygenase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GSK-3 β	Glycogen Synthase Kinase-3 β
GTT	Glucose Tolerance Test
HF	Heart Failure
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HTx	Heart Transplantation
IL	Interleukin
IP	Intraperitoneal

I-R	Ischemia – Reperfusion
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LV	Left Ventricle
LVDP	Left Ventricular Developed Pressure
mPTP	Mitochondrial Permeability Transition Pore
MI	Myocardial Infarction
OXPHOS	Oxidative Phosphorylation
Pre-Db	Prediabetes
RISK	Reperfusion Injury Survival Kinase
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RTK	Receptor Tyrosine Kinase
SAFE	Survivor Activating Factor Enhancement
SR	Scavenger Receptor
STAT3	Signal Transducer and Activator Transcription 3
STZ	Streptozotocin
TMA	Trimethylamine
TMAO	Trimethylamine N-Oxide
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
T2D	Type II Diabetes

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Chapter 1. Literature Review

Foreword

While the detrimental impacts of diabetes have been established, we are only beginning to unravel the possible role that TMAO plays on overall health. With growing rates of CVD, obesity, and diabetes we are yet to fully understand how these diet-related risk factors may influence the cardiovascular system. In the context of HTx, donor shortage is one of the biggest challenges we have to overcome to achieve successful organ transplantation. To increase the pool of viable organs, an understanding of the impact that increased TMAO levels and diabetes have on organ preservation with CSS for HTx is required. Currently, there is very little research assessing the post-transplant outcomes associated with using hearts from unhealthy individuals as transplant donors...

The following literature review introduces and describes the relationships between diabetes, TMAO and organ preservation, whilst highlighting the effects that these risk factors may have on HTx success. Firstly, it will introduce CVD and the risk factors that lead patients to require HTx. It will then describe the influence that a Western diet has on TMAO levels and diabetes in the context of cardiovascular health. Finally, these risk factors will be linked to organ preservation, uncovering their relationships with cardiac ischemia and reperfusion injury.

1.1 Introduction to Cardiovascular Disease

Despite advances in research and medicine, CVD continues to increase in prevalence and is the leading cause of global mortality (1). It takes approximately 18 million lives and makes up a 1/3 of all deaths globally (2). From 1990 to 2016, incidence of CVD has increased from 257 million to 550 million cases respectively (2). Additionally, disability-adjusted life years (DALYs) were doubled from 17.7 million to 34.4 million years during this period (1).

Described as a group of disorders of the heart and blood vessels, CVD takes the form of ischemic heart disease, stroke, HF, peripheral arterial disease, and several other conditions (3). It is a multifaceted disease with a number of known preventable risk factors that have been established to play a significant role (4). Many of these risk factors are linked to unhealthy lifestyles, such as smoking, physical inactivity and unhealthy dietary habits (4).

Clinically, CVD often begins with a reduction of blood flow to the coronary circulation due to formation of atherosclerotic plaques. These blockages then create an imbalance between oxygen supply and demand causing myocardial ischemia. Acutely, these plaques can rupture, occluding smaller vessels and cause myocardial infarction (MI) (5). Chronically, exposure to myocardial ischemia due to coronary occlusion plays a large role in the pathogenesis of HF (6).

HF is a complex syndrome that affects over 26 million people worldwide. In Australia alone, HF kills one person every 2 and a half hours (7). These numbers are predicted to continue to increase in parallel with increasing global age. As a condition with a significant burden and poor quality of life for patients, a systematic plan must be put in place to ensure education about cardiovascular risk factors and treatment is available (8).

Currently, treatment of HF is limited to treating symptoms rather than the cause. Various pharmacotherapies aim to improve blood flow, regulate rhythm and reduce LDL cholesterol. Additionally, dietary modification, exercise prescriptions and lifestyle counselling

are also provided. When patients are in end-stage HF, damage is often irreversible. Hence, a heart transplant (HTx) can be the only option to increase the patients chances of survival (9).

1.2 Diet and Cardiovascular Disease

The probability of developing CVD is greatly associated with consumption of an unhealthy diet. Both CVD and poor dietary habits often coincide with multiple comorbidities, such as, obesity, diabetes, hypertension, or dyslipidaemia. These 4 risk factors represent 4 of the 10 largest risks of all-cause mortality globally (10). Hence, food choices can be described as one of the most important factors in well-being, playing a role in 50 % of all CVD deaths (11). Fortunately, diet is easily modifiable and working with a nutritionist can be an effective tool in preventing and reversing chronic disease.

1.2.1 Western Diets

A Western diet is characterised by being rich in saturated fats, refined carbohydrates, processed meat, red meat, and salt. It is also often low in fruit and vegetables and consists of large portion sizes (12). Consumption of a modern Western diet has been linked to obesity and various other diseases (13). In preclinical studies, mice fed a Western-type diet have also demonstrated weight gain and poor cardiovascular outcomes (14, 15). Evidence shows that an unhealthy Western-type diet has a significant impact in the pathogenesis of atherosclerosis and can be used as a predictor of severe CVD. The progressive evolution of atherosclerosis from endothelial dysfunction to atherosclerotic plaque formation transpires due to systemic inflammation, oxidative stress and dyslipidaemia; factors that can all be modified by diet (16).

Dietary fatty acids have long been understood to play a role in CVD development (17). However, a meta-analysis assessing high and low intake of saturated fats has displayed inconsistencies with CVD associations (18). Nevertheless, replacing dietary saturated fats with

polyunsaturated fatty acids seems to reduce coronary heart disease (CHD) risk and death (19). Hooper *et al.* in a meta-analysis of 48 randomised clinical trials also found that reduction and modification of total dietary fat reduced risk of cardiovascular events by up to 14 % (20). For improved cardiac outcomes, reduction of saturated fats through replacement with polyunsaturated fats may be a beneficial alternative.

High intake of refined carbohydrate (such as corn syrup) within a Western diet is associated T2D, obesity and poor cardiac outcomes (21). One prospective study found that replacing saturated fats with refined carbohydrates increased risk of MI by 33 % (22). Assessing carbohydrate consumption with different glycaemic index (GI) values, a meta-analysis found that replacing saturated fatty acids with carbohydrates with high-GI values produced significantly increased CHD and MI risk (23). GI is a measure used to characterize how rapidly various carbohydrate foods alter blood glucose levels (23). High-GI carbohydrates can influence blood glucose rapidly and are representative of those found in a Western-type diet (12). Thus, clear links between highly refined carbohydrate consumption in a Western-type diet and cardiovascular risk have been made.

The consumption of fried and processed foods must be considered when assessing the impact of a Western diet on cardiovascular health. Frying food is a method of cooking that promotes oxidation and hydrogenation, as well as increases in fat content (24). Consuming fried foods more than 2-4 times per week has been shown to increase risk of dyslipidaemia (25) and hypertension (26), with Cahill *et al.* (27) reporting an association with development of CVD (27). Processing food is the process of modifying food from their original fresh or whole state. Highly processed foods seen in a Western-type diet undergo significant levels of processing so they may no longer resemble the raw ingredients they are made from. These foods often contain preservatives, emulsifiers, sweeteners, and artificial colours and flavours - exerting their adverse effects through increased sodium, trans fatty acid and saturated fatty acid

content (28). Clear links between the consumption of highly processed foods and CVD have been reported (29, 30). Hence, frying and processing foods are key components in worsening cardiac outcomes from a Western diet.

Specifically, consumption of 50 g/day (daily serving) of processed meat (i.e. bacon, sausages) has been found to be strongly associated with CVD in several studies (31-33). However, evidence about the relationship between red meat consumption and CVD is not well understood. One meta-analysis of 1 674 272 individuals found that consuming 100 g/day of red meat was associated with a 16 % increase in CVD risk (34). Conversely, two other studies showed no relationship (31, 33). More research is required to understand the dose dependant nature of red and processed meat consumption and CVD risk.

1.2.2 Other Diets

Various other diets have received attention due to their alleged ability to reduce CVD risk. The well-recognised Mediterranean diet is a modern take on the traditional dietary patterns of Greece and Italy. It consists of consumption of olive oil, legumes, vegetables, cereals; moderate consumption of fish, dairy and wine; and low consumption of red and processed meats (35). Supporting the claims, this traditional diet has been found to reduce the risk of 10-year fatal and non-fatal CVD (36). Additionally, vegan and vegetarian dietary patterns and their effect on cardiovascular health have been examined. A meta-analysis from 2022 showed that vegetarians have a 15 % reduction in risk of CVD compared to non-vegetarians. However, no clear association between veganism and CVD risk was found (37).

1.3 Type 2 Diabetes

Type 2 diabetes is a multifactorial disease characterised by insulin resistance and elevated blood glucose levels. Like CVD, it has many modifiable risk factors such as diet,

inactivity and obesity that can lead to various medical conditions involving multiple organ systems. This chronic, metabolic disorder if left unchecked causes damage to the heart, vasculature, kidneys, and other organ systems. T2D makes up approximately 95 % of all diabetes patients with approximately 425 million people suffering from the condition (38). Despite our improved understanding of the risks surrounding this metabolic disorder, case numbers of T2D have increased 4-fold since 1980 (39). Hence, approaches must be considered to reduce the overall burden of this multifactorial disease.

The impairment of insulin secretion and insulin resistance tend to contribute jointly to the pathophysiology of T2D. The impairment of insulin secretion decreases responsiveness to glucose, leading to chronic hyperglycaemia (40). It is usually progressive, resulting in formation of glycation end products, increased oxidative stress, chronic inflammation and platelet hyperactivity (38). When left untreated, impaired insulin secretion has been found to reduce pancreatic β -cell function and further negate the long-term control of glucose. To assess glucose handling and impairment of insulin secretion, oral glucose tolerance tests (GTT) are used (40). Conversely, insulin resistance occurs when insulin is inefficient in controlling blood glucose levels. The combination of genetic and modifiable risk factors works together to impair insulin signalling and nullify its action. Insulin resistance is often assessed using a homeostasis model assessment for insulin resistance (HOMA-IR). This indirect method of quantifying insulin resistance is calculated from fasting glucose and insulin levels (40).

To clarify, insulin secretion is controlled by the pancreas, a glandular organ located in the retroperitoneal cavity. This organ is responsible for roles in food digestion and control of hormones maintaining glucose homeostasis. Within the pancreas, islets of Langerhans are randomly distributed containing various cell types. β -cells are the most abundantly found cell and secrete insulin. α -cells are also located within pancreatic islets and secrete glucagon (41). Simply, insulin helps to control uptake of glucose to insulin-sensitive peripheral tissue,

promote glycogenesis and inhibit glucagon secretion from α -cells. Conversely, glucagon plays a major role in maintaining blood glucose through stimulation of hepatic glucose production (42).

In T2D, pancreatic β -cell mass is typically found to be significantly reduced when compared to healthy individuals. This reduction in mass and dysfunction has long been attributed to apoptotic pathways, driven by the adverse environmental conditions exposed to the body (43). Though, it is now understood that the interaction of T2D and β -cell dysfunction is far more complex. In chronic hyperglycaemia and free fatty acid (FFA) excess, these cells are subject to the pressures of inflammation, oxidative stress, and lipotoxicity. The combination of these stressors leads to endoplasmic reticulum (ER) and mitochondrial stress, upregulating cell death signalling (44).

In combination with cohort and retrospective human studies, it is common practice to use non-genetic animal models of T2D to study the condition. Commonly, the alkylating antineoplastic agent, streptozotocin (STZ) has been used to induce diabetes in rodents and other animals (45-49). STZ works by causing direct damage to pancreatic β -cell through intracellular methylation, free radical production and nitric oxide production.

1.3.1 Type 2 Diabetes and Cardiovascular Disease

CVD is the leading cause of death in diabetes patients. Overall risk of CVD, specifically CHD is increased by 2-to-3-fold and 4-to-6-fold in diabetic men and women respectively (50). Additionally, up to 80% of all T2D patients will die from MI, stroke and peripheral arterial disease (51). It has long been understood that T2D is a distinct condition, linked to chronic systemic inflammation, atherosclerosis, CVD and other related disorders. However, literature has recently been interpreted that these conditions may be a consequence of T2D rather than just exacerbated by it (52).

There is a complex relationship between diabetes and factors affected by T2D including blood constituents, myocardium, and plaque formation; all of which are vulnerable to this chronic disease state. Therefore, not only is the incidence of CVD increased by T2D, but the damage caused as well (51).

T2D plays a role in increasing risk and incidence of myocardial ischemia, decreasing myocardial tolerance to ischemia-reperfusion (I-R) injury and eliminating cardioprotective mechanisms (53). Supporting this claim, Russel *et al.* (46, 54) found that T2D significantly worsened I-R injury, abolished protection from ischemic preconditioning and eliminated the effects of injury mediators (Bax expression and cardiac nitrosylation) in a mouse model (46, 54). These findings of ischemic intolerance and loss of cardioprotection in murine diabetic myocardium are consistent with prior publications (53, 55-58). Conversely, certain novel pharmacotherapies have been found to decrease infarct size and myocardial damage caused by chronic T2D (59-61). Pioglitazone, an insulin-sensitizing agent was found to increase insulin sensitivity, reduce infarct volume and suppress inflammatory responses after reperfusion in leptin deficient (db/db) mice (59). Additionally, Wang *et al.* (60) demonstrated that propofol inhibited excessive autophagy and oxidative stress in T2D rat myocardium, whilst reducing I-R injury (60). Resveratrol, a polyphenol found in grapes, was found to induce cardioprotection against I-R injury in T2D female rats (61). Hence, there is potential therapeutic uses for various medications to improve the outcome of human cardiovascular outcomes in T2D.

In a human study, extensive echocardiography data indicated that T2D was associated with increased left ventricle (LV) wall thickness, decreased LV internal diameter and diastolic function (62). Another study found that incidence of arrhythmias was significantly increased in diabetic patients, thought to be caused via the aforementioned cardiac remodelling (63). Additionally, myocardial triglyceride levels were increased in T2D patients with metabolic syndrome, providing a potential insight into prediction of diabetic cardiomyopathy (64).

Hence, the literature provides a clear link between cardiac dysfunction, CVD and T2D, though fails to identify a complete solution in humans once damage has occurred. Although cardioprotective mechanisms such as local and remote ischemic and pharmacological pre-conditioning, post-conditioning, and pre-conditioning have been extensively researched, these concepts have not been transferred to a clinical setting. Further study is required involving diabetes and I-R to apply these therapeutic processes to human trials.

1.3.2 A Western Diet and Type 2 Diabetes

In the last 50 years, the number of T2D patients has increased in parallel with our increase of obesity. Driven by the consumption of a modern Western-type diet, increased simple carbohydrate intake is at the forefront of this epidemic (65). In Australia, obesity prevalence has increased from 10% in 1983, to 28% in 2012. This has been accompanied with a significant increase in carbohydrate consumption across this time period (66). Similarly, US macronutrient consumption between 1965 to 2011 showed a clear correlation between carbohydrate consumption and obesity (67). Interestingly, both studies found that fat consumption had decreased, irrespective of increases in obesity (66, 67). An association between body weight and T2D incidence has also been identified (13-15). Though, individuals that were initially lean also showed a significantly increased risk of T2D when subject to weight gain from poor dietary behaviours (68). Furthermore, links between sugar intake and T2D diagnosis have been made irrespective of weight gain and obesity (69). As a Western diet is often the cause of hyperglycaemia, a clear relationship exists between the onset and progression of T2D and consumption of this unhealthy diet. Whilst diet plays a role in the pathogenesis of T2D, it also plays a role in the progression of the disease and in Pre-Db.

1.3.3 Prediabetes

Pre-Db is the condition preceding T2D and often precedes the development of the fully evolved metabolic condition. It is defined as an intermediate state of hyperglycaemia with blood glucose levels above normal range but below the diabetic threshold (70). Between different international organisations, the diagnostic criteria for Pre-Db have not been uniformly established. However, fasting blood glucose between 7.8 mM and 11.1mM has been used as an estimated range for the diagnosis in humans (71). Like T2D, measurement of fasting blood glucose and oral glucose tolerance tests are common methods of assessing impaired glucose homeostasis in Pre-Db patients. These glucose abnormalities in Pre-Db patients can often be caused by insulin resistance (72). However, there are well documented instances in which both T2D and Pre-Db has developed in non-obese humans devoid of insulin resistance (72, 73). Other factors that may induce Pre-Db and diabetes progression include; diet, family history, age, obesity, sedentary lifestyle and pancreatic dysfunction (74).

In Australia alone, 2 million individuals have Pre-Db and are at a high risk of developing T2D (71). This number is believed to reach approximately 471 million globally by the end of 2035 (75). With a yearly conversion rate of developing diabetes being a staggering 5-10 % (70), it is imperative that individuals at risk monitor glucose homeostasis to prevent downstream complications. It has also been found that Pre-Db is associated with increased risk of CVD development in humans (76-78). However, due to the high conversion rate from Pre-Db to T2D, it is unclear whether Pre-Db or the fully evolved condition elevate this risk. More research surrounding Pre-Db is required to get an understanding of its effects on various organ systems compared to fully evolved T2D.

1.4 Gut Microbiota and Disease

There has been a recent focus on the role of gut microbiota in non-communicable diseases. It is thought that gut microbiota may be a major player that links many modifiable risk factors to diverse chronic diseases (79). It has long been established that pathogenesis of CVD involves several known environmental factors. Though, what is less clearly understood is the involvement of natural microflora and disease. Exposure to certain bacteria and their substrates has been linked to adverse cardiovascular effects and developing research continues to uncover the mechanisms involved in disease (80).

1.4.1 Trimethylamine N-Oxide and Gut Microbiota

TMAO has been at the forefront of many investigations involving gut health and pathology. It is generated from various dietary substrates including choline, carnitine and betaine from red meat, poultry, fish dairy and eggs – food often seen in a Western diet. These dietary precursors are firstly converted into trimethylamine (TMA) via gut microbial metabolic pathways in the colon, before being taken up into the systemic circulation and being oxidised to TMAO by hepatic flavin monooxygenase 3 (FMO3) (81). TMAO is then transported via the blood to the rest of the body (Figure 1.1).

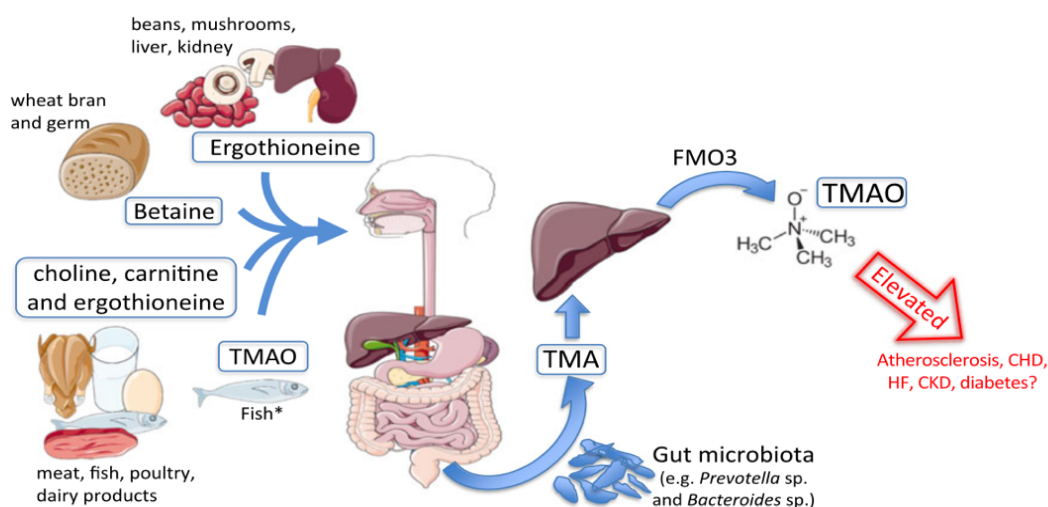


Figure 1.1. TMAO formation. Process of TMAO intake or generation from dietary sources. Substantial elevations in circulating TMAO are hypothesised to promote cardiometabolic and renal diseases. Figure from Naghipour et al (81).

Various microbial metabolic processes are involved in the conversion of the aforementioned dietary precursors to TMA. Several commensal bacteria of the human intestine such as *Firmicutes* and *proteobacteria* have been found to consume choline, carnitine and betaine as a potential source of TMA. Hence, with the use of their own bacterial enzymes, the conversion can occur. Choline and its derivatives are converted to TMA via action of TMA Lyase (CutC). Additionally, carnitine is converted to TMA via carnitine monooxygenase (CntA/B) in a multistep chemical reaction (82). Whereas, betaine relies on the action of selenocysteine-containing glycine betaine reductase (GrdH) (83).

As the production of TMAO requires these microbial metabolic pathways to be activated excessively, it unlocks a potential target for therapeutics. Previous studies have found that using broad spectrum antibiotics can be used to reduce overall TMAO levels in animal models (80, 84, 85). Additionally, pharmacotherapies such as 3,3-dimethyl-1-butanol (DMB) (86) and iodomethylcholine (87) have been utilised to specifically target the TMA lyase

enzyme. Hence, further research is required to assess whether these methods of TMAO reduction can be used as a potential therapeutic for disease.

1.4.2 Trimethylamine N-Oxide and Cardiovascular Disease

Initially identified as a waste product with non-specific metabolic action, TMAO is associated with cardiovascular events and increased CVD risk (80). Evidence has suggested that high levels of TMAO play a role in atherosclerotic plaque formation, myocardial dysfunction and other actions promoting CVD. However, dose-dependence has been a major issue, showing inconsistencies between experimental studies. In fact, pathological concentrations found *in vivo* may only be seen in end-stage CVD, HF or renal disease (81).

Recent clinical and preclinical studies have suggested relationships between high TMAO levels and increased CVD risk. A 2011 study by Wang *et al.* (80) showed that choline, betaine and TMAO were identified as predictors of CVD risk in a large clinical cohort (80). A prospective study of patients who had already had a stroke showed that high TMAO levels were correlated with increased risk of subsequent cardiovascular problems (88). Various other large cohort studies have also confirmed this proposed link (89, 90). This leaves the question of whether high TMAO levels are the cause of CVD or just an outcome of disease.

1.4.2.1 Atherosclerosis and Vascular Dysfunction

Specifically, there has been focus on the roles of TMAO in atherosclerosis and vascular dysfunction. At extremely high concentrations, TMAO has been found to be pro-inflammatory, upregulate scavenger receptors (SR) and inhibit reverse cholesterol transport (RCT) (84, 91). Additionally, exposure to TMAO was proven to cause platelet hyperreactivity and increased thrombotic potential via amplified intracellular Ca^{2+} release (92). It is believed that these actions may play a role in the late stages of vascular disease when TMAO levels are high; in

diseases such as HF and chronic kidney disease. Though, the involvement in initial pathogenesis is less understood (81).

In a murine model predisposed to disease, it was shown that large elevations in TMAO from carnitine supplementation promotes atherosclerosis; an effect reversed by DMB treatment (86) and broad spectrum antibiotic use (80). A study from Geng *et al.* (93) also demonstrated that direct TMAO supplementation (1 mM in drinking water) could increase plaque formation in ApoE^{-/-} mice. They then demonstrated that TMAO exaggerated changes in cluster of differentiation 36 (CD36) and foam cell formation via p38 Mitogen-activated protein kinases/c-Jun N-terminal kinases 1/2 (MAPK/JNK1/2) signalling in a concentration-dependant manner; TMAO exaggerated changes in CD36 and foam cell formation via p38 MAPK/JNK1/2 signalling (93). Although promising, the translation of ApoE^{-/-} murine models should be viewed with caution as they lack the ability of RCT (94). Conversely, various studies have claimed that TMAO levels have no role in atherogenesis and that it may even be protective. For example, one study found that TMAO levels were inversely related to size of aortic lesions in the genetically modified ApoE^{-/-} mice (94). Another study relying on FMO knockout mice found no correlation with TMAO production or urinary TMAO and atherosclerosis (95).

In a human population, 2235 patients with stable CHD were examined to assess fasting plasma TMAO and all-cause mortality over 5-year follow-up. It was found that patients with higher TMAO levels had up to a 4-fold increase in mortality risk (96). Additionally, a small human study of patients undergoing choline supplementation with high TMAO levels showed increased platelet reactivity and thrombosis rates, attenuated by aspirin (97). Although much of the literature demonstrates an interaction between TMAO, atherosclerosis and vascular dysfunction, a direct mechanism is yet to be identified. Further insight could provide a potential target to limit plaque burden in unhealthy individuals.

1.4.2.2 Heart Failure

Recently, there has been a growing understanding that TMAO is a powerful prognostic marker of HF progression. A prospective study of patients with stable cardiac disease showed that the median TMAO levels in HF patients was significantly higher than the healthy control cohort (98). Additionally, patients with increased serum TMAO were more likely to have elevated brain natriuretic peptide (BNP), and increased use of diuretics (98). Hence, in combination with a mechanistic link, the prognostic value of TMAO may be invaluable in the assessment and treatment of HF.

In a commonly used murine model of pressure-overload and HF, mice underwent transverse aortic constriction and were provided choline or carnitine supplementation. After 12 weeks, LV dilation, reduced left ventricular ejection fraction (LVEF), increased BNP, increased pulmonary edema and increased myocardial fibrosis were found compared to control mice (99). A similar study, showed that DMB supplemented in water (1% v/v) reduced TMAO levels and decreased HF risk by regulating transforming growth factor β 1/Smad3 signalling pathway and p65 nuclear factor kappa B (NF- κ B) signalling pathway (100). Hence, these findings suggest that DMB may play a role in reducing LV structural and electrical remodelling. Another study that fed mice a Western-type diet found that increased TMAO led to cardiac inflammation, perivascular fibrosis, decreased cardiac function and decreased cardiac compliance (101).

Influencing inflammation, TMAO may play another role in the severity of HF. One study showed that physiological levels of TMAO could induce expression of pro-inflammatory cytokines and adhesion molecules. This was controlled via NF- κ B signalling pathways, leading to vascular smooth muscle cell inflammation (102). There has also been evidence that TMAO may promote mitochondrial reactive oxygen species (ROS) accumulation by inhibiting sirtuin 3 (SIRT3) expression and superoxide dismutase 2 (SOD2) activity. This inhibition leads

to activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasomes, generating the pro-inflammatory Interleukin 1 β (IL-1 β) and IL-18 and causing subsequent endothelial inflammation (91, 103). Additionally, a study from Zhang *et al.* (104) showed that administration of TMAO (120 mg/kg) in drinking water can reverse the cardioprotective effects of voluntary exercise in western diet fed mice. They found that the pro-inflammatory tumour necrosis factor- α (TNF- α) had increased expression and anti-inflammatory IL-10 had decreased expression in the TMAO supplemented mice (104).

There is also recent evidence that TMAO may influence HF through mitochondrial dysfunction. A murine model providing mice with chronic exposure to TMAO effected mitochondrial function and energy metabolism, leading to ventricular remodelling and HF development. Using an Oxygraph-2k device (O2k, OROBOROS Instruments, Innsbruck, Austria), it was found that TMAO reduces pyruvate oxidation and fatty acid oxidation in cardiac mitochondria. This insufficient energy metabolism may be a cause of consequent HF (105). Further study on TMAO, HF and mitochondrial function are required to paint a more comprehensive outline of the mechanisms involved.

1.4.3 Trimethylamine N-Oxide and Type 2 Diabetes

In line with our growing understanding of gut microbiome and disease, it is understood that gut dysbiosis is linked to several metabolic diseases such as diabetes (106). As T2D patients often present with a number of abnormal metabolic products associated with this dysbiosis, TMAO has been a major player in many ongoing investigations. Various investigations in human participants have demonstrated that plasma TMAO and its precursors were associated with greater risk and mortality due to cardiac events in T2D patients (107-109). Nevertheless, conflicting evidence has been found. Shan *et al.* (110) evaluated plasma

TMAO and T2D while looking at the effects of polymorphisms in the FMO3 enzyme. The results found that higher plasma TMAO is associated with increased odds of T2D diagnosis in humans. Recently, a study of CVD and renal disease individuals with T2D and albuminuria were analysed to investigate TMAO precursors and all-cause mortality. It was found that higher plasma levels of carnitine, choline and other TMAO precursors were collectively associated with deteriorating renal function in T2D and albuminuria. Though, this investigation failed to find associations with CVD risk and TMAO levels in T2D patients (111). In a cohort study of 4442 older adults, it was found that plasma TMAO levels and its precursors were not associated with the risk of T2D. Though, increases were found to affect fasting insulin and insulin resistance (112). Overall, a more comprehensive analysis of various study populations is required to determine the role that TMAO may play in T2D risk and outcomes.

Various studies in animal models have also been completed to assess this complex relationship and identify the mechanisms involved. One study found that FMO3 knockdown reduces hyperglycaemia in a murine model of hepatic insulin resistance (113). Conversely, Liao *et al.* (114) reported that FMO3 had beneficial effects on glucose levels independently of the insulin-signalling pathway (114). Additionally, hepatic TMAO concentration declines and renal concentrations are unchanged in diabetic mice (115). Assessing the influence of metformin - a first-line therapeutic agent for T2D, choline-supplemented mice were treated daily with the drug for 9 weeks. Interestingly, it was found that the therapy significantly reduced serum TMA and TMAO compared to the control group. Another diabetic murine study assessed how probiotic and prebiotic supplementation to stimulate *Lactobacillus* growth would affect mechanisms involved in vascular dysfunction. From their findings, they suggest that diabetes-induced FMO3 and Intracellular adhesion molecule (ICAM) expression and potential vascular impairment occur via enteric dysbiosis-related JNK pathways. However, this was reversed by stimulation of *Lactobacillus* growth which restored diabetes induced dysregulation

(116). Although some relationships have been identified, the effect of TMAO on diabetes still requires further characterisation.

1.4.3.1 Trimethylamine N-Oxide and Prediabetes

There is limited research addressing the interactions of Pre-Db and TMAO. Recently, Roy *et al.* (117) looked at the effects of TMAO on impaired glucose regulation in diabetes-free individuals. They reported that TMAO levels were not associated with insulin resistance, HbA1c or fasting plasma glucose. Though, it was found that TMAO levels were associated with a moderate rise in Pre-Db incidence (117). Another study found that a healthy diet with high pistachio intake altered gut-microbiota-related metabolites such as TMAO in Pre-Db patients through differential gut microbiota modulation. (118). Hence, a pistachio-rich diet could be implemented to reduce this potential biomarker for disease. More research surrounding Pre-Db onset and involvement in disease is required to assess the mechanisms surrounding TMAO and this intermediate condition.

1.5 Pre-transplant Organ Preservation

For patients suffering from end-stage CVD, HTx can often be the only viable option for treatment. Over the past half century organ transplants have evolved from an experimental process to a lifesaving procedure for many critically ill patients. Advances in organ preservation are at the forefront of this practice and continue to improve outcomes and reduce mortality (119).

The issue of organ preservation was first recognised well before the first successful kidney transplant by Murray in the 1950s. In 1908, Carell used a perfusion machine to harvest organs, but could not be utilised due to contamination (120). However, he later developed a sterile perfusion pump with the help of Lindbergh to study hypothermia on organs *ex vivo*

(121). Nevertheless, it wasn't until 1960 that the first canine kidney reimplantation took place using hypothermic blood (122). This critical research would provide an understanding that human organs could be preserved for a longer period of time, prior to implantation.

Another key advancement in organ preservation was the development of the University of Wisconsin Solution. This, and the use of other similar cardioplegic solutions allowed cold static preservation to be utilised more effectively to improve patient outcomes (119). Cardioplegia is a pharmacological therapy administered during cardiac surgery and transplant to intentionally arrest the heart and reduce ischemic damage. Additionally, more modern *ex vivo* perfusion machines are currently used with both cold and warm preservation. With the improvement of diagnostics, machinery and reagents, the efficacy of organ preservation continues to improve (119). Nevertheless, with all this modern technology, organ preservation for HTx is still a very simple process.

Since the first successful HTx in 1967, cold ischemic storage or CSS at 4 °C has been the most widely used technique for heart retrieval and storage (123). As the HTx procedure continues to evolve, CSS remains a significant limitation of the procedure. Increased storage time of the recipient organ increases the risk of graft dysfunction (123). As mentioned, various preservation fluids have been developed to attenuate the damage caused by ischemia to the organ (124). These preservation solutions may differ in composition, but they share similar qualities. All solutions have energy substrates, free radical scavengers and buffering capacity (125). The primary goal of cardioplegic solutions is to reduce myocardial oxygen demand through electrical quiescence and provide osmotic support to stop cells rupturing as ATP levels decline (126). The influx of ions such as potassium, sodium, calcium and magnesium from the solution all participate in reducing cardiac contractility and inducing diastolic arrest. Previously, there has been no standardized guidelines regarding the volume of solution and type used (127). Further analysis on preservation solution use may help to refine the process

of CSS. Hence, further understanding the effects of CSS is an important tool for refining the process of HTx.

1.5.1 Heart Transplantation

HTx are most frequently indicated for patients suffering from chronic HF. Typically, patients that follow the guideline-directed medical therapy exhibiting advanced HF, refractory angina, and intractable ventricular arrhythmias will be referred for HTx (128). Patients suffering from acute HF may also require HTx if other therapies are unsuccessful. Currently, the most common implantation procedure is known as orthotopic HTx (129). This technique begins by opening the chest with a sternotomy and pericardiotomy. The aorta and pulmonary veins are separated, and vena cava is secured. While waiting for the donor heart, cardiopulmonary bypass is started. Once the donor heart arrives, anastomosis of the donor and recipient aorta, pulmonary artery, and atria to atrial cuffs occurs. This is followed by excision of the recipient's right atrium and anastomosis of the vena cava to the donor hearts right atrium (129). To limit the risk of complications post-surgery, patients are carefully monitored and provided with immunosuppressive drugs (128). Although this highly complex procedure has been refined over the last half century, the issue of organ shortages is still a concern for many individuals stuck on the waiting list.

In 2021, there were 112 HTx performed in Australia (130). According to Heart Foundation Australia, there are currently not enough donor hearts for the number of people who need transplants. Consequently, more than 100 people can be on the waiting list at any one time, often for up to two years (131). Hence, it has long been argued that the standard criteria used to assess donor heart acceptability for HTx is restricting HTx rates globally (132). To counter this, shifts in donor characteristics have shifted resulting in the acceptance of older, more obese donors with various comorbidities (133). Prior analysis has indicated that high-risk

donors are also more likely to be matched up with high risk recipients (134-136). Additionally, the previously strict criteria have been extended to include donation after circulatory death (137). Although required, these organs with extended-criteria have an even greater potential to cause post-transplant complications.

In addition to the extended criteria, predictive risk models have been developed specifically to optimise donor selection and donor-recipient matching (132). Though not widely accepted, these risk models help predict donor mortality using various donor and recipient characteristics (138-140). Smits *et al.* (140) found that donor age was the only significant risk factor for mortality after looking at multiple risk factors. Whilst assessing donor-recipient matching, another risk model found that there was no benefit to matching age, ethnicity, or BMI. Though, avoiding female donors with male recipients was found to increase risk of mortality (141). Overall, further research is required surrounding the current risk models and population specific data should be generated by various local institutions.

In a review from Sathianathan & Bhat. (132), the authors proposed several risk factors that are the most crucial to evaluate in potential heart donors. In healthy individuals, donor age, sex and time of ischemia were found to be some of the best estimators of associated risk. Across numerous studies, donor age (140, 142, 143) and sex (144, 145) has been found to have a significant impact on HTx success. These donor characteristics cannot be controlled as sex and age cannot be changed to suit a recipient. However, a risk factor that can be somewhat controlled is ischemic time. According to the International Society of Heart and Lung Transplants (ISHLT) 2018, recommended total ischemic time should be limited to less than 4 hours to increase long-term recipient survival (146). In 2020, the ISHLT showed that mortality was further worsened for ischemic time over 4 hours when paired with increased donor age, hypertension, or reduced LV ejection fraction (133). Conversely, smaller cohort studies have shown that mortality and risk may be unchanged with ischemic time ranging from 5-8 hours

(147, 148). Understanding limitations of increased ischemic time is a useful tool in predicting risk of short-term mortality after HTx. For individuals living in remote communities, research involving the risk of prolonged ischemia on I-R damage may be crucial for the success of surgery.

1.5.2 Myocardial Ischemia-Reperfusion Injury

In organ preservation, the cost of prolonged ischemia is an increased severity of the I-R injury (149). Characterised by an absence of oxygen supply followed by a subsequent restoration of blood flow, it can cause cellular dysfunction and death as well as irreversible tissue damage (150). As the heart is a contractile organ with a high metabolic rate, it is very susceptible to oxygen supply deficiencies (151).

The initial cellular injury is caused by a lack of blood flow, which leads to deficiency in oxygen, glucose and other important substrates required for metabolism (149). These deficiencies result in a decrease in mitochondrial oxidative phosphorylation and a switch from aerobic to anaerobic metabolism. This switch causes a lowering of intracellular pH, leading to activation of the sodium hydrogen exchanger and accumulation of Na^+ . Additionally, sarcolemma calcium uptake mechanisms are impaired leading to Ca^{2+} accumulation. This is exacerbated by sarcolemmal fragility and disruption due to high concentration of intracellular solutes; worsened by Ca^{2+} -dependant protease activation. If blood flow isn't restored, this ischemic period can lead to myocyte atrophy and tissue necrosis (151).

The re-establishment of blood flow to an organ is essential in salvaging the ischemic tissue. Though, it can paradoxically cause further damage to the heart itself (149). Originally, it was believed that reactivation of the electron transport chain (ETC), solely stimulated reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, resulting in endothelial dysfunction, DNA mutations and sarcolemma damage promoting more Ca^{2+}

accumulation (152). Though, Chouchani *et al.* (153) showed that selective accumulation of succinate is responsible for mitochondrial ROS production during the reperfusion stage (153).

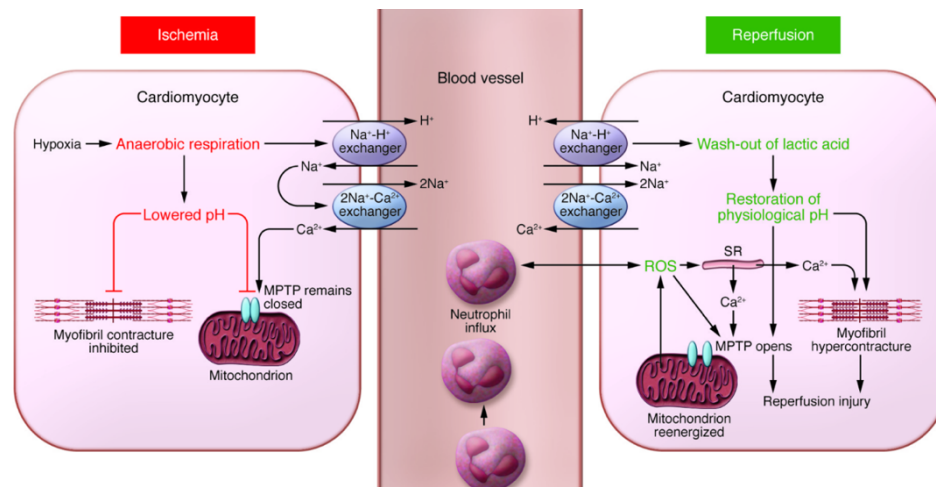


Figure 1. 2. Metabolic and biochemical changes during I-R. During ischemia, anaerobic respiration leads to inhibited myofibril contracture, mPTP inactivation and Na^+ influx. Restoration of oxygen and pH, with calcium overload triggers myocyte hypercontracture, mPTP opening and reactive oxygen species (ROS) production from oxidative phosphorylation and inflammatory cells. Figure from Hausenloy *et al.* (154).

The restoration of adenosine triphosphate (ATP) then activates the sodium calcium exchanger; which removes Na^+ from the cell and worsens the Ca^{2+} overload. The Ca^{2+} overload, ROS/RNS damage and increasing intracellular pH trigger formation and opening of the mitochondrial permeability transition pore (mPTP). Hence, accelerated cell death occurs (149).

Although cold ischemic time for HTx has been extensively analysed in humans, there are currently limited studies assessing how I-R injury in CSS can be further reduced. Cai *et al.* (155) compared a novel preservation solution called SBI-SEIKU II containing various antioxidant reagents with a clinically used preservation solution in mice. They found that the novel therapeutic prolonged acceptable ischemic time by reducing myocardial fibrosis and ROS damage (155). Another study in mice looking at *ex vivo* cold storage found that implantation of mesenchymal stem cells prolonged static storage time and assisted in miRNA transport (156). In a recent clinical trial, the SheraPak Cardiac Transport System has been tested to prevent temperature fluctuation and freeze damage that can occur during hypothermic

organ preservation (157). Limited research surrounding other CSS devices have recently been developed. Yet, one study assessing 120 patients undergoing orthotopic HTx found that ischemic preconditioning reduces cardiac troponin I levels 6 hours after aortic declamping compared to patients who didn't undergo the therapy. Nevertheless, there was no change in overall mortality and morbidity between groups (158). As modern technology advances, a push for normothermic perfusion machines has been made (119). However, as CSS is still the gold standard for heart preservation, it is important that further research is completed to help reduce damage to I-R injury. Hence, mitochondrial energy metabolism may be a potential therapeutic target to improve success of HTx.

1.5.2.1 Oxidative Phosphorylation

Integral for normal cellular function, mitochondria are responsible for energy production in all eukaryotic cells, including the synthesis of phospholipids and heme, calcium homeostasis, apoptotic activation and cell death. In healthy cells, cellular metabolism via the mitochondria is comprised of the utilization of carbohydrates, fats and proteins to derive energy (159). In the processes of glycolysis, pyruvate oxidation, fatty acid β -oxidation, deamination, and transamination - several molecules are derived to be used in the tricarboxylic acid (TCA) cycle. The TCA cycle can then generate the precursors required for oxidative phosphorylation (OXPHOS) within the electron transport chain (ETC) (159).

The ETC is located within the mitochondrial inner membrane, close to the mitochondrial matrix to retrieve substrates from the TCA cycle. To provide electrons for the ETC, the TCA cycle reduces NAD^+ to NADH and FADH to FADH_2 (160). These reducing equivalents will then donate electrons to Complex I (ubiquinone oxidoreductase) or Complex II (succinate dehydrogenase) respectively. At Complex I, the transfer of electrons from NADH is linked to proton translocation from the matrix to the intermembrane space. At Complex II,

electrons donated from FADH₂ are not accompanied by the transfer of protons. The donated electrons from both Complex I-II are then transferred through the Q-Cycle to Complex III (cytochrome c reductase) and consequently Complex IV (cytochrome c oxidase), where more protons are transferred to the intermembrane space. O₂ also binds and is reduced to H₂O at Complex IV, in a process known as mitochondrial respiration. Driving the phosphorylation of adenosine diphosphate (ADP) to ATP, the translocated protons from Complex I, Complex III and Complex IV accumulate and generate an electrochemical proton gradient known as the mitochondrial membrane potential. This membrane potential combined with reduced pH generates a proton motive force to couple electron transport to Complex V (ATP synthase), where protons re-enter the matrix. To complete the process of OXPHOS, the movement of these protons allows a mechanical motor to rotate at ATP Synthase and permit the production of ATP (159). Within cardiomyocytes, this active process is essential in meeting their high metabolic demand. Though, in I-R injury, it has been found that the mechanisms of OXPHOS are disrupted, causing ATP depletion and production of mitochondrial reactive oxygen species (ROS) (161).

During CSS of a donor organ, variable periods of ischemia are inescapable. With the associated lack of oxygen, the mitochondrial ETC is disrupted, reducing ATP production and resulting in a gradual decrease in ATP:ADP ratio – leading to eventual cell death (162). Hence, the organ must rely on anaerobic glycolysis to maintain the ATP:ADP ratio and keep cells alive. When oxygen is absent, ATP synthase will act in a retrograde manner, hydrolysing ATP generated by glycolysis to maintain proton translocation. The reason for this is not well understood and significantly reduces ATP during organ preservation (163). Both of these metabolic processes contribute to reductions in ATP and subsequent cell death. Although the cooling of organs during HTx slows metabolism and ATP use, metabolic processes still

continue. Therefore, it is important to understand how ischemic time will influence overall graft function.

Upon implantation and reperfusion of the donor organ within the recipient, mitochondria become the main contributor to I-R injury. This damage occurs through the generation of ROS causing cellular dysfunction and tissue damage (126). Accumulated succinate, a hallmark of ischemia, is rapidly oxidised during reperfusion to drive superoxide production (164). The associated oxidative damage can then damage mitochondria and induce the mitochondrial permeability transition pore (mPTP) opening (165). Additional damage during reperfusion has been found to release damage-associated molecular patterns (DAMPs), activating the innate immune response and associated inflammatory mediators – all contributing to organ dysfunction and rejection (166).

Overall, the extent of mitochondrial damage is indicative of the myocardial I-R injury (161). Due to this, mitochondria have been identified as potential therapeutic targets against I-R injury using compounds such as respiratory complex II inhibitors to reduce succinate-dependent superoxide, amobarbital to protect cellular survival, various mitochondrial-targeted antioxidants, and a range of other agents (161). Recently the understanding of how mitochondrial metabolism contributes to general cell damage (167-169) and poor outcomes during transplant has improved (162, 170, 171). Hence, it is imperative to analyse mitochondrial function in situations of organ preservation to understand the exact mechanisms involved and potential therapeutics available.

To assess mitochondrial OXPHOS activity and ETC function, various methods can be used. The gold standard for direct assessment of the mitochondrial ETC function in humans, animals and cell culture is a measurement of mitochondrial oxygen consumption. A Clarke-type electrode within the Oroboros O2k system has been used as it provides amperometric measurements of O₂ with high sensitivity (159). In a typical protocol, basal oxygen

consumption is measured to determine the turnover of the ETC using pyruvate, glutamate, malate and succinate (OXPHOS State). A protonophore such as carbonylcyanide-3-chlorophenylhydrazone (CCCP) will then be added to uncouple respiration, yielding maximal oxygen consumption (ETS state). Finally, a mitochondrial inhibitor is added such that the rate of non-mitochondrial oxygen consumption is revealed (172). Another method of analysing mitochondrial activity is by directly assessing the protein expression at each OXPHOS complex (173-175). This can potentially provide insight into why function may be altered during tissue insult and disease. Finally, assessment of upstream protein pathways may also be an effective method for assessing the effects of I-R injury during HTx.

1.5.2.2 Reperfusion Injury Survival Kinase Pathway

The reperfusion injury survival kinase (RISK) pathway is a name that has been dedicated to various pro-survival protein kinases that have been found to confer cardioprotection when activated during myocardial reperfusion (176). First discovered in the late 1990s, the concept of the RISK pathway emerged whilst discovering that apoptotic cell death contributed to reperfusion injury (177), whilst protein kinases such as protein kinase b (AKT) and extracellular signal-regulated kinase (ERK1/2) acted as formidable cardioprotectants (178, 179). As time has progressed, it is now understood that this pathway encompasses two independent cascades; phosphatidylinositol 3-kinase/Akt (PI3K-AKT) and mitogen-activated extracellular signal-regulated kinase (MAPK-ERK1/2) (180). This pathway is engaged by two types of receptors; either G-protein coupled receptors (GPCR) such as receptors activated by adenosine (181), or receptor tyrosine kinases (RTK) such as receptors activated by cytokines and insulin (179). Although the RISK pathway is separated into two routes, its modulation always triggers inhibition of mPTP opening with subsequent anti-apoptotic outcomes (182, 183).

Activation of the RISK pathway begins with binding of adenosine, bradykinin or opioids to GCPRs or insulin, cytokines or erythropoietin to RTKs. Simply, activation of AKT and ERK1/2 permits the phosphorylation of glycogen synthase kinase-3 beta (GSK-3 β) and inhibition of GSK-3 β -mediated mPTP opening (182)(Figure 1.3). Other proteins such as protein kinase c epsilon (PKC- ϵ), protein kinase G (PKG) and endothelial nitric oxide synthase (eNOS) also play direct and indirect roles in inhibiting the opening of the mPTP during RISK activation (176, 184).

Preclinical research has demonstrated that the RISK pathway can be activated using pharmacological therapeutics and mechanical interventions such as ischemic pre- and post-conditioning to reduce MI size significantly (178). Though, no studies have assessed how the RISK pathway is affected during CSS. Hence, further research is required to assess how expression of RISK pathway proteins may be enhanced during organ preservation to reduce I-R injury severity.

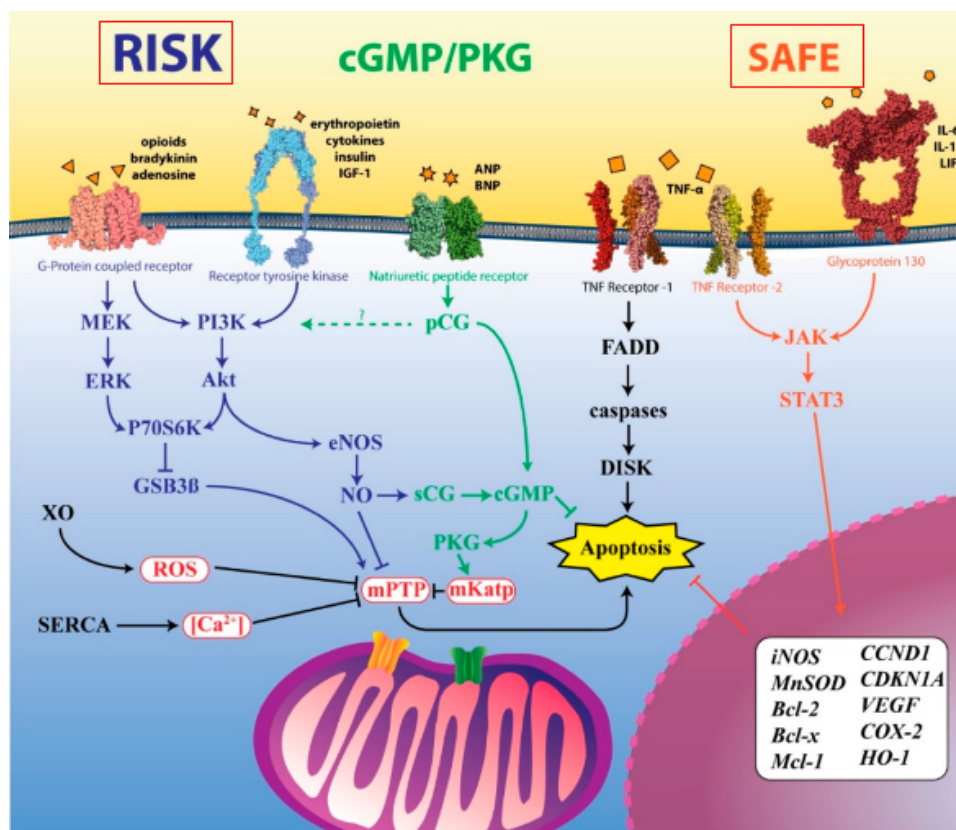


Figure 1. 3. Overview of cell survival pathways activated during reperfusion highlighting RISK (blue) and SAFE (orange) pathways. Figure from Soares et al (182).

1.5.2.3 Survival Activating Factor Enhancement Pathway

In addition to the RISK pathway, other signalling cascades have been found to play a role in mediating protecting during I-R injury. In 2009, the survival activating factor enhancement (SAFE) pathway was discovered while Lecour. (185) was describing the protective role that TNF- α seemed to play during I-R injury. TNF- α is known to exert its effects through binding as a trimer to two members of the TNF receptor superfamily (186). Binding to the 55 kDa, TNFR-1 leads to activation of the Fas-associated death domain (FADD), caspase activation and apoptosis. However, when bound to the 75 kDa, TNFR-2, activation of Janus kinase/signal transducer and activator of transcription 3 (JAK-STAT3) via the SAFE pathway leads to cell survival (181). The SAFE pathway is also activated by inflammatory cytokines such as IL-6, IL-1 and leukemia inhibitory factor (LIF). Once activated, JAK-STAT3 signalling leads to nuclear transcription of cell survival and proliferation genes, growth factors and other transcription factors (182)(Figure 1.3). Between the SAFE and RISK pathways, there also seems to be some interplay that occurs during I-R injury. In mice given cardiomyocyte specific inhibitors of SAFE or RISK pathway mediators, cardioprotection from both seems to be lost (187-189). The cGMP/PKG pathway has also been identified as a potential therapeutic target that interacts with both the RISK and SAFE pathways (182) Overall, these pathways act to prevent apoptosis and limit mPTP opening during reperfusion.

Like the RISK pathway, preclinical research has shown that the SAFE pathway may be a potential therapeutic target for cardioprotection and ischemic pre- and post-conditioning (190-193). Though, in humans, demonstration of the protective role of STAT has remained to be a challenge. Interestingly, one study found that STAT5 (as opposed to STAT3) is activated during ischemic stimulus in humans (194). Another study showed activation of both STAT3

and STAT5 in children receiving remote preconditioning for tetralogy of Fallot repair surgery (195). Although this pathway may offer a target for limiting I-R injury, further research is required in the human population. Previously, there has been no research regarding the SAFE pathway and cardiac CSS. Further research is required to see if this pathway can be targeted prior to HTx to prevent associated I-R damage during organ preservation.

1.5.3 Variability of the Donor Pool

With limitations in available donor organs, it is important to understand the impacts of using hearts from an expanding donor pool. ISHLT 2020 reports found that donor BMI increases are correlated with donor heart size increasing (146). Oversizing of hearts may be favourable as smaller hearts can be prone to cause increased pulmonary resistance and be prone to graft failures (196, 197). The use of opioid and non-opioid drugs has also increased in recent years. Hence, leading to increased rates of brain death from overdose. These organs have not been found to be adversely impacted and should remain in the current donor pool (146). Coinciding with an increase in opioid and other drug use, the number of hepatitis C positive donors has also increased (146). With the advancements of antiviral drug therapy, these organs are still able to be included and do not increase risk of 1-year mortality (132). More caution should be taken if donors are hepatitis B positive as there is an increased risk of transmission post-transplant. This can be prevented using lamivudine and is relatively safe if care is taken (198). With the donor population changing, it is important that we understand the risks of using these extended criteria organs.

1.5.3.1 Type 2 Diabetes and Donor Heart Viability

In our globally ageing population, the prevalence of diabetes continues to grow. Since 1995, donor heart diabetes has increased from 1.1% to 4.5% as recently as 2018 (146). Due to

limited history of donor diabetes, conflicting evidence has been found. As of 2020, ISHLT has stated that donor diabetes does not influence 1-year mortality but can induce coronary artery vasculopathy (146). Retrospective studies have also confirmed these findings in small cohorts (199, 200). Though, conflicting evidence was found by Stehlik *et al.* (201) finding that diabetes and hypertension impacted long term mortality in males, but not females (201). More research surrounding long-term mortality and coronary artery vasculopathy is required to elucidate the effects of diabetes in donor hearts.

When brain death occurs, the cardiovascular system undergoes immense stress leading to marked activation of the sympathomimetic axis and release of epinephrine, corticosteroids, glucagon and various cytokines (202, 203). The release of these agents initiates gluconeogenesis, impaired release of insulin from the pancreas and hyperglycaemia (204). In a clinical setting, donor hyperglycaemia is now managed but there is little evidence suggesting that this provides improved outcomes (203). As the exact effects of donor diabetes are not completely understood, there has been limited studies modelling this issue. As T2D has been found to reduce cardioprotection and limit tolerance to I-R injury (45, 46, 205, 206), studies modelling CSS or HTx with diabetes may fill this gap in the literature. Interestingly, Tonnesen *et al.* (207) found that inhibition of succinate dehydrogenase and subsequent reduction of succinate reduced infarct size and diabetes severity in diabetic rats (207). Another study assessing Pre-Db rats found that pharmacological inhibition of mitochondrial fission attenuates I-R injury, offering potential novel therapeutic targets (208). Additionally, no research involving Pre-Db and I-R injury during CSS have been completed. In a society with rapidly increasing health concerns, understanding the effects of donor diabetes or Pre-Db may expand the donor pool and give individuals with end-stage CVD a second chance at life.

1.5.3.2 Trimethylamine N-Oxide

Currently, there is no data on donor TMAO levels and risk during organ preservation. With minimal information on TMAO and I-R injury, it is difficult to extrapolate the effects that this gut metabolite may play. Future studies are required to map out any influence that TMAO may have during I-R injury. This could be modelled by inhibition of TMAO using a TMA lyase inhibitor such as DMB to prevent downstream TMAO formation. Overall, research involving CSS and TMAO may uncover how a Western diet and associated microbial pathways may influence success of HTx in patients with end-stage CVD.

Chapter 2. Aims & Hypotheses

This project aims to investigate the effects of TMA lyase inhibition (using DMB) and Pre-Db on the outcomes of CSS. CSS is a clinically relevant model of I-R injury in the setting of HTx. Hence, the project aims to assess how STZ and diet-induced Pre-Db affects CSS outcomes and whether TMA lyase inhibition alter these outcomes in healthy control and Pre-Db hearts.

We will use a murine model to assess, body weight, glucose tolerance, cardiac recovery from CSS, mitochondrial function, and specific protein expression in CTRL, Pre-Db, CTRL + DMB and Pre-Db + DMB hearts.

2.1 Aims

1. To determine whether TMA lyase inhibition modifies Pre-Db progression.

H₀: TMA lyase inhibition does not significantly affect Pre-Db progression.

H₁: TMA lyase inhibition does significantly affect Pre-Db progression.

2. To determine whether chronic TMA lyase inhibition influences cardiac tolerance to ischemia-reperfusion after CSS in Pre-Db hearts.

H₀: TMA lyase inhibition does not significantly influence cardiac tolerance to ischemia-reperfusion after CSS in Pre-Db hearts.

H₁: TMA lyase inhibition does significantly influence cardiac tolerance to ischemia-reperfusion after CSS in Pre-Db hearts.

3. To examine whether chronic TMA lyase inhibition influences cardiac mitochondrial function and expression of proteins associated with mitochondrial function in Pre-Db.

H₀: Chronic exposure to TMA lyase inhibition does not significantly influence cardiac mitochondrial function and expression of proteins associated with mitochondrial function in Pre-Db.

H₁: Chronic exposure to TMA lyase inhibition does significantly influence cardiac

mitochondrial function and expression of proteins associated with mitochondrial function in Pre-Db.

2.2 Overall Hypothesis

Pre-Db will adversely affect cardiac ischemic tolerance and mitochondrial function after CSS and reperfusion whilst chronic TMA lyase inhibition will reverse these negative outcomes through reduction of circulating TMAO in Pre-Db animals.

Chapter 3. Research Methods

3.1 Animal Model

All investigations undertaken were approved in accordance with the policy guidelines “The Animal Care and Protection Act 2001”, of the Animal Ethics Committee of Griffith University, which is accredited by the Queensland Government. Male C57BL/6 mice were supplied by the Animal Resource Centre (Perth, Australia) and housed in the Griffith University Animal Facility for the duration of the study. Animal ethics number AEC:MSC/04/20.

12-week-old C57BL/6 male mice were obtained from the *Animal Resources Centre* (Perth, Australia). They were housed with 5 animals per cage with a 12-hour dark/light cycle (7:00 a.m. – 7:00 p.m.) at 21 °C (40 % humidity), in Green Line GM500 individually ventilated cages stored in DGM racks (Tecniplast S.p.A, Varese, Italy). This animal model was separated into 3 batches of 40 mice: Baseline, Cold Static Storage, Normal Ischemia-Reperfusion,. Baseline hearts were collected after the 14-week feeding protocol and before hearts are subjected to I-R or CSS. Post sacrifice, this project will focus on data collected from baseline and CSS hearts. Normal I-R heart data will be incorporated into an ongoing PhD project. Once housed and habituated, mice were randomly assigned to one of four groups: CTRL, CTRL + DMB, Pre-Db or Pre-Db + DMB groups (Figure 3.1).

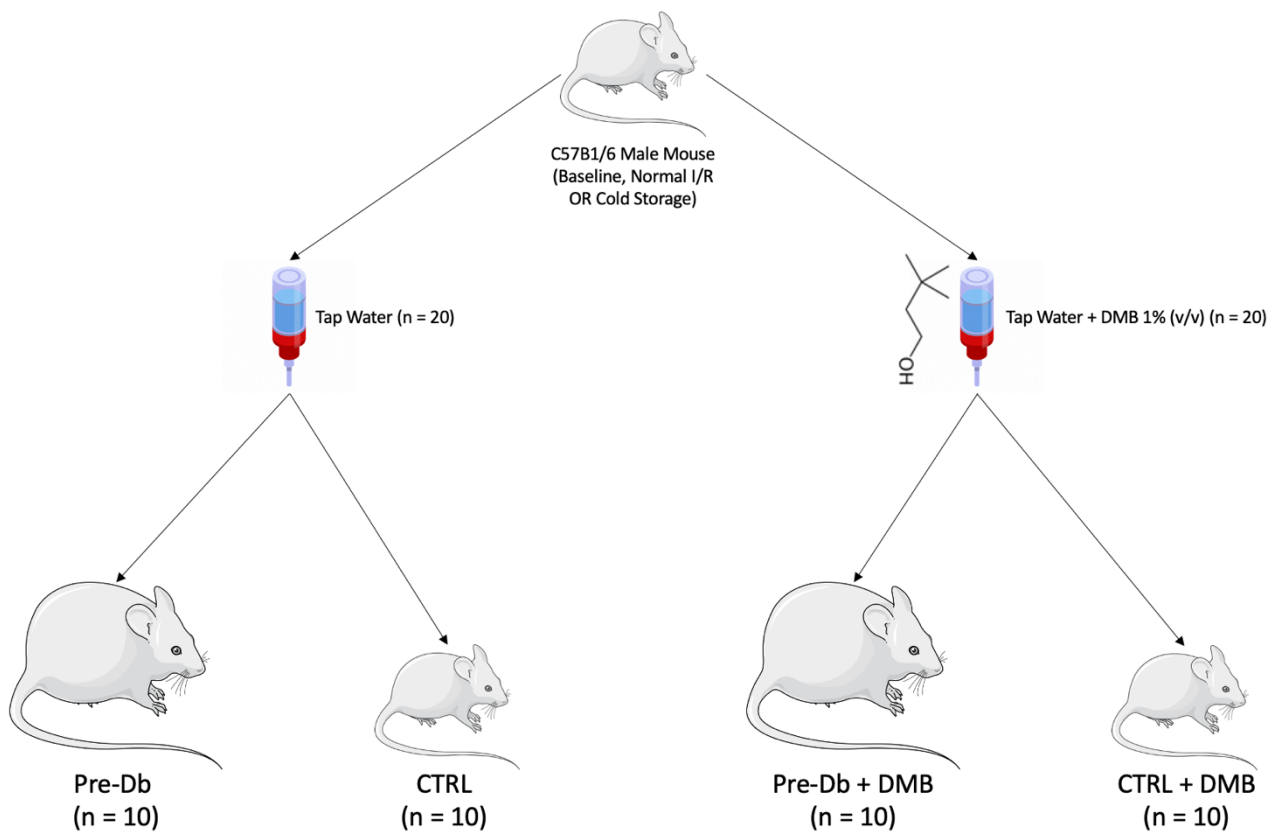


Figure 3.1. Animal model experimental groups. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control.

3.1.1 Prediabetic Murine Model & Composition of Diet

All CTRL mice (n = 20) were provided with a single dose of Na-Citrate (vehicle) injections and fed a standard rodent chow (18.8 % protein, 63.4 % carbohydrates, 17.8 % fat; Speciality Feeds, Glen Forrest, Western Australia). Mice in the Pre-Db groups were administered with a single intraperitoneal (IP) injection of STZ (75 mg/kg) and fed a ‘Western’ Diet (12.7 % protein, 29.7 % carbohydrates, 43.2 % fat) for 14 weeks to produce a Pre-Db phenotype (n = 20). The purpose made high-fat, high-carbohydrate diet (SF18-004) was purchased (Speciality Feeds, Glen Forrest, Western Australia) and refrigerated. Prior studies within our lab have found that a purpose made ‘Western’ diet causes increased body mass, visceral fat accumulation, insulin resistance, and decreased myocardial tolerance to

ischemia/reperfusion in C57BL/6 mice (14, 209). Food and water were available ad libitum for all mice (Appendix Table 1 for detailed dietary breakdown).

3.1.2 3,3-Dimethyl-1-Butanol Water Supplement

Mice in the DMB groups were supplemented with 3,3-Dimethyl-1-Butanol (1.0 % v/v) in their drinking water. DMB is a TMA lyase inhibitor, hence is used to inhibit the production of TMAO (86, 100). All CTRL mice were provided with normal tap water from the lab. Crude weekly water consumption was calculated by measuring the total water consumed from each bottle in a cage and determining the average for the 5 mice.

3.2 Phenotypic Characteristics

Body weight and water consumption was assessed weekly using a laboratory scale (A&D Weighing GX-2000 precision scale, A&D Australasia Pty. Ltd, Adelaide, South Australia) from 12 weeks of age until sacrifice. Glucose and insulin were assessed at week 6 and 12 to track diabetes progression (see below).

3.2.1 Glucose Tolerance Tests

Glucose tolerance tests (GTT) were performed to assess glucose clearance and diabetic phenotype progression at week 6 and 12 of the feeding protocol. Mice were fasted 4-6 hours prior to blood sampling via tail tipping. Glucose was measured using an Accu-check II glucometer (Roche Diagnostics, Castle Hill, Australia) to assess fasting blood glucose. Mice then received an IP administration of 20% glucose (2 g/kg) and blood glucose was measured after 15, 30, 60, 90, 120 and 180 minutes. Glucose area under the curve (AUC) was calculated to compare glucose clearance between groups. Half of the mice from each group were randomly selected to undergo GTT (n = 5).

3.2.2 Biochemical Assessment

For assessment of biochemical changes, fasted and non-fasted blood was collected into EDTA coated tubes via tail tipping. Whole blood was stored on ice for 55 ± 5 minutes before being centrifuged at 1000RPM for 10 minutes. Samples were stored at $-80\text{ }^{\circ}\text{C}$ for ELISA analysis according to manufacturer's instructions (insulin: Crystal Chem Inc, Elk Grove Village, USA), using a Tecan or BioPlex plate reader. Fasting insulin was assessed at week 12 and HOMA-IR was used as an indirect measure of insulin resistance. HOMA-IR was calculated as: $\text{fasting insulin } (\mu\text{U/L}) \times \text{fasting glucose } (\text{mg/dL}) / 405$. Other samples were then transferred to centrifugal filter units and centrifuged at 14000RCF for 40 minutes at $4\text{ }^{\circ}\text{C}$. Filtered serum lighter than 3 kDa was then transferred into collection tubes for future biochemical analysis.

3.3 Cardiac Outcomes

3.3.1 Heart Perfusion and Post I-R Assessments

Assessment of cardiac function and response to I-R has been developed in the Heart Foundation Research Centre Laboratory using a Langendorff perfusion mouse model and has been replicated numerous times in our laboratory (15, 210).

At the end of the feeding protocol hearts were removed and Langendorff perfused for assessment of function and I-R tolerance during CSS. Mice were anaesthetised by intraperitoneal injection of sodium pentobarbital (60 mg/kg IP). Level of consciousness and function of anaesthesia was confirmed by assessing pedal withdrawal reflexes at 5-minute intervals. Mice were thoracotomised, hearts rapidly excised and the aorta immediately cannulated for perfusion of the coronary circulation at 80 mmHg with modified Krebs-Henseleit buffer, gassed with 95 % O₂/ 5 % CO₂, maintained at $37\text{ }^{\circ}\text{C}$ (pH 7.4) and containing: 119 mM NaCl, 11 mM glucose, 22 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.2 mM EDTA, 0.5 mM and 2.5 mM CaCl₂.

Contractile function was monitored via a fluid-filled balloon in the left ventricle, inflated to an end-diastolic pressure (EDP) of 5 mmHg (15). Balloons were connected by fluid-filled tubing to a P23 XL pressure transducer (Viggo-Spectramed, Oxnard, CA), permitting continuous assessment of contractile function. Coronary flow was measured via ultrasonic flow- probe proximal to the aortic cannula and connected to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA). A 4 channel MacLab system (ADInstruments Pty Ltd., Castle Hill, Australia) connected to an Apple iMac computer was used for continuous acquisition (1 KHz sampling rate) and processing of data, including: systolic and end diastolic pressures, +dP/dt and -dP/dt, heart rate and coronary flow. Temperature of perfusate was continuously monitored via a thermal probe connected to a Physitemp TH-8 digital thermometer (Physitemp Instruments Inc, Clifton, NJ, USA).

Hearts were stabilised over 20 minutes, with hearts exhibiting abnormal or unstable function excluded. Hearts were then switched to ventricular pacing at 420 beats/min (via silver wires attached to an SD9 stimulator; Grass Instruments, Quincy, MA, USA) for 10 minutes before undergoing 3 hours of cold ischemia 4-6 °C (Appendix Figure 1), followed by 45 minutes of aerobic reperfusion at 37 °C.

3.3.2 Cell Death via Lactate Dehydrogenase Efflux

To estimate myocardial cell death, total lactate dehydrogenase (LDH) efflux was measured for each heart after 15 minutes (E1) and 45 minutes (E2) of reperfusion. Post ischemic coronary effluent was collected on ice at the two time points and assayed at 37 °C using 3 mL cuvettes at 340nm (Tecan Sunrise). During this enzymatic coupling reaction, LDH released from the cell oxidizes lactate to generate NADH. Absorbance was then quantified by the amount of NADH present. The average change in absorbance per minute was calculated

and applied to the effluent volume to discern the quantity of LDH (IU). LDH efflux were further normalised by heart mass (IU/g).

3.3.3 Heart Tissue Dissection

Post-Langendorff perfusion protocol, hearts were dissected on frozen Krebs-Henseleit buffer. For hearts undergoing Oxygraph-2k analysis, a section of left ventricle was removed and prepared. Two samples of both left and right ventricles were then extracted and snap frozen in liquid nitrogen and then stored at -80 °C for future protein analysis or for any further studies that may be completed.

3.3.4 Other Tissue Dissection

Following heart excision, non-fasted blood was extracted using a 1 mL Tuberculin syringe for assessment of neuroendocrine and inflammatory changes. It was separated into a 300 µL EDTA-coated tube (plasma) and a 1.5 mL Eppendorf tube (serum). Centrifugation of plasma was completed immediately at 4 °C for 10 minutes at 2000 RCF while serum was allowed to coagulate for 30-60 minutes and then spun at 4 °C for 10 minutes at 1000 RCF. Samples stored at -80 °C until ELISA and Mass Spectrometry Analysis.

Brain, liver, and gut tissue were excised and snap frozen immediately. All samples stored at -80 °C for future protein and RNA analysis. Additionally, stool samples were collected for future bacterial analysis and Mass Spectrometry.

3.3.5 Mitochondrial Respirometry

After removal from the chest or Langendorff apparatus, hearts were dissected and immediately prepared for mitochondrial analysis. Approximately 8-10 mg samples were then sectioned from the whole heart and blot dried on filter paper prior to being dissected into 3-4

smaller pieces with a scalpel. The myocardial samples were added to a glass dounce and immediately suspended in Mir06 media to a concentration of 1 mg/mL. The tissue was kept ice cold and homogenised.

Left ventricular oxygen consumption was quantified using a Oxygraph-2k instrument (Oroboros Instruments, Innsbruck, Austria). Chambers A and B of the apparatus were cleaned with ethanol and milliQ water prior to stabilisation with MiR06 media containing: 0.5 mM EDTA, 3 mM MgCl₂.6H₂O, 60 mM K- lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g BSA (pH to 7.1 at 37 °C). After stabilisation, 2.2mL of heart tissue homogenate (1 mg/mL) was added to each chamber and analysis of mitochondrial respiration at 37 °C. Datalab software (Oroboros Instruments) was used to undertake the quantitative analysis.

Analysis of maximal mitochondrial respiration and complex I, complex II and complex IV function was achieved upon the stepwise utilisation of the following mitochondrial complex I and complex II substrates: 5 mM pyruvate, 2 mM malate, 10 mM glutamate, 10 mM succinate, 10 µM cytochrome c, 1 mM ADP. Additionally, mitochondrial uncoupling was achieved using 0.5 µM CCCP. As a quality control measure, cytochrome c was used to validate mitochondrial integrity, and various mitochondrial complex inhibitors were used to determine individual complex functions: 0.5 µM rotenone, 5 mM malonic acid, 2.5 µM antimycin A. Additionally, residual oxygen consumption (ROX) was determined and subtracted from all readings to ensure non-mitochondrial respiration was accounted for.

3.4 Protein Detection & Analysis

3.4.1 Subcellular Fractionation

Protein isolation was completed on tissue sections of left ventricles of excised mouse hearts. Tissue samples were sectioned into smaller pieces prior to homogenisation in a glass

dounce with ice-cold lysis buffer containing: 1 % Triton X, 1 mM PMSF, 10 μ M leupeptin, 3 mM benzamidine, 5 μ M pepstatin A, 1 mM NaO and KINEXUS (20 mM MOPS, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 50 mM β -glycerophosphate, 50 mM NaPP).

Whole homogenate samples were removed and stored in lysis buffer for future analysis. Homogenate was then centrifuged at 600 g for 10 minutes at 4 °C. The nuclear fraction was then removed, pellet resuspended in mitochondrial-isolation buffer containing: 1 mM PMSF, 10 μ M leupeptin, 3 mM benzamidine, 5 μ M pepstatin A, 1 mM NaO, 70mM sucrose, 190mM Mannitol, 20mM HEPES, 0.2mM EDTA and KINEXUS (20 mM MOPS, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 50 mM β -glycerophosphate, 50 mM NaPP).

The resuspended sample (mitochondria) was then centrifuged at 10,000 g for 30 minutes at 4 °C. An isolated mitochondrial supernatant was then separated and stored at -80 °C. The remaining pellet was then resuspended in lysis buffer (211, 212).

The final supernatant was centrifuged at 100,000 g for 1.5 hours at 4 °C. The pellet left over (membrane rich) was resuspended in lysis buffer. The supernatant (cytosol) was then transferred to a new tube and stored at -80 °C for future protein analysis (213).

3.4.2 Western Immunoblotting

Once thawed, protein aliquots were prepared in required volumes with loading dye and denatured at 95 °C for 5 minutes in a heating block. A 30 μ L volume of each sample containing 20 μ g of protein was loaded into hand-cast 10 % acrylamide gels. Protein separation was achieved by running gels at 120 V for 90 minutes. The transfer of proteins was achieved using a polyvinylidene difluoride fluorescent (PDVF) membrane at a constant 75 V for 2-2.5 hours, and then blocking with Odyssey fish serum for an additional 1.5 hours at room temperature.

The membranes containing transferred proteins were incubated with the following primary antibodies overnight at 4 °C with gentle agitation: total AKT 1:1000 (9272S, Cell

Signalling); phosphorylated AKT 1:1000 (9271S, Cell Signalling); total ERK1/2 1:1000 (ab4782, abcam); phosphorylated ERK1/2 1:1000 (9101S, Cell Signalling); total GSK-3B 1:1000 (9315S, Cell Signalling); phosphorylated GSK-3B 1:1000 (9336S, Cell Signalling); total STAT3 1:1000 (12640, Cell Signalling); phosphorylated STAT3 1:1000 (9415, Cell Signalling); OXPPOS Cocktail 1:500 (ab110413, abcam).

The PVDF membrane was washed 4 times in TBST (5 minutes each) and again in TBS for 10 minutes before incubation with corresponding secondary antibodies at room temperature in the dark: IRDye® 680RD donkey anti-mouse 1:30,000 (925-68072, LI-COR); or IRDye® 680RD goat anti-rabbit 1:30,000 (925-68071, LI-COR). Membranes were then washed again 4 times in TBST (5 minutes each) and in TBS for 10 minutes, before drying overnight between paper towels in the dark. Membranes were visualised on a Licor Odyssey Infrared Imaging System (Millenium Science, Mulgrave, Australia) and protein densitometry data for each sample normalised to GAPDH, β -Actin or total protein as a loading control. To compare protein expression both within and between gels, sample data was also normalised to an internal standard. For cytosolic and whole cell analysis, a mixed whole cell internal standard was used. For mitochondrial analysis, a mixed mitochondrial fraction internal standard was used.

3.5 Statistical Analysis

All data analysed using GraphPad Prism 8 and presented as mean \pm SEM. Grubb's tests were used to determine all outliers within datasets. Student's T-tests were used to determine differences between two groups, while one-way ANOVAs were used to compare 3 or more groups. Fisher's LSD post-hoc test was used following ANOVA. P-value of < 0.05 was considered statistically significant throughout.

Chapter 4. Results

4.1 Phenotype Characteristics

Several phenotype characteristics were analysed during the 14-week feeding protocol to map Pre-Db progression, cardiometabolic risk factors and effect of TMAO inhibition. To stratify the data, baseline, normal ischemia-reperfusion, and CSS mice were included in the phenotype analysis.

4.1.1 Body Weight

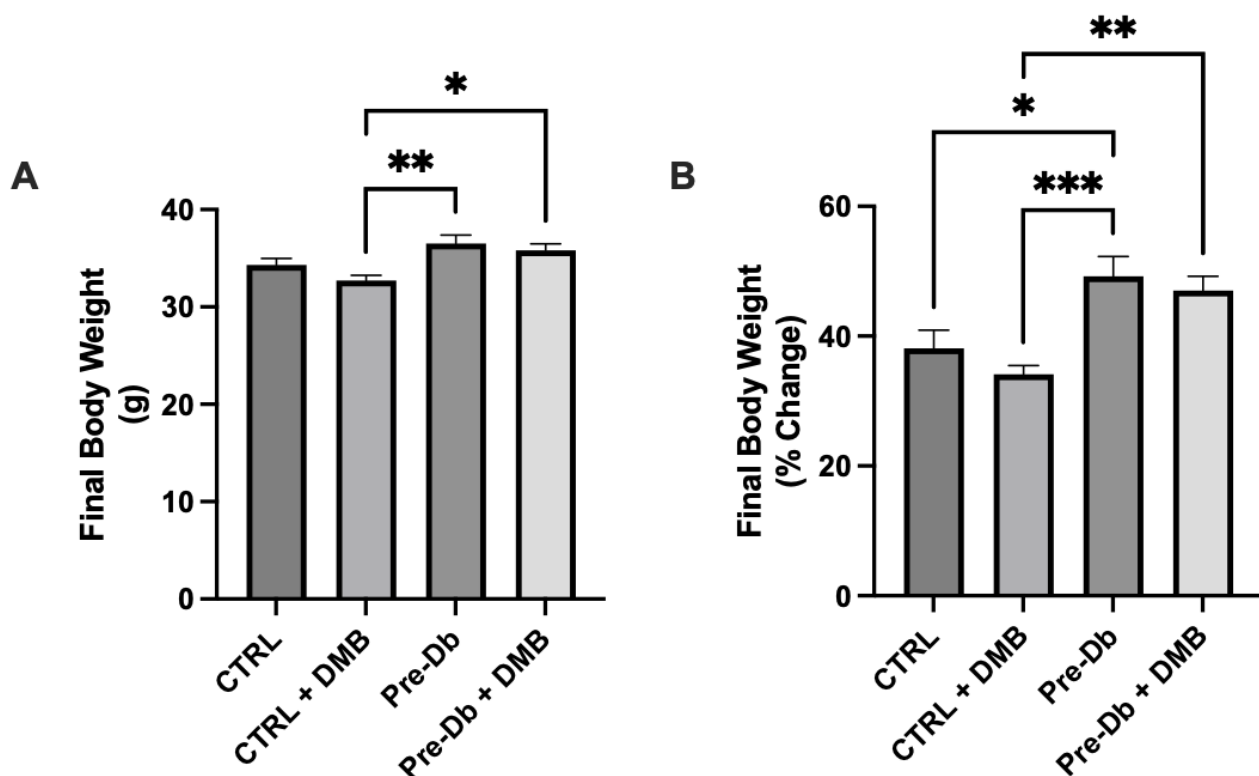


Figure 4.1. Final Body Weights (post-14-week feeding protocol). (A) Final Body Weight (g). (B) Final Body Weight (% Change). DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 29-31$.

After a single STZ-injection in non-fasted animals followed by 14 weeks of a high-fat Western-type diet feeding, Pre-Db (36.5 ± 0.9 g) mice displayed no change in final body weight compared to CTRL (34.4 ± 0.7 g, $p > 0.05$) mice. However, Pre-Db ($p < 0.01$) and Pre-Db + DMB (35.8 ± 0.7 g; $p < 0.01$) animals presented with 11% and 9% increases in mass vs CTRL + DMB (32.7 ± 0.5 g) animals respectively (Figure 4.1A).

When comparing the percent change in body weight from week 1 to week 14, Pre-Db ($49.2 \pm 3.1 \%$, $p < 0.05$) mice percent change in body weight was increased compared to CTRL ($38.1 \pm 2.8 \%$) animals. The CTRL + DMB ($34.1 \pm 1.4 \%$) group also presented with significantly reduced body weight changes compared with Pre-Db ($49.2 \pm 3.1 \%$, $p < 0.001$) and Pre-Db + DMB ($47.0 \pm 2.2 \%$, $p < 0.01$) animals (Figure 4.1B).

4.1.2 Prediabetic Phenotype

To map the progression of Pre-Db in the animals; fasted glucose, fasted insulin, and glucose tolerance tests were measured. Fasted glucose and glucose tolerance were assessed at week 6 and week 12. Insulin was assessed at week 12 only.

4.1.2.1 Blood Glucose

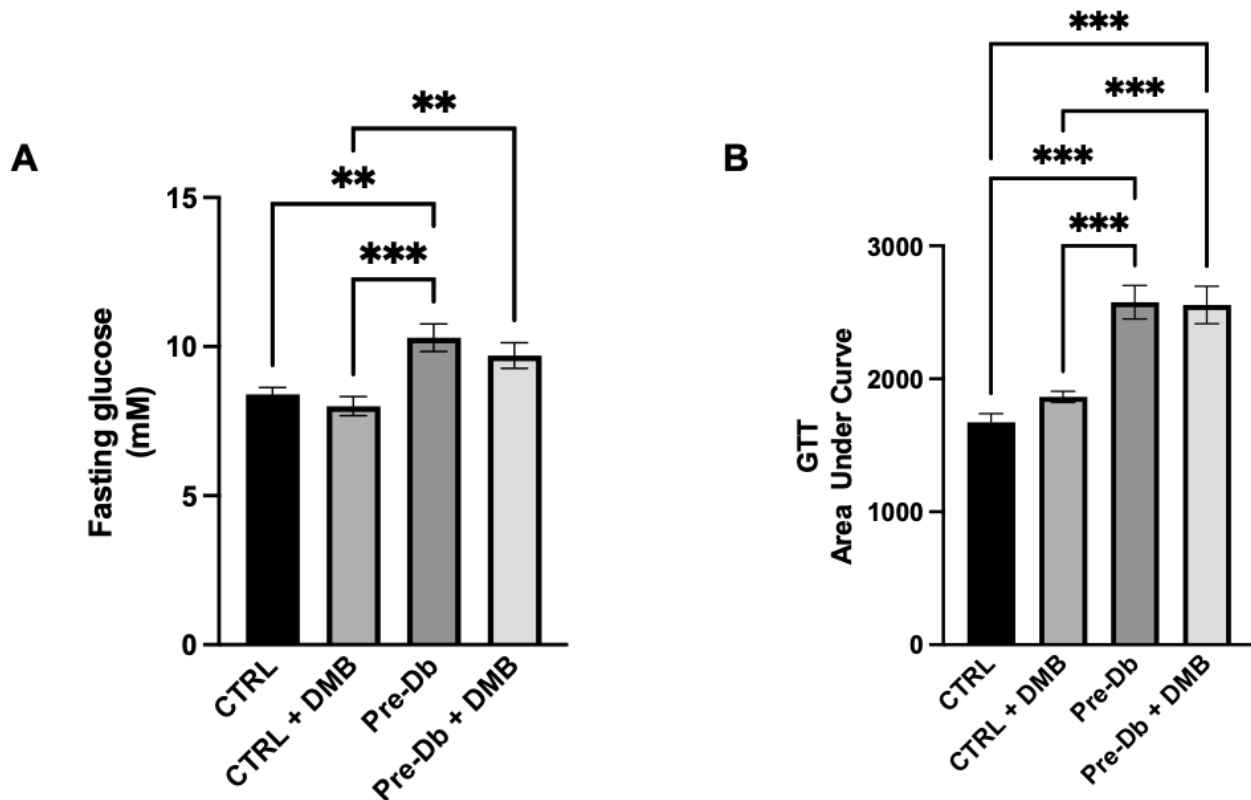


Figure 4.2. Combined Fasting Glucose and GTT at week 6. (A) Fasting blood glucose of mice fasted for 4-6 hours. (B) AUC from GTT. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 14-16$.

After 6-weeks of the feeding protocol, Pre-Db (10.6 ± 0.5 mM) had higher fasting glucose levels than CTRL (8.4 ± 0.2 mM, $p < 0.01$) and CTRL + DMB (8.0 ± 0.3 mM, $p < 0.01$) animals. Pre-Db + DMB (9.7 ± 0.4 mM) was significantly higher than CTRL + DMB ($p < 0.01$) mice but not the CTRL ($p > 0.05$) animals. Fasting glucose in Pre-Db + DMB mice was unchanged from Pre-Db ($p > 0.05$) alone (Figure 4.2A).

When assessing the week 6 GTT, the Pre-Db (2575 ± 126.0) and Pre-Db + DMB (2555.8 ± 141.6) animals had a significantly larger AUC when compared to both the CTRL (1673.9 ± 64.8 , $p < 0.001$) and CTRL + DMB (1864.9 ± 41.9 , $p < 0.001$) groups (Figure 4.2B).

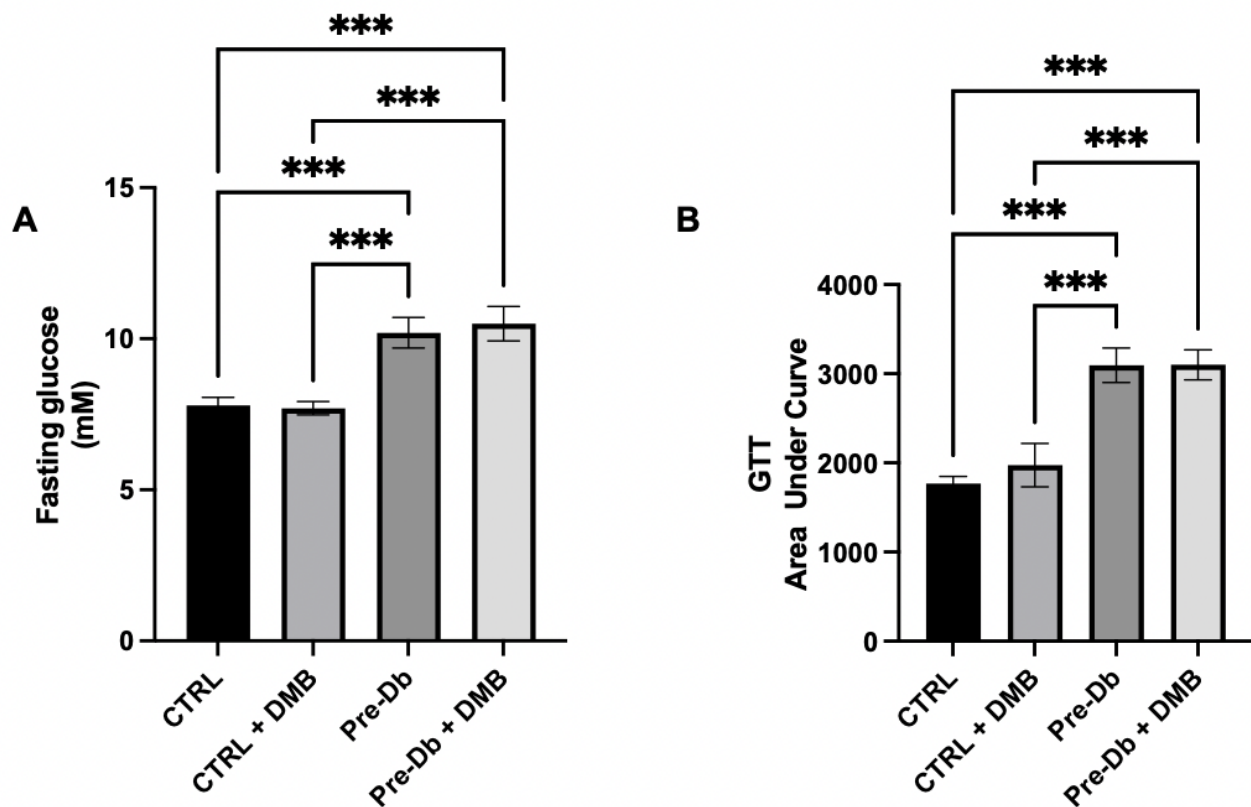


Figure 4.3. Combined Fasting Glucose and GTT at week 12. (A) Fasting blood glucose of mice fasted for 4-6 hours. (B) AUC from GTT. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 13-16$.

At week 12 of the feeding protocol, fasting glucose was approximately 30 % higher in the Pre-Db (10.2 ± 0.6 mM) group compared to both CTRL (7.8 ± 0.3 mM, $p < 0.001$) and CTRL + DMB (7.7 ± 0.2 mM, $p < 0.001$) groups. Additionally, Pre-Db + DMB (10.5 ± 0.5 mM) animals also had greater fasting glucose than CTRL ($p < 0.001$) and CTRL + DMB ($p < 0.001$) groups. Again, Pre-Db + DMB mice presented with fasting glucose unchanged from the Pre-Db group (Figure 4.3A).

When assessing the week 12 GTT, there was a larger AUC calculated in the Pre-Db groups (Pre-Db: 3094.5 ± 194.4 ; Pre-Db + DMB: 3101.4 ± 168.4 ; $p < 0.001$) compared to both CTRL diet groups (Figure 4.3B).

4.1.2.2 Insulin

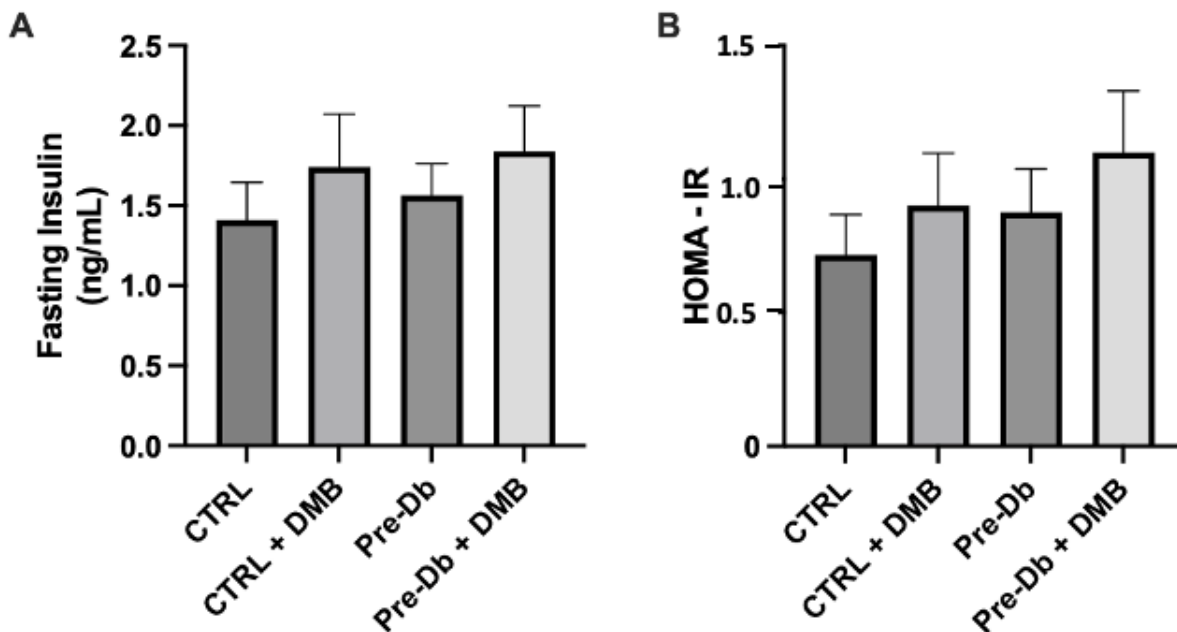


Figure 4.4. Combined Fasting Insulin at week 12. (A) Fasting blood insulin of mice fasted for 4-6 hours. (B) HOMA-IR. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 14-15.

Fasting insulin and HOMA-IR was unchanged compared to CTRL animals at week 12 ($p > 0.05$, Figure 4.4A & Figure 4.4B).

4.1.3 Water Consumption

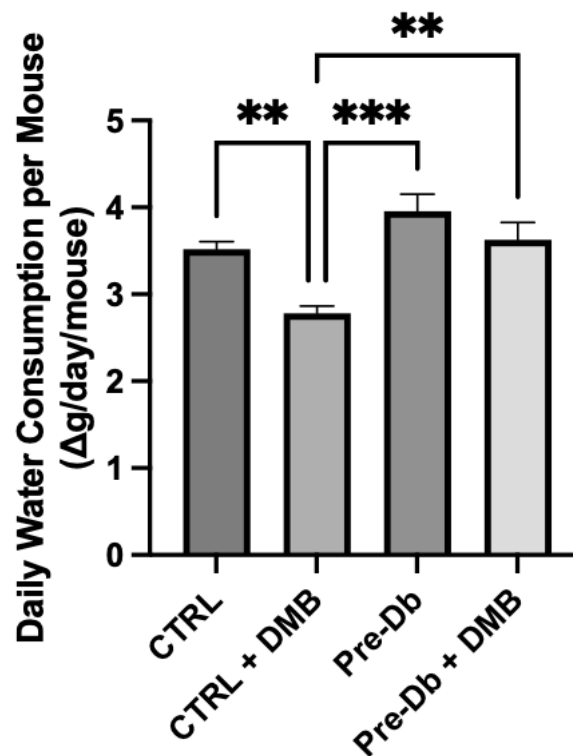


Figure 4.5. Daily Bottle Weight Changes per Mouse. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 29-31$.

After the addition of DMB to the treatment group's water, daily water consumption in CTRL + DMB (2.8 ± 0.08 g) animals was decreased compared to CTRL (3.5 ± 0.09 g, $p < 0.005$), Pre-Db (4.0 ± 0.2 g, $p < 0.001$) and Pre-Db + DMB (3.6 ± 0.2 g, $p < 0.005$) groups (Figure 4.5).

4.2 Cardiac Ischemic and Post-ischemic Function

The following data is relevant to the Baseline and CSS hearts. Normal I-R hearts were excluded for the purpose of this project. Several variables were measured during ischemia and at post-ischemic time points to assess cardiac function, tolerance, and recovery from cold ischemia.

4.2.1 Cardiac Perfusion and I-R Response

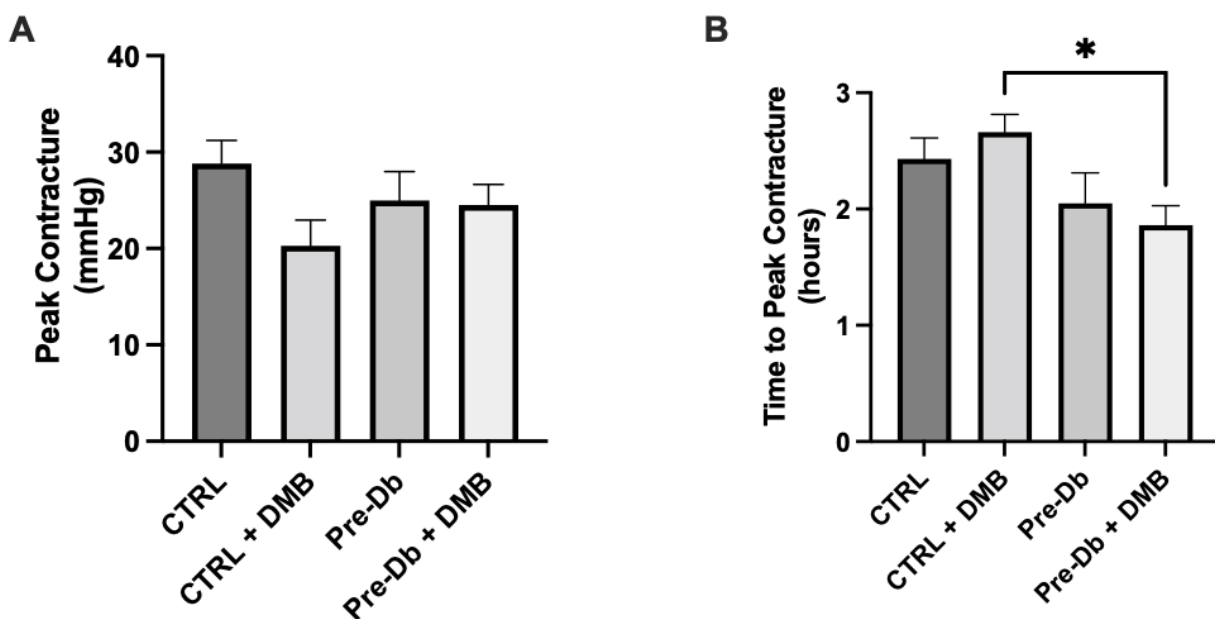


Figure 4.6. Extent and rate of cardiac ischemic contracture. (A) Peak contracture. (B) Time to peak contracture. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$. $n = 9-10$.

No significant difference in peak ischemic contracture was found compared to CTRL (28.8 ± 2.4 mmHg). Interestingly, CTRL + DMB (20.3 ± 2.7 mmHg) animals trend towards a lower peak contracture compared to all other groups ($p > 0.05$, Figure 4.6A).

When assessing the time to peak ischemic contracture, no difference was found vs CTRL (2.43 ± 0.18 hours). However, CTRL + DMB (2.66 ± 0.15 hours) mice took an average

of 48 minutes longer to reach peak contracture compared to the Pre-Db + DMB (1.86 ± 0.17 hours, $p < 0.05$) mice (Figure 4.6B).

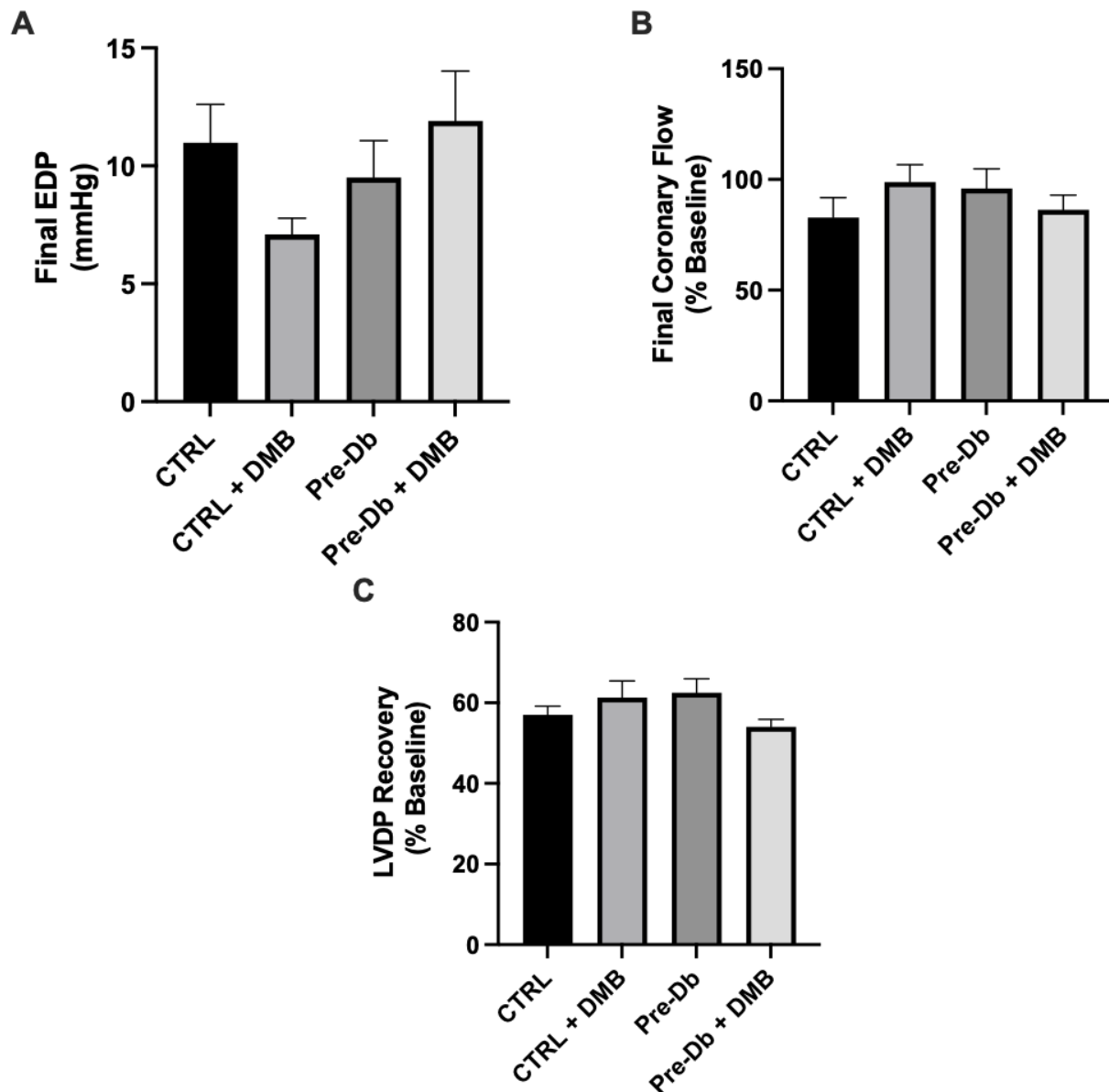


Figure 4.7. Post-ischemic outcomes following 3 hours of cold ischemia/ 45 minutes of reperfusion. (A) Final End Diastolic Pressure (EDP). (B) Final Coronary Flow. (C) Left Ventricular Developed Pressure (LVDP) Recovery. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 9-10.

There were no differences in Final EDP between groups. (Figure 4.7A). Additionally, no significant differences were found between groups for Final Coronary Flow or LVDP Recovery (Figure 4.7B & Figure 4.7C).

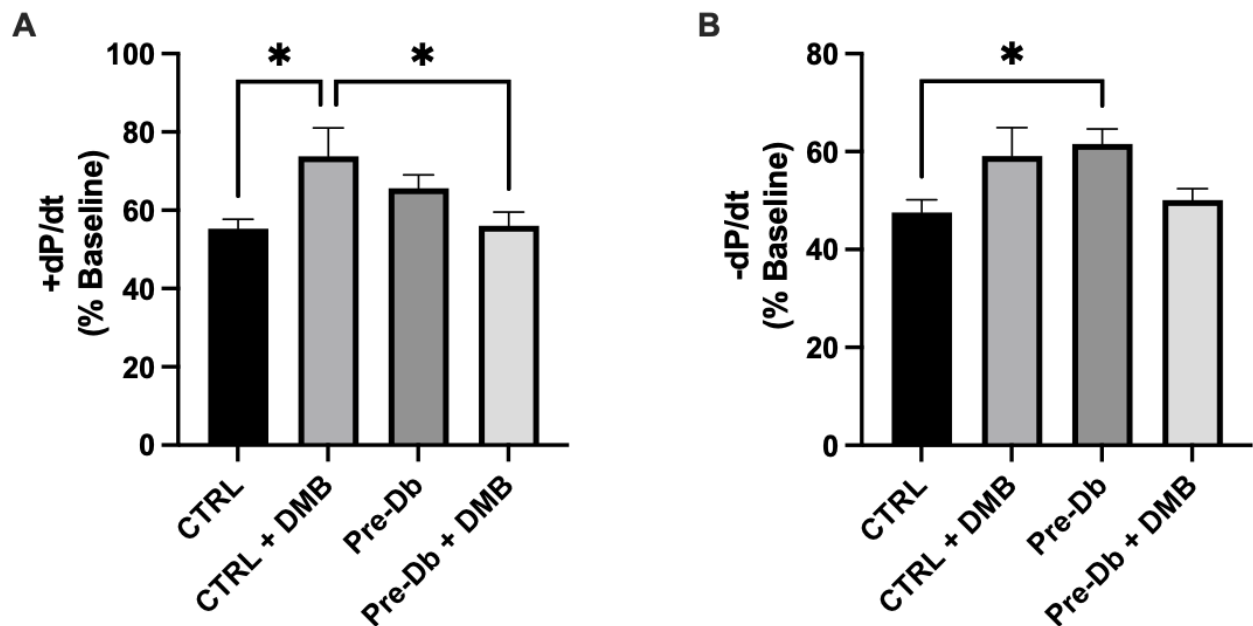


Figure 4.8. Change in pressure over change in time as a percentage of baseline. (A) +dP/dt Recovery (B) -dP/dt Recovery. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$. $n = 9-10$.

There was a significant increase in recovery of +dP/dt after ischemia between CTRL ($55.3 \pm 2.4\%$) vs CTRL + DMB ($73.8 \pm 7.2\%$, $p < 0.05$) mice. However, no change was noted when comparing CTRL to other groups. Additionally, the +dP/dt recovery was significantly higher in the CTRL + DMB group when compared to Pre-Db + DMB ($56.0 \pm 3.5\%$, $p < 0.05$) group (Figure 4.8A).

When assessing recovery for -dP/dt after ischemia, Pre-Db ($61.6 \pm 3.1\%$, $p < 0.05$) animals had elevated recovery compared to CTRL ($47.6 \pm 2.6\%$) mice. No other statistically significant changes were noted (Figure 4.8B).

4.2.2 Lactate Dehydrogenase Release

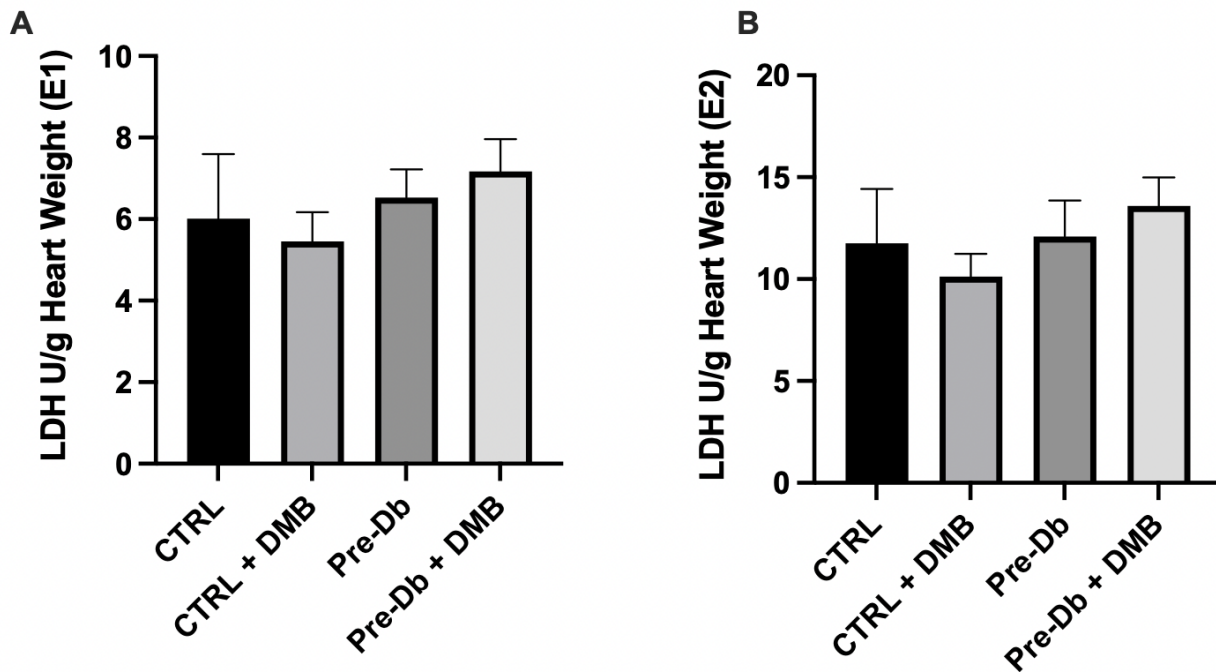


Figure 4.9. LDH release from coronary effluent after CSS. Units of LDH enzyme present per gram of heart weight in coronary effluent. (A) Coronary effluent taken at 15 minutes of reperfusion (B) Coronary effluent taken at 45 minutes of reperfusion. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 9-10.

When assessing the cell death marker, LDH release was consistent between groups at both 15 minutes and 45 minutes of reperfusion (Figure 4.9A & Figure 4.9B).

4.2.3 Mitochondrial Function

4.2.3.1 Baseline Mitochondrial Respiration

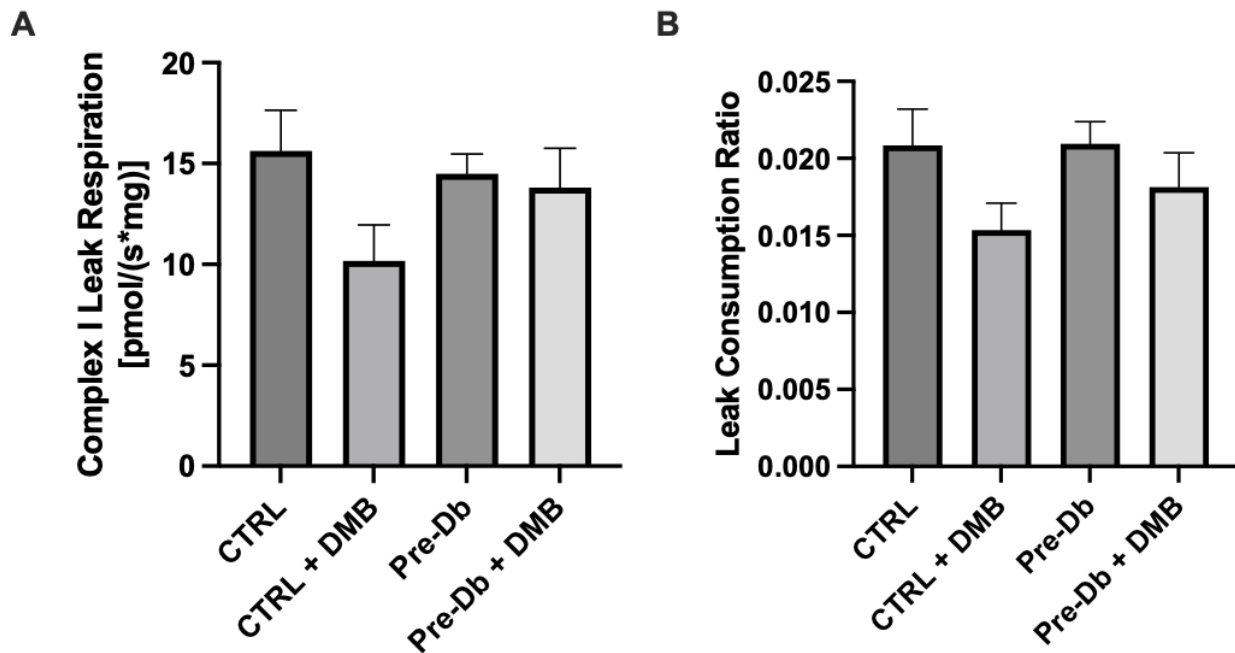


Figure 4.10. Left ventricular oxygen consumption in murine hearts at baseline. (A) Complex I Leak respiration (B) Leak Consumption Ratio. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 7-10.

In the current study, baseline hearts are collected at the conclusion of the 14-week feeding protocol, prior to CSS. For hearts at baseline, there was no significant change between groups for Complex I Leak Respiration (Figure 4.10A).

The Leak Consumption Ratio for all groups was also measured as unchanged compared to CTRL animals. This is calculated by normalising the Complex I leak respiration to maximal respiration (Figure 4.10A & Figure 4.10B).

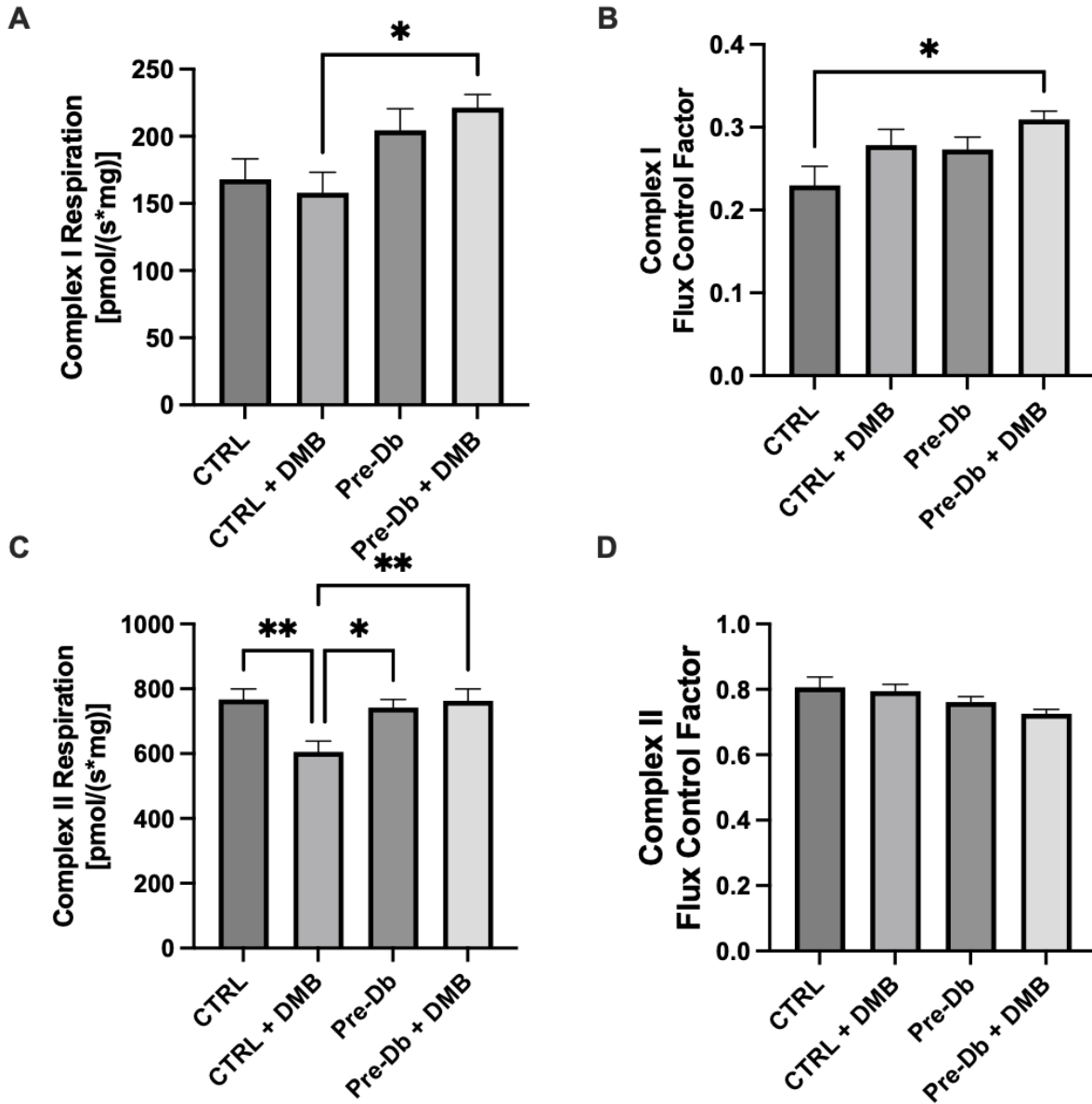
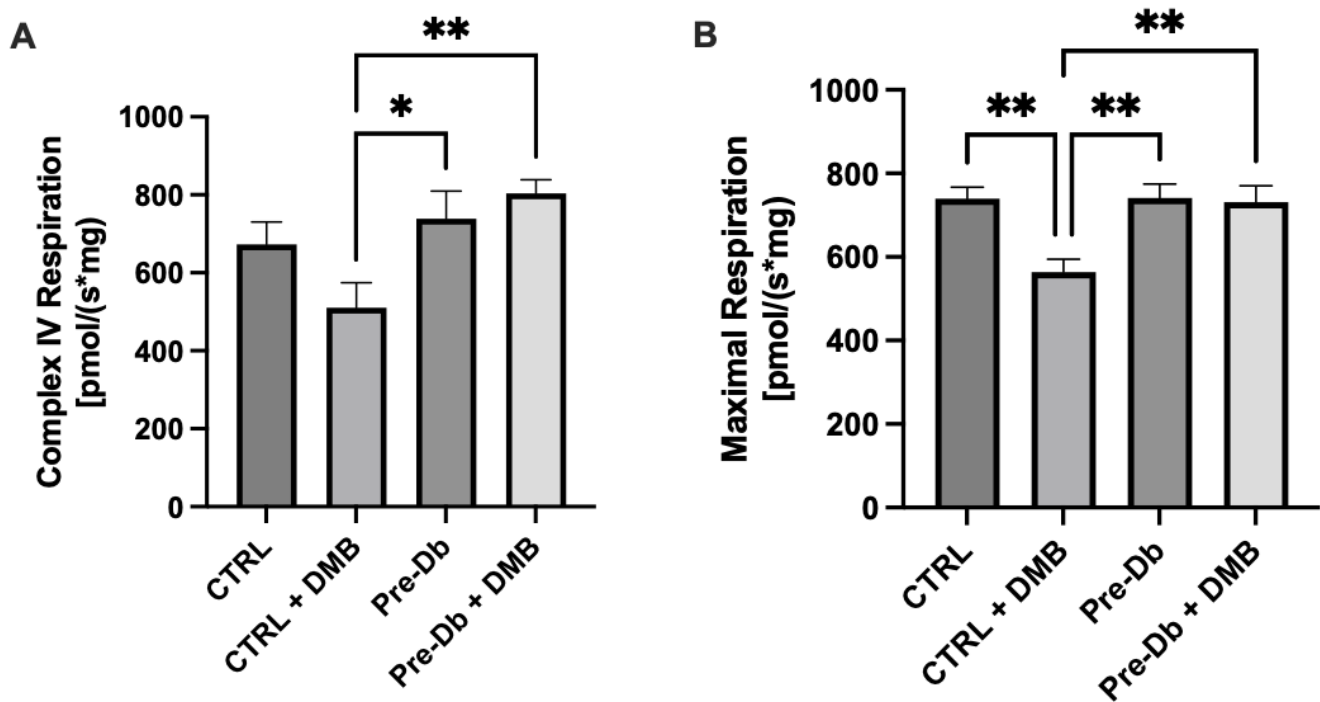


Figure 4.11. Left ventricular oxygen consumption in murine hearts at baseline. (A) Complex I respiration (B) Complex I Flux Control Factor (C) Complex II respiration (D) Complex II Flux Control Factor. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. $n = 7-10$.

There was no significant change in Complex I respiration at baseline between groups (Figure 4.11A). However, when normalised to maximal respiration and converted to Flux Control Ratio, complex I respiration of CTRL (0.23 ± 0.02) mice was reduced compared to Pre-Db + DMB (0.31 ± 0.01 , $p < 0.05$). No change was noted for other comparisons (Figure 4.11B).

Complex II respiration was considerably decreased in CTRL + DMB (605.4 ± 33.6 pmol/(s*mg)) vs CTRL (767.4 ± 32.3 pmol/(s*mg), $p < 0.01$) animals. All other comparisons to CTRL were not significant. Additionally, it was found that CTRL + DMB mice also had lower Complex II Respiration when comparing to Pre-Db (742.3 ± 24.4 pmol/(s*mg), $p < 0.05$) and Pre-Db + DMB (762.8 ± 36.7 pmol/(s*mg), $p < 0.01$) groups (Figure 4.11C). However, when normalised to maximal respiration, these changes were negated (Figure 4.11D).

Figure 4.12. Left ventricular oxygen consumption in murine hearts at baseline. (A) Complex



IV respiration (B) Maximal Respiration. DMB – 3,3-Dimethyl-1-butanol, Pre-Db - Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. n = 7-10.

With regards to Complex IV, there was no significant change when comparing to CTRL (673.1 ± 56.9 pmol/(s*mL)) animals for baseline hearts. There was however a decrease in respiration in CTRL + DMB (510.2 ± 64.8 pmol/(s*mL)) compared to Pre-Db (736.9 ± 70.6 pmol/(s*mL), $p < 0.05$) and Pre-Db + DMB (803.5 ± 35.6 pmol/(s*mL), $p < 0.01$) mice (Figure 4.12A).

Maximal Respiration in CTRL (740.0 ± 27.3 pmol/(s*mL)) animals was 31% higher than CTRL + DMB (564.1 ± 30.2 pmol/(s*mL), $p < 0.01$) mice at baseline. Additionally, CTRL + DMB mice also had a decline in Maximal Respiration when compared to both Pre-Db (741 ± 33.0 pmol/(s*mL), $p < 0.01$) and Pre-Db + DMB (731.5 ± 38.8 pmol/(s*mL), $p < 0.01$) groups (Figure 4.12B).

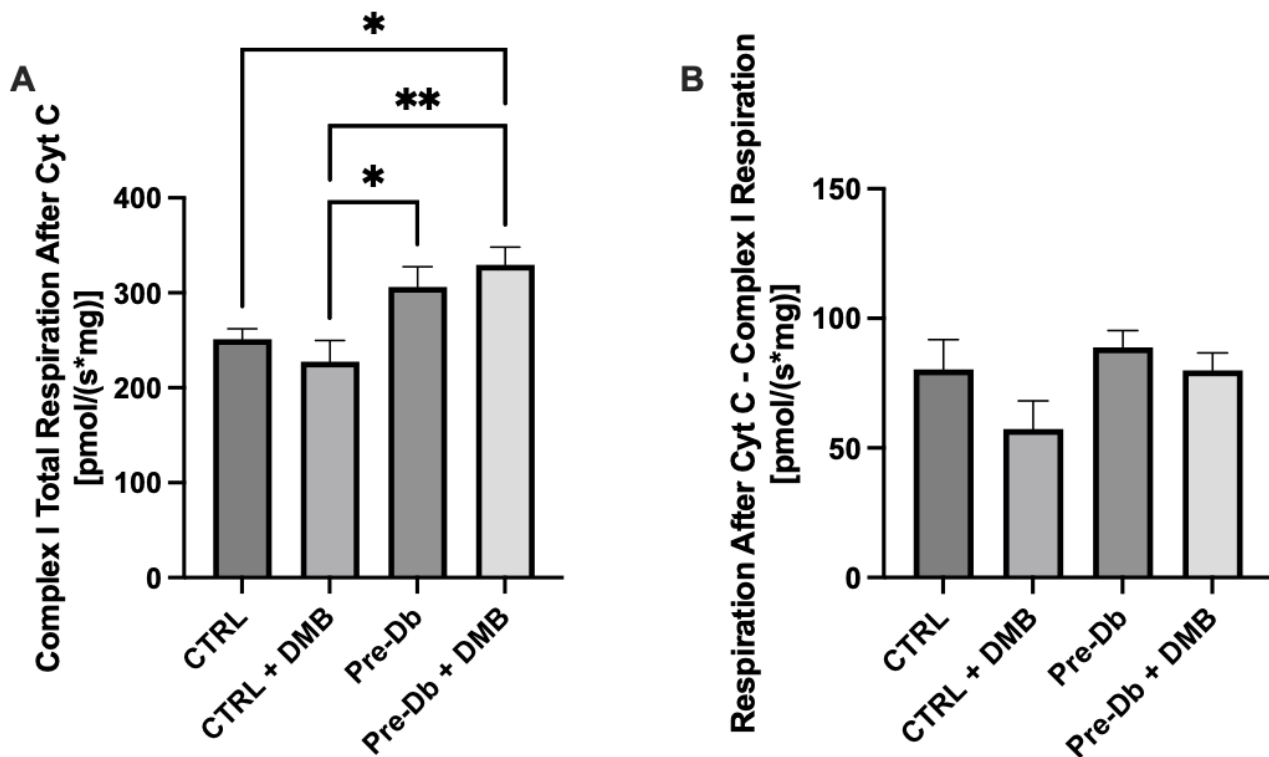


Figure 4.13. Left ventricular oxygen consumption in murine hearts at baseline. (A) Complex I Total Respiration After Addition of Cytochrome C (B) Complex I Respiration After Addition of Cytochrome C. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. $n = 7-10$.

After the addition of Cytochrome C, Complex I respiration was increased in T2D + DMB (329.3 ± 18.7 pmol/(s*mg), $p < 0.05$) mice vs CTRL (251.2 ± 10.9 pmol/(s*mg)) group. At baseline, CTRL + DMB (227.4 ± 22.5 pmol/(s*mg)) animals had a significant decrease vs Pre-Db (306.0 ± 21.4 pmol/(s*mg), $p < 0.01$) and Pre-Db + DMB ($p < 0.01$) study groups (Figure 4.13A).

However, when respiration after cytochrome C addition was subtracted by Complex I respiration, all significance was lost. This calculation provides a true representation of the exact change occurring via cytochrome C addition (Figure 4.13B).

4.2.3.2 Mitochondrial Respiration After Cold Static Storage and Reperfusion

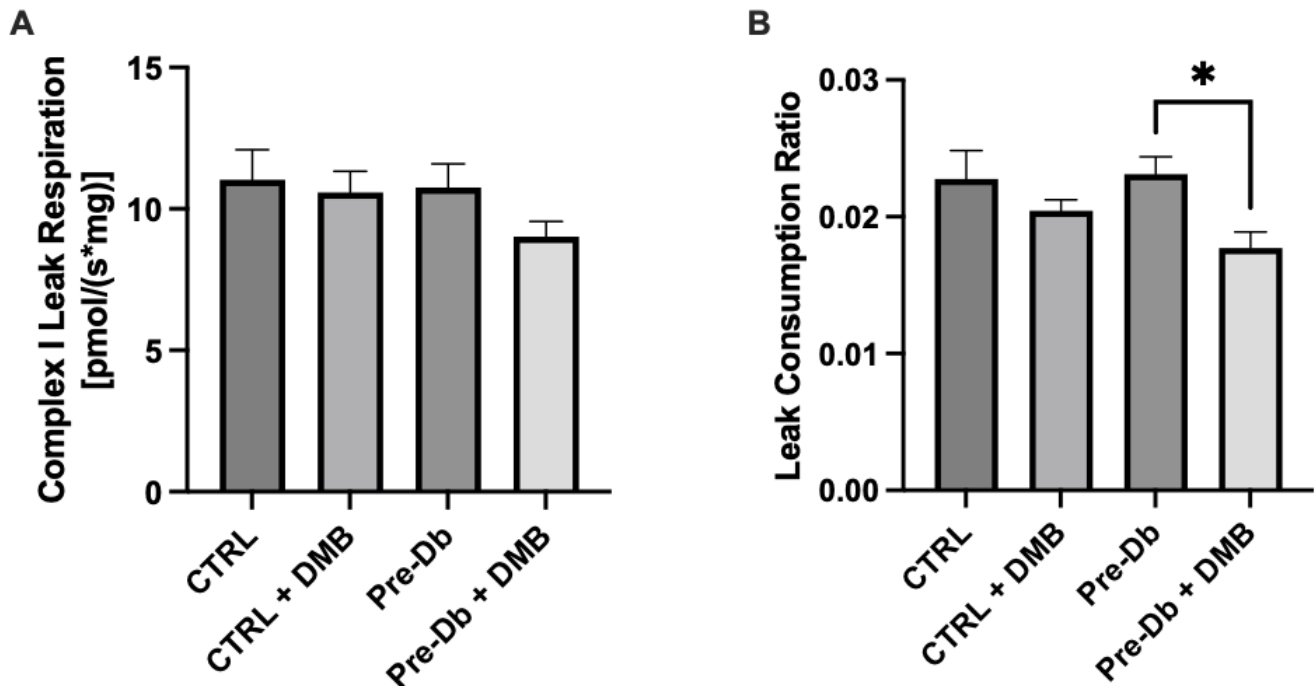


Figure 4.14. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion. (A) Complex I Total Respiration After Addition of Cytochrome C (B) Complex I Respiration After Addition of Cytochrome C DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. $n = 7-10$.

After CSS and reperfusion, there were no difference in Complex I Leak Respiration between groups when compared to CTRL animals (Figure 4.14A).

When calculated as Leak Consumption Ratio, there was no difference vs CTRL mice. However, there was an increase in Pre-Db (0.023 ± 0.001) vs Pre-Db + DMB (0.018 ± 0.001 , $p < 0.05$, Figure 4.14B).

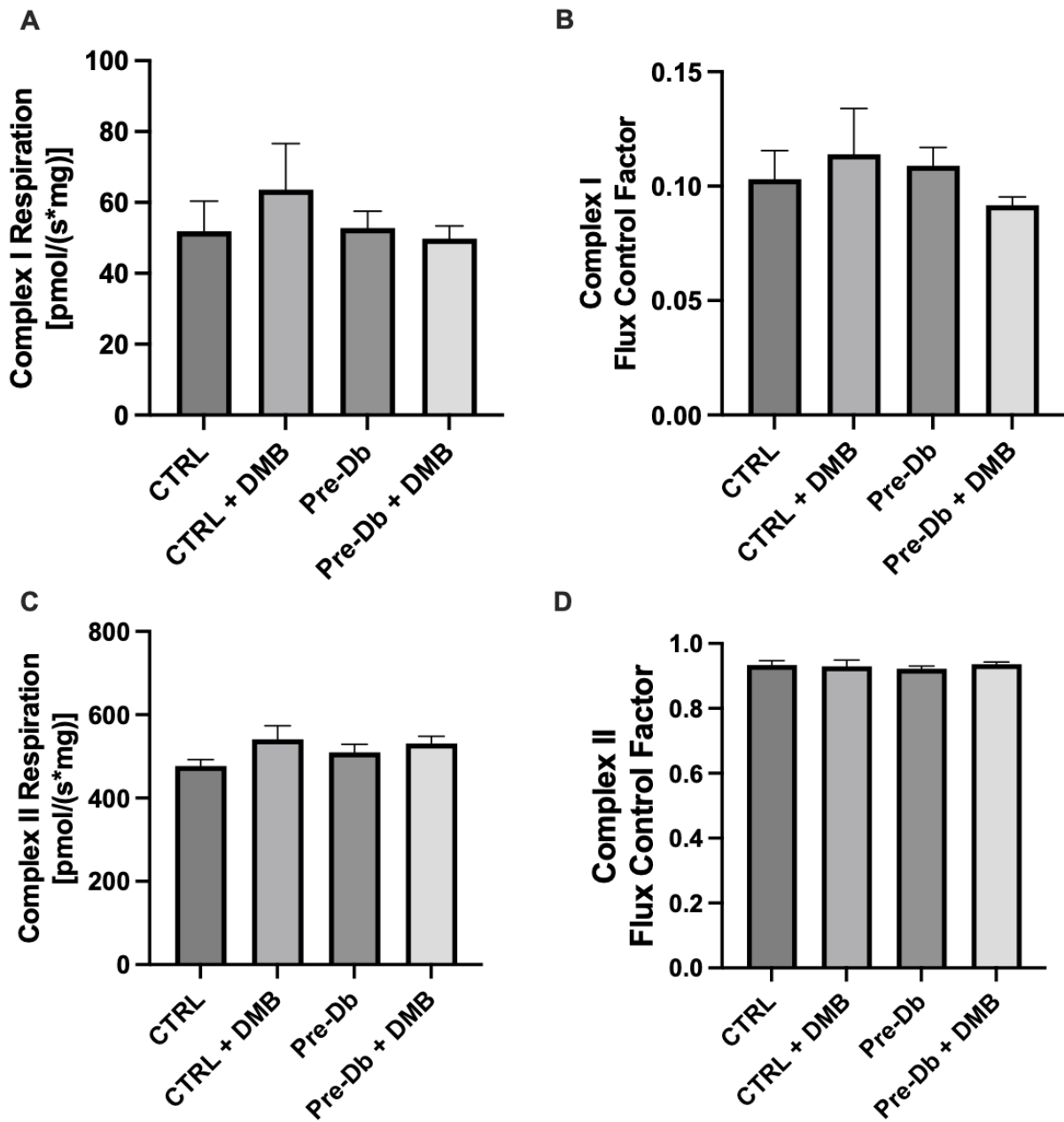


Figure 4.15. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion. (A) Complex I respiration (B) Complex I Flux Control Factor (C) Complex II respiration (D) Complex II Flux Control Factor. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 7-10.

There was no change from the CTRL in Complex I Respiration, Complex I Flux Control Factor, Complex II Respiration or Complex II Flux Control Factor in CSS hearts (Figure 4.15).

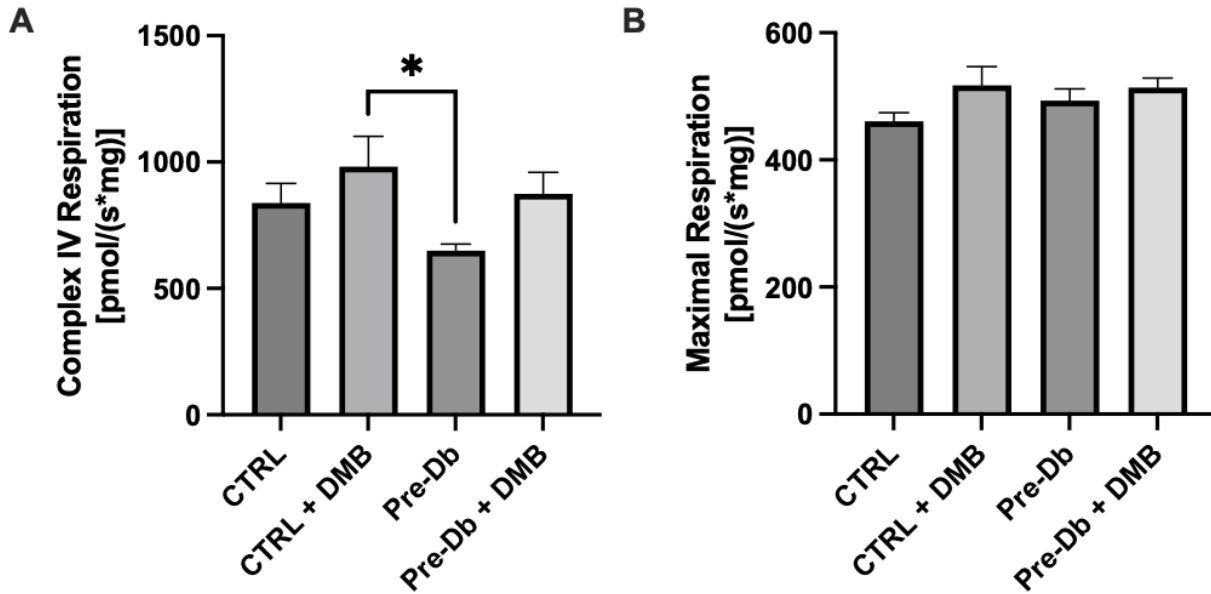


Figure 4.16. Left ventricular oxygen consumption in murine hearts after CSS and reperfusion. (A) Complex IV respiration (B) Maximal Respiration. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.01$. $n = 7-10$.

After CSS and reperfusion, there was no change in Complex IV Respiration between groups compared to the CTRL (838.6 ± 77.2 pmol/(s*mL), $p > 0.05$). There was however a 51% rise in CTRL + DMB (982.3 ± 119.4 pmol/(s*mL)) animals compared to Pre-Db (649.4 ± 26.0 pmol/(s*mL), $p < 0.05$) mice (Figure 4.16A).

The change measured in Maximal Respiration between groups after CSS and reperfusion was negligible (Figure 4.16B).

4.3 Protein Expression

4.3.1 Protein Expression in Hearts at Baseline

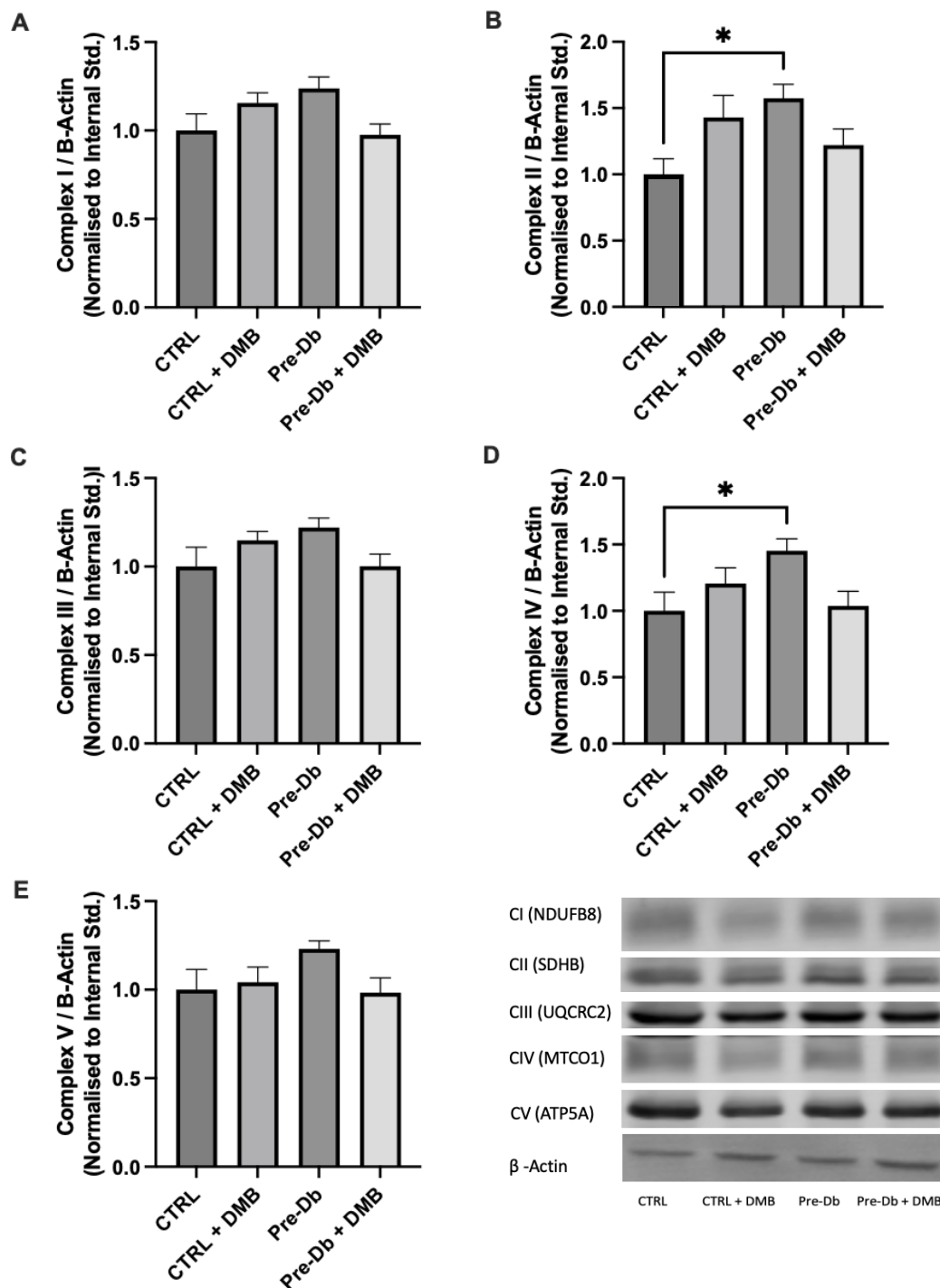


Figure 4.17. Whole Homogenate OXPHOS complex subunit expression in left ventricles of murine heart at baseline. (A) Complex I (NDUFB8) expression (B) Complex II (SDHB) expression (C) Complex III (UQCRC2) expression (D) Complex IV (MTCO1) expression (E) Complex V (ATP5A) expression. Protein expression is normalised to β -actin and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$. $n = 9-10$.

At baseline, there was no significant change in protein expression in mitochondrial oxidative phosphorylation Complex I, Complex III and Complex V in any groups compared to CTRL. However, Complex II (SDHB) expression was greater in Pre-Db (1.57 ± 0.10) mice compared to CTRL (1.00 ± 0.10 , $p < 0.05$) mice. Complex IV (MTCO1) also had increased protein expression in Pre-Db (1.45 ± 0.09) animals when compared to the CTRL (1.00 ± 0.10 , $p < 0.05$) group. Though not statistically significant, Pre-Db mice also presented with increased Complex IV expression compared to Pre-Db + DMB (1.04 ± 0.11 , $1 < p < 0.05$; Figure 4.17).

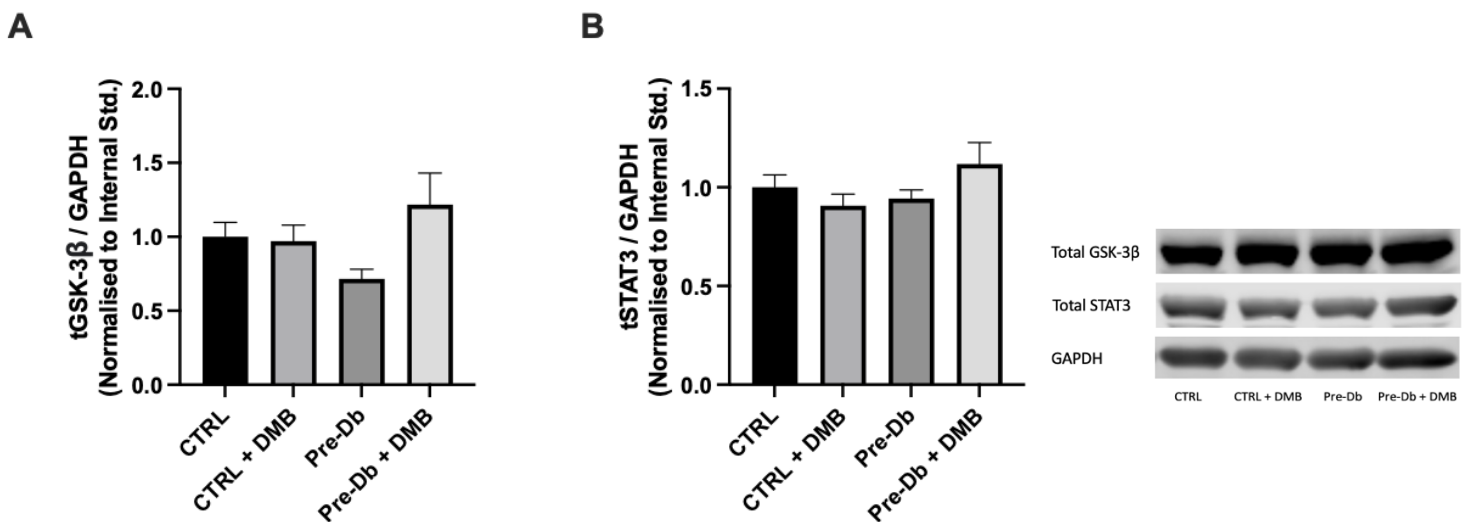


Figure 4.18. Cytosolic protein expression at baseline. (A) Total GSK-3 β expression. (B) Total STAT3 expression. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 8-10.

At baseline, there was no difference in the protein expression of total GSK-3 β or total STAT3 compared to CTRL mice. There was however a noticeable reduction in total GSK-3 β expression in Pre-Db (0.72 ± 0.07) mice compared to Pre-Db + DMB (1.22 ± 0.21) animals ($1 > p > 0.05$; Figure 4.18A and Figure 4.18B).

4.3.2 Protein Expression in Hearts After Cold Static Storage and Reperfusion

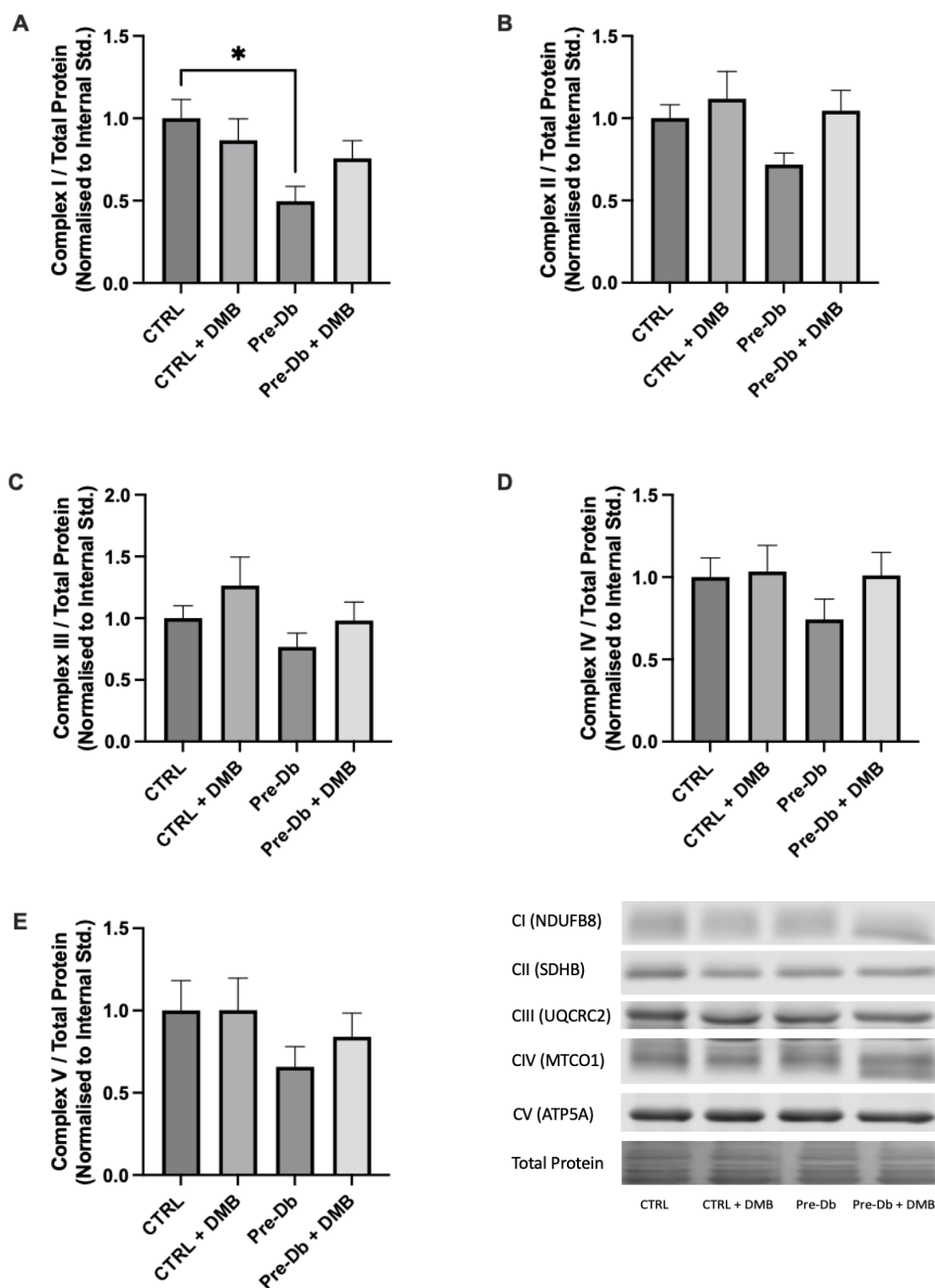


Figure 4.19. Mitochondrial OXPHOS complex subunit expression in left ventricles of murine heart after CSS and reperfusion. (A) Complex I (NDUFB8) expression (B) Complex II (SDHB) expression (C) Complex III (UQCRC2) expression (D) Complex IV (MTCO1) expression (E) Complex V (ATP5A) expression. Protein expression is normalised to total protein and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$. $n = 8-10$.

After CSS and reperfusion, we found that Complex I (NDUFB8) expression was halved in T2D (0.50 ± 0.09) vs CTRL (1.00 ± 0.11 , $p < 0.05$) mice (Figure 4.22A). However, there were no other changes in mitochondrial OXPHOS subunit expression at Complexes II-V. It should be noted that Pre-Db mice had decreased expression across all complexes (Figure 4.19).

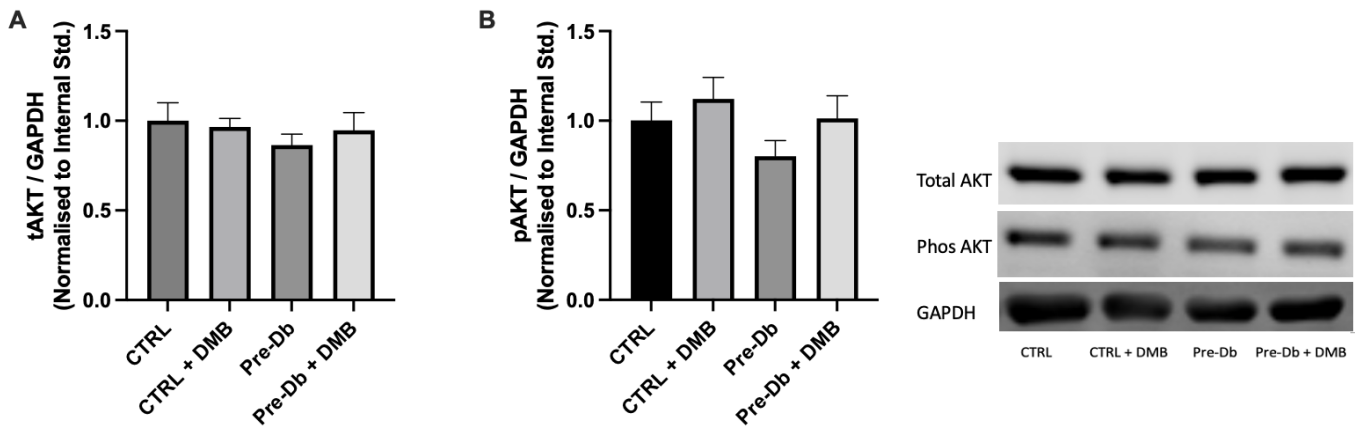


Figure 4.20. Cytosolic AKT expression in left ventricles of murine hearts after CSS and reperfusion. (A) Total AKT expression (B) Phosphorylated AKT expression. Protein expression is normalised to GAPDH and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. $n = 8-10$.

There was no change in total or phosphorylated AKT expression between groups after CSS and reperfusion. Interestingly, both total and phosphorylated AKT expression was slightly reduced in Pre-Db mice compared to all other groups ($p > 0.05$). This change was however not significant (Figure 4.20).

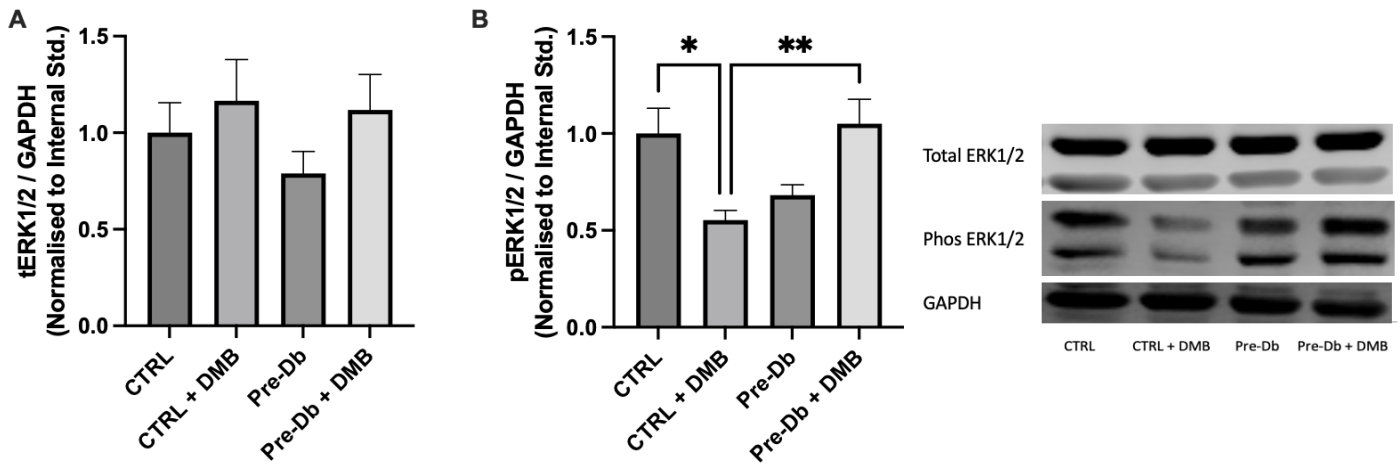


Figure 4.21. Cytosolic ERK1/2 expression in left ventricles of murine hearts after CSS and reperfusion. (A) Total ERK1/2 expression (B) Phosphorylated ERK1/2 expression. Protein expression is normalised to GAPDH and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. * $p < 0.05$, ** $p < 0.01$. $n = 8-10$.

There was no change in expression of total cytosolic ERK1/2 ($p > 0.05$, Figure 4.21A). However, CTRL (1.00 ± 0.13) mice nearly 2-fold more phosphorylated ERK1/2 compared to the CTRL + DMB (0.55 ± 0.05 , $p < 0.05$) group. Additionally, Pre-Db + DMB (1.05 ± 0.15) had almost 2-fold more phosphorylation than CTRL + DMB ($p < 0.01$) mice (Figure 4.21B).

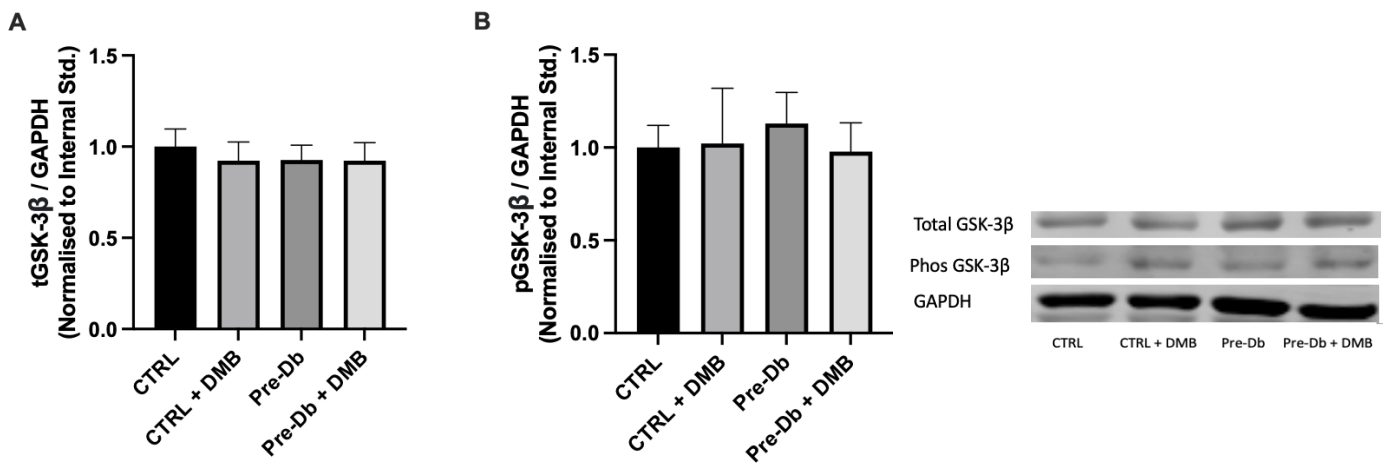


Figure 4.22. Cytosolic GSK- 3β expression in left ventricles of murine hearts after CSS and reperfusion. (A) Total GSK- 3β expression (B) Phosphorylated GSK- 3β expression. Protein expression is normalised to GAPDH and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. $n = 8-10$.

Both total and phosphorylated GSK- 3β expression was unchanged between groups after CSS and reperfusion ($p > 0.05$, Figure 4.22).

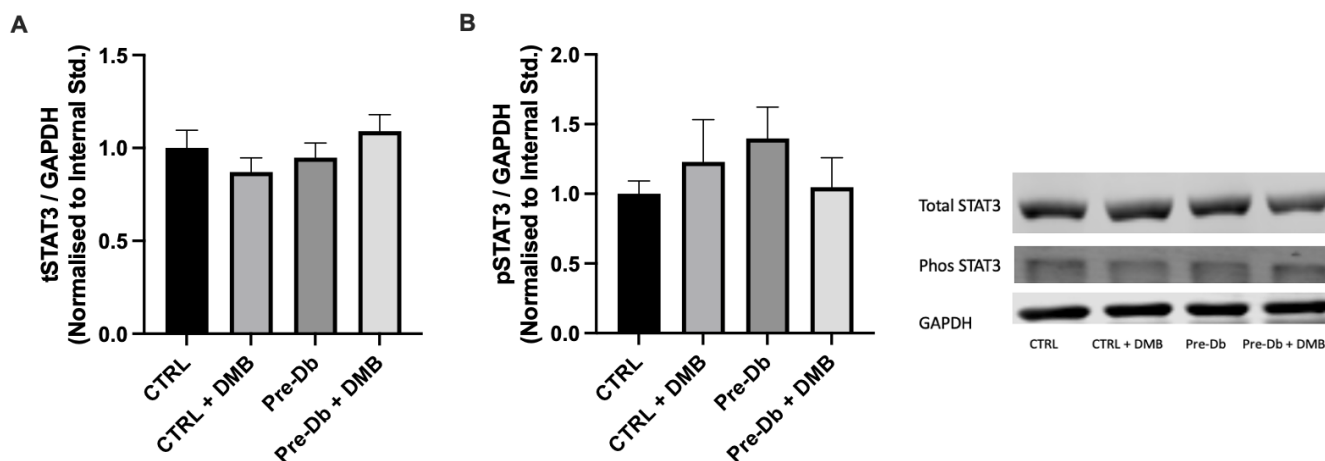


Figure 4.23. Cytosolic STAT3 expression in left ventricles of murine hearts after CSS and reperfusion. (A) Total STAT3 expression (B) Phosphorylated STAT3 expression. Protein expression is normalised to GAPDH and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 8-10.

There was no change in total or phosphorylated STAT3 expression between groups after CSS and reperfusion ($p > 0.05$, Figure 4.23).

4.3.3 Liver FMO3 Expression

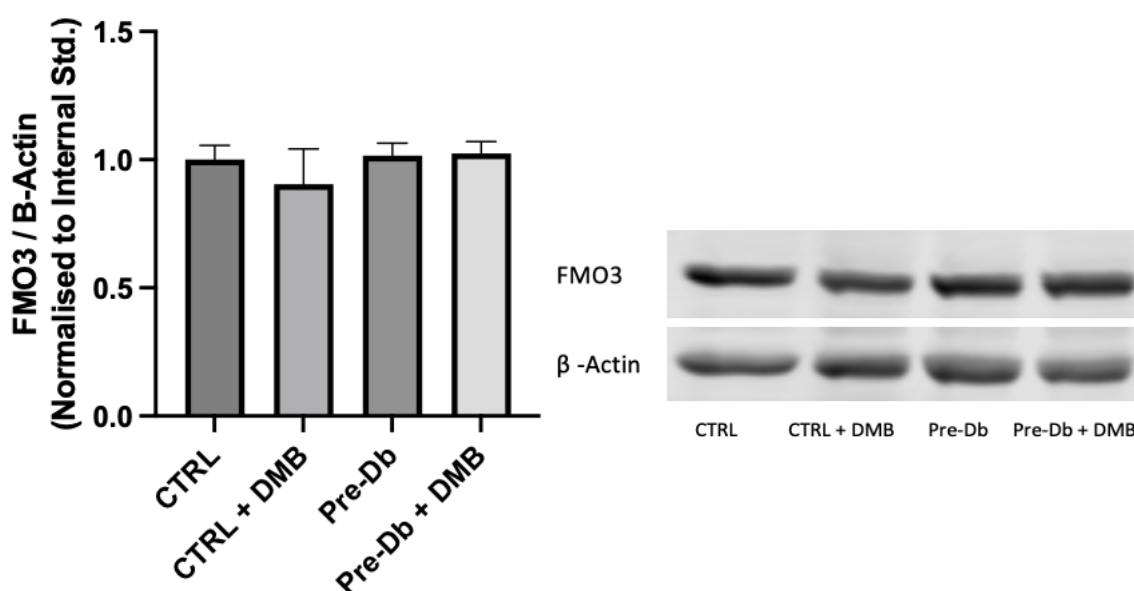


Figure 4.24. Whole homogenate FMO3 expression in murine livers. Total FMO3 expression. Protein expression is normalised to β -actin and internal standard and relative to CTRL. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 10.

Hepatic FMO3 is the primary enzyme that facilitates the conversion of TMA to TMAO. Prior studies have suggested potential links with FMO3 expression and TMAO levels (95). In the current study, no difference in FMO3 expression in murine livers was found when compared to the CTRL ($p>0.05$, Figure 4.24).

Chapter 5. Discussion

The current study investigated the effects of Pre-Db and TMA Lyase inhibition on cardiac outcomes after CSS and reperfusion. The endpoints investigated included assessment of body weight, markers of Pre-Db, cardiac tolerance to CSS and reperfusion, mitochondrial respiration, and cardiac protein expression. A modified Langendorff perfusion model was used to replicate clinical practice of organ preservation for HTx and quantify the impact on the heart. Our data reveals that a combination of STZ and a high fat Western-type diet induced a Pre-Db phenotype, with increases in body weight and blood glucose. In mice, this metabolic protocol often generates a T2D phenotype. As the animals were not overtly diabetic as evidenced through a lack of change to circulating insulin, the model has been defined as Pre-Db. The study also reveals that DMB treatment in healthy animals provides improved cardiac contracture outcomes during hypothermic ischemia. This protection afforded by DMB is however lost in Pre-Db mice. Additionally, our findings show that DMB treatment was detrimental to cardiac mitochondrial respiration at baseline, but potentially protective in Pre-Db as seen with improved post-CSS outcomes. Interestingly, the Pre-Db had a negative effect on mitochondrial respiratory complex protein expression, while DMB had no effect. Upstream signalling pathways of cell death including the RISK and SAFE pathways were relatively unchanged after CSS and reperfusion in all groups. Overall, this data suggests that DMB treatment may protect the heart during CSS prior to HTx and that this protection is lost with Pre-Db.

5.1 Phenotypic Effects of Western Diet, STZ and TMA Lyase Inhibition

5.1.1 Body Weight

Previously, murine models of T2D using STZ in combination with a standard rodent chow have shown minimal increases in body mass and overall body fat (214). High and repeated doses of the drug have even been found to decrease body weight in models of type 1

diabetes (215). Conversely, non-diabetic models using a high fat Western diet from our lab have found that weight gain can be induced with this diet (14, 15). When combining this alkylating antineoplastic drug with a Western-type diet, overall weight gain has also been demonstrated (46). In the present study, Pre-Db was found to cause an overall increase in body weight change across the 14-week feeding protocol. As initial body weights were not consistent between cages, percent change across the 14-week feeding protocol was assessed. Hence, it was found that Pre-Db mice had increases in overall weight change (%) over the course of the study. Thus, our findings are consistent with previous literature that demonstrated the combination of both STZ and consumption of the Western diet in mice induces an increase in weight gain.

A large body of data suggests that an increase in body weight is generally associated with an increased risk of metabolic diseases such as T2D (13). Data also suggests that patients suffering from Pre-Db or T2D are commonly overweight or obese (216, 217). There is an understanding that excessive caloric intake relative to energy expenditure is the mechanistic cause of this increase in body weight. However, the aetiology of weight gain is highly complex involving genetic, social, psychological, and economic factors (218). As the present study employed genetically identical mice and consistent diets between groups, these factors were controlled. Therefore, the observed weight gain can be attributed to the excess caloric intake from the Western diet.

In contrast, mice supplemented with DMB demonstrated no difference in body weight when comparing them to the CTRL and Pre-Db mice without DMB. This is consistent with other literature showing that TMAO may not play a role in the pathogenesis of obesity and weight gain (101). Although slight increases in serum TMAO have been found to be associated with obesity, any mechanistic involvement has not been defined (81). Independent of weight gain, a Western-type diet has been found to significantly increase TMAO levels in mice when

compared to a normal chow diet (101). Additionally, Western diet consumption has been found to alter microbiota composition, influencing TMA production and TMAO synthesis (84). In humans, it has been demonstrated that consumption of a Western-type diet can increase circulating TMAO levels (219). However, no studies have discussed any potential mechanisms involving these increases in TMAO and weight gain. Overall, it is proposed that TMA lyase inhibition does not influence body weight, as TMAO may be a dietary outcome rather than a causal factor in obesity. Further studies are required involving TMAO and a Western-type diet to confirm these claims.

These results along with current literature confirm that consumption of a Western diet can lead to increased body weight and potential obesity if not rectified. To validate the model and confirm diabetic progression, glucose homeostasis was then assessed.

5.1.2 Glucose Homeostasis

Models using STZ and a Western diet has long been used to induce a mild and stable form of diabetes, resembling human Type II diabetes in rodents (92). Along with increased body weight change, the Pre-Db mice in this model presented with hyperglycaemia and glucose intolerance. However, they lacked any changes in fasting insulin and HOMA-IR that is a common hallmark of T2D.

By week 6 of the protocol, metabolic changes were already noticeable in Pre-Db mice. Interestingly, Pre-Db animals supplemented with DMB presented with fasting blood glucose levels that were unchanged from CTRL animals. However, they still presented with noticeable glucose intolerance compared to healthy mice as measured by the GTT. By week 12, Pre-Db mice presented with hyperglycaemia and glucose intolerance compared to their CTRL counterpart. By this timepoint there was no longer evidence of DMB providing a protective effect on glucose homeostasis. Despite these significant changes in blood glucose, there was

no difference in fasted plasma insulin and HOMA-IR across the study. Though, it was clear that STZ combined with a Western-type diet had a negative impact on blood glucose control. As this model presented with altered glucose handling, whilst failing to demonstrate changes in fasting insulin, the mice were defined as Pre-Db.

This is the first study to look at the effects of DMB on a model of Pre-Db. Previous animal models assessing glucose homeostasis and TMAO have provided contradictory results showing that TMAO may or may not be associated with worsened diabetic outcome (113, 114). As conflicting evidence has also been found in human cohort studies (110, 111), further research must be completed to decide whether the relationship between TMAO and diabetes is causal or coincidental. The present data suggests that DMB treatment may play a role in diabetes progression. This assumption can be made as fasting glucose was unchanged from healthy mice at week 6. As many links to TMAO and disease have been made, a decrease in this gut metabolite due to DMB supplementation may slow both the onset of Pre-Db and rate of progression of T2D. This may be beneficial in allowing individuals more time to change lifestyle factors such as diet and physical inactivity to prevent progression of T2D. Further investigation looking at circulating TMAO levels, DMB supplementation and diabetic progression are required to confirm this causal relationship.

In previous rodent models of Pre-Db (220, 221) and T2D (45-47), blood glucose levels and glucose tolerance were markedly affected. Increases in fasting insulin and insulin resistance is another marker often reported in similar models of T2D (45-47). Though, some studies fail to report insulin levels and only look at measures of hyperglycaemia (48, 49). Marciniak *et al.* (47) demonstrated that changes to fasting insulin are not necessarily a reliable indication of insulin resistance in C57BL6/J mice and that further analysis may be required. They assessed circulating insulin pre- and post-GTT and performed an insulin tolerance test (47). Fasting insulin was unchanged between groups, whilst pancreatic β -cells in T2D mice

were not responsive to the subsequent influx of glucose. The insulin tolerance test also demonstrated the ability of STZ to prevent adequate glucose disposal after insulin injection. Additionally, they found that STZ altered islet size in pancreatic β -cells via immunohistochemistry to influence insulin resistance (47). In our present study, fasting serum insulin in a randomly selected sample of mice was the only measure assessed to confirm insulin resistance. Without the assessment of an insulin tolerance test, it can be proposed that the mice in the Pre-Db and Pre-Db + DMB groups presented with features of Pre-Db, progressing towards a T2D phenotype by week 12 of the study. Thus, lengthened duration of feeding may have been necessary to elicit overt T2D.

In future studies, serum TMAO should be measured at various time points to inform us on the efficacy of DMB and its potential impacts on diabetic progression. Further assessment of metabolic homeostasis should also be completed to confirm the presence of diabetes. By performing insulin tolerance tests and pancreatic immunohistochemistry, the effects of STZ on glucose homeostasis can be completely analysed. Additionally, increased duration of feeding may induce T2D, limit cage-to-cage variation and ensure the model is robust (Appendix Figure 2). Although lacking overt T2D, imbalances in blood glucose in Pre-Db may have influenced thirst and water consumption, a characteristic of hyperglycaemia.

5.1.3 Implications of Changes in Water Consumption in Groups

During this study CTRL mice supplemented with DMB consumed less water each week than the other groups. In previous research, no concerns about overall water consumption have been reported in similar models of TMA lyase inhibition in mice (86, 100, 222, 223). However, as DMB has a strong alcohol-like odour (224), it is proposed that the smell may have influenced how readily water was consumed. As mice in this intervention group were drinking less water than other groups, drug efficacy and dehydration may play a role the various cardiac outcomes

that have been assessed. Previously, hypohydration has been suggested to impair vascular function, reduce cardiac endothelial function and alter blood pressure regulation (225). Additionally, chronic lifelong hypohydration has been shown to increase of LV hypertrophy and HF (226, 227).

Surprisingly, Pre-Db mice supplemented with DMB did not demonstrate any changes in water consumption compared to the CTRL and Pre-Db groups. Prior studies using STZ-induced diabetes have shown that increased thirst may be an outcome of the drug intervention (228). In humans, polydipsia is a symptom that frequently occurs due to imbalances in blood glucose in Type 1 diabetics (229). It has also been found to arise in some Pre-Db and T2D patients due to blood glucose imbalances (230-232). Therefore, it is reasonable to propose that the overall influence on thirst in the Pre-Db mice outweighed the resistance to drink the water due to its odour.

5.2 Effects of Prediabetes and TMA Lyase Inhibition on Ischemic Tolerance to CSS

5.2.1 Ischemic Contracture

Ischemic contracture development is a direct indicator of ischemic insult and damage (233). It is understood that the magnitude of- and time to peak ischemic contracture is dependent on intracellular calcium concentrations. During ischemia, ionic imbalances and acidosis lead to excessive intake of Ca^{2+} leading to intracellular calcium overload (210, 234). When intracellular calcium is increased, more severe contracture will develop more quickly, compromising function during reperfusion and causing overall damage to the heart itself (235).

The current study demonstrates that mice supplemented with DMB may have improved ischemic tolerance during CSS. Clearly, the reduction in water consumption had no deleterious effects on outcomes during ischemia. CTRL mice supplemented with DMB were found to have less severe ischemic contracture than mice without the treatment. This protection appears to

have been lost with Pre-Db as they reached ischemic contracture at a faster rate. Thus, our findings reveal that DMB treatment may be protective during CSS in healthy mice but not Pre-Db mice.

This study is the first (to our knowledge) to look at TMA lyase inhibition and its effects on ischemic outcomes during or after CSS. Our results show that during ice cold ischemia, healthy mice exposed to TMA lyase inhibition had reductions in ischemic contracture. Previously, TMAO exposure has been shown to amplify intracellular Ca^{2+} release (92). Hence, when TMA production is reduced, the subsequent reduction of TMAO may reduce intracellular Ca^{2+} and provide overall cardioprotection. Hence, our data suggest that the reduction of TMAO may lead to a reduction in intracellular calcium. Overall, measurement of TMAO may be a predictor of CSS outcomes prior to HTx. Future research relating to intracellular calcium and TMA lyase inhibition is required to assess the potential mechanisms involved. Nevertheless, it seems that Pre-DB may negate the beneficial effects associated with TMA lyase exposure.

Research involving myocardial I-R tolerance and cardioprotection in diabetes has become a topic of great interest in recent years. Previous studies have found that reduced I-R tolerance and loss of cardioprotection is caused by Pre-Db (208, 236) and T2D (45, 46, 205). Additionally, imbalances in blood glucose have been found to have major adverse effects on the cardiovascular system through oxidative stress, direct endothelial harm and activation of the polyol pathway (237). Overall, the therapeutic protection afforded by DMB supplementation seems to be lost in the current model of Pre-Db. Savi *et al.* (57) recently reported that mice given a single dose of STZ (60 mg/kg) had worsened ventricular calcium dynamics and impaired contractility compared to healthy mice. The authors attributed this to poor calcium reuptake into the sarcoplasmic reticulum due to decreases in SERCA2 activity and reduced phosphorylation of PLB (57). The current data demonstrates that intracellular calcium may have been increased in Pre-Db mice with DMB treatment compared to CTRL

mice with DMB treatment. Hence, increases in intracellular calcium in models of STZ-induced diabetes and Pre-Db may provide a potential mechanistic link between I-R intolerance and diabetes during CSS. In the context of organ transplantation, these changes to ischemic contracture may lay the foundation for outcomes during reperfusion of the organ within the recipient (206).

5.2.2 Myocardial Tolerance to CSS and Myocardial Function During Reperfusion

The current study revealed that after 3 hours of CSS, Pre-Db and DMB supplementation had no effect on final EDP, final coronary flow and LVDP recovery. Simply, EDP describes the LV pressure at the end of diastole. Whereas LVDP is a measure of the difference between the LV systolic and diastolic pressure. When assessing cardiac contractility, healthy mice supplemented with DMB had improved $+dP/dt$ recovery compared to both CTRL and Pre-Db + DMB mice. This increase in $+dP/dt$ recovery in CTRL + DMB mice may be further indicative that therapeutic effects of DMB supplementation are lost in Pre-Db. Conversely, when assessing cardiac relaxation Pre-Db mice demonstrated an improved $-dP/dt$ recovery compared to healthy CTRL mice. $+dP/dt$ has long been used as a measure of rate of cardiac contractility, whereas $-dP/dt$ is used as a measure of rate of cardiac relaxation (238).

No studies have looked at associations between myocardial ischemic tolerance and TMA lyase inhibition. It is also the first of its kind to assess these parameters after a clinically relevant model of organ preservation for transplant. Previously, numerous studies have assessed links between CVD and TMAO. Although a single causal mechanism has not been identified, several factors are thought to play a potential role. Reports have identified TMAO to be a contributor to atherosclerosis (96, 97) and HF (98) through inflammation and vascular dysfunction (91, 102, 103). Although they were not assessed, these factors have the potential to negatively affect myocardial tolerance to I-R. Hence, reasons for the protective effects of

DMB treatment could be due to the downstream reduction of TMAO levels. Again, assessment of reduced TMAO in models of CSS and HTx could unveil a potential marker for surgery success. This relationship requires further investigation to evaluate how TMAO and TMA lyase inhibition may influence tolerance to I-R.

Improved contractility after DMB supplementation could be considered a direct consequence of the improved ischemic tolerance during CSS in this model. Current evidence relating to changes in pressure over changes in time in murine models seems to be subject to variability. A recent study using a model of T2D (STZ 65 mg/kg) showed I-R injury was worsened in rats and stimulation of the endoplasmic reticulum is a useful method of preconditioning to prevent damage (58). Similar to our findings, research shows that rodents consuming a high fat diet (without STZ treatment) presented with improved $-dP/dt$ compared to baseline (208). Additionally, in STZ-induced T2D *in vivo* recovery of ventricular contractility is maintained, whilst recovery of ventricular relaxation is lost (239). This is consistent with a study using a Pre-Db model that proposed that the associated cardiomyopathy is caused by inflammation and oxidative stress due to insulin resistance (240). Although mice in the Pre-Db group seemed to recover relatively well in the current study, it seems like the protective effects of DMB was lost when combined with the metabolic disruption associated with Pre-Db. Hence, using hearts from Pre-Db donors may have little effect on the outcome of HTx, but will limit the cardioprotective effects of reduced TMAO. Dose-dependent studies using TMA lyase inhibition may help to uncover its mechanistic relationship to cardiac recovery from I-R. Additionally, future models comparing recovery from cold (4 °C) ischemia and recovery from warm (37 °C) ischemia could be helpful in understanding why the Pre-Db group maintained normal function. As Pre-Db mice had similar post-CSS outcomes to CTRL mice, cell death was also assessed during reperfusion to ensure that Pre-Db donor hearts can be used for HTx.

5.2.3 LDH Release and Cell Death

A common surrogate marker of cardiac cell death is the assessment of LDH content in the coronary effluent. LDH is a stable cytoplasmic enzyme found in almost all eukaryotic cells and is rapidly released upon membrane damage during necrosis or apoptosis (241). Overall, the current data shows that LDH release in coronary effluent was similar between all groups at both time points during reperfusion. As no change in LDH levels occurred, our data suggest that DMB treatment and Pre-Db had no effect on cardiac cell death after CSS in this model.

No previous research has outlined the effect of TMA lyase inhibition or TMAO on cardiac cell death after CSS. However, the effects of these two variables on cardiac cell death and fibrosis after MI has been described. In humans, an increased risk of cardiac events as well as poor outcomes has been associated with increased TMAO levels (80, 88-90). Additionally, rodent models have been used to uncover the mechanisms that may be involved in this pathological process. Yang *et al.* (242) found that DMB treatment reduced TMAO levels and subsequently limited cell death after ligation of the left anterior descending coronary artery in male C57BL/6 mice (242). The results suggested that increased TMAO led to increased myocardial fibrosis and enhanced inflammation related to cardiomyocyte death. Various other studies have also demonstrated the effects of TMAO on myocardial fibrosis after acute ischaemic events. Although not a direct measure of cell death, increased fibrosis has been associated with increasing TMAO levels (104, 242). Due to our conflicting results, it may be speculated that CSS limits cell death attributed to rising TMAO levels. Comparing CSS to normothermic ischemia with TMA lyase inhibition may help fill this gap in the literature. Similarly, our findings regarding Pre-Db and its effects on LDH release conflict with other studies.

Although numerous studies have assessed how T2D may influence myocyte death after I-R, this study is the first to consider the effects of Pre-Db and myocardial cell death after CSS. T2D promotes pro-apoptotic signalling and apoptosis in reperfused human myocardium (243-245). However, models of Pre-Db generate conflicting outcomes with regards to cell death due to the more modest metabolic disruption caused by this condition (208, 246, 247). Analysis of I-R injury and cell death in human ex vivo models also reveals increased apoptosis in T2D patients (248), with evidence that mitochondrial-dependant cell death may be the cause (249). Prior rodent models have also demonstrated that myocardial cell death is exacerbated by T2D during reperfusion (250, 251). Since our model failed to show any differences in LDH release/cell death, it seems that CSS limited any Pre-Db-induced injury during reperfusion. Additionally, the Pre-Db phenotype may also explain the modest differences in LDH release between Pre-Db and healthy CTRL mice. Hence, this further approves that Pre-Db donor hearts may be a useful tool for increasing donor pool size. Further studies assessing Pre-Db and I-R injury during CSS is required to validate our findings. With no changes in cell death and apoptosis between groups, mitochondrial function was assessed to potentially explain why mice with Pre-Db lost protection afforded by DMB supplementation during and after CSS.

5.2.4 Mitochondrial Function

The current study shows that chronic TMA lyase inhibition via DMB treatment may have negative effects on basal pre-ischaemic cardiac mitochondrial function. Complex I, complex II and complex IV respiration, respiration after cytochrome C and maximal respiration were all reduced in CTRL mice treated with DMB. Although not significant, CTRL + DMB mice had reduced complex I leak respiration compared to the CTRL group. Additionally, Pre-Db mice supplemented with DMB had an increased complex I flux control factor. As this study is the first of its kind, there is limited literature to compare to our findings.

The present study assessed the effects of chronic TMA lyase inhibition and mitochondrial function using respirometry. Previous research shows that TMAO has a potential impacts on cardiac energy metabolism as suggested by TMAO-dependant inhibition of mitochondrial pyruvate and fatty acid oxidation (105). Specifically, investigators showed that acute exposure of cardiac mitochondria to TMAO decreased LEAK and OXPHOS mitochondrial respiration with pyruvate and impaired substrate flux via pyruvate dehydrogenase. However, long-term TMAO administration decreased mitochondrial LEAK state respiration with pyruvate by 30% without affecting OXPHOS state respiration (105). Contradicting past findings, the current study demonstrated that chronic DMB treatment and subsequent reduction of TMAO reduces mitochondrial respiratory capacity in LEAK, OXPHOS and ETS states. Interestingly, it appeared that mitochondrial inner membrane integrity was improved in CTRL + DMB mice. When accounting for the reduction in overall respiration, the changes to membrane integrity were no longer significant. Again, contrary to our hypothesis, Pre-Db mice supplemented with DMB had an increased complex I flux control factor. Further investigation is required to understand the mechanisms associated with DMB treatment, mitochondrial oxygen consumption and disease.

The present model demonstrated that STZ-induced Pre-Db combined with a Western diet had a limited influence on cardiac oxygen consumption at baseline. Previously, Jespersen *et al.* (45) showed that diabetes compromised mitochondrial respiratory capacity and may explain a decreased responsiveness to cardioprotective mechanisms. Similarly, another study showed that diabetic mice had significantly reduced oxidative capacity along with fatty acid induced mitochondrial uncoupling (252). Conversely, there were no noticeable changes in mitochondrial respiration at baseline in the current study. The maintained mitochondrial function could be due to the Pre-Db phenotype being too mild to induce robust changes in mitochondrial respiration. This lack of insult in Pre-Db mice is consistent with outcomes during

CSS and reperfusion. Models of Pre-Db with high fat feeding absent of STZ could be used to confirm these baseline findings.

After 3 hours of CSS and reperfusion, mitochondrial respiratory capacity seemed less variable than at baseline. No changes were found between groups in complex I, complex II or maximal respiration. There was however a reduced leak consumption ratio in Pre-Db + DMB mice compared to Pre-Db mice, suggesting potential protective effects for DMB. Complex IV respiration was reduced in Pre-Db mice, but this change was no significant compared to CTRL animals.

As stated, to our knowledge no previous research has looked at the effects of STZ-induced Pre-Db and DMB treatment on mitochondrial function after CSS. It seems that DMB treatment in Pre-Db mice was protective and reduced leak consumption ratio which is an indication of how much proton leak is occurring and whether the cardiomyocytes are intact. Hence, this means there was improved mitochondrial function and reduced cell damage after DMB treatment in our Pre-Db mice. It may be speculated that Pre-Db increased complex IV sensitivity to CSS since respiration was reduced. As this wasn't the case at baseline, it is possible that there was Pre-Db increased sensitivity to damage caused by CSS. To further assess any changes in mitochondrial function, expression of mitochondrial ETC and OXPHOS proteins was evaluated.

5.3 Effects of Prediabetes and TMA Lyase Inhibition on Myocardial Protein Expression

5.3.1 Oxidative Phosphorylation Protein Expression

At baseline, there were some unexpected changes to mitochondrial OXPHOS protein expression. There were no changes in expression of complex I, complex III or complex V proteins between groups. However, there was increased expression of complex II and IV, in the Pre-Db groups when compared to CTRL. Previous literature has found that diabetes is

correlated with a decreased expression in mitochondrial respiratory gene expression in various tissue types (253). However, conflicting findings have demonstrated that diabetic animals may have increased cardiac mitochondrial respiration governed by availability of certain substrates at the time (254, 255). Although these mechanisms may not be directly comparable, they can be drawn upon as potential predictors of mitochondrial function within the Pre-Db heart. Hence, prior to the ischemic insult of CSS, it is possible that upregulation of OXPHOS protein expression was required to increase the myocardial energy demand within the diseased heart.

After 3 hours of CSS and reperfusion, it was found that the Pre-Db had a negative effect on mitochondrial oxidative phosphorylation protein expression. At complex I, the Pre-Db group presented with reduced protein expression of the targeted subunit. When animals were supplemented with DMB, this reduction in protein expression was negated. No other significant changes were present, however, Pre-Db mice presented with minor reductions in protein expression at all complexes.

No previous studies have addressed the effects of TMA lyase inhibition using DMB treatment on mitochondrial OXPHOS protein expression after CSS. The data reveals that DMB treatment had no effect on mitochondrial OXPHOS protein expression in healthy animals. However, it seems to prevent the loss of complex I expression that occurs with Pre-Db.

Prior research has demonstrated that Pre-Db and T2D have detrimental effects to mitochondrial function (45, 252). Jespersen *et al.* (45) have however shown that mitochondrial protein expression within the oxidative phosphorylation pathway was unchanged by T2D in mice after ischemic insult. Another group also demonstrated that high fat, high sugar feeding altered mitochondrial function via post-translational modifications, without altering oxidative phosphorylation protein expression (256, 257). In the current model, complex I protein expression was reduced in Pre-Db, whilst oxygen consumption and function were maintained. Similarly, complex IV oxygen consumption and function were reduced whilst protein

expression was unchanged. This may indicate that OXPHOS subunit expression is not the primary cause of altered mitochondrial mechanics after CSS. In the present study, ensuring a moderate to severe T2D phenotype may uncover a link between OXPHOS expression and implications to overall mitochondrial function after CSS. Overall, progression of this metabolic condition may play a detrimental role in donor heart success after organ preservation. In addition to investigation of ETC proteins, the cytosolic RISK pathway was evaluated to further understand how Pre-Db influences cardiac tolerance to I-R after CSS.

5.3.2 RISK Pathway Protein Expression

This study is the first to assess RISK pathway protein expression at baseline and after CSS. It is also the first of its kind to assess how Pre-Db and DMB treatment may alter this signalling pathway. In the present study, no significant changes to the RISK pathway protein expression or activity was found at baseline. Total GSK-3 β expression was measured, and no significant change was found compared to CTRL animals. However, a trend was noted as Pre-Db mice supplemented with DMB demonstrated a trivial increase compared to Pre-Db mice without treatment. As the RISK pathway is most active during early reperfusion (182) and our samples were collected before ischemia, large changes were not expected. In the absence of I-R it has been demonstrated that GSK-3 β plays an important role in a myocytes ability to respond to stretch, a component that governs the Frank-Starling mechanism (258). As T2D can lead to poor cardiac outcomes and remodelling (62, 63), it is possible that this reduction in GSK-3 β expression may have some influence. Additionally, increased TMAO levels have been associated with reduced cardiac compliance and increased fibrosis (99-101). Therefore, as Pre-Db mice treated with DMB had increased expression of this protein, it is possible that the drug has protective effects by increasing GSK-3 β expression in response to reductions in TMAO levels. Due to the lack of consensus surrounding whether increased GSK-3 β is protective or

harmful within the heart during remodelling and HF (259-261), further research is required to uncover any underlying mechanisms. Additional research exploring the protein's association to TMAO levels may also help to explain the potential therapeutic impact after DMB treatment.

After 3 hours of CSS and reperfusion, the RISK pathway expression and activity was mapped out by measuring expression of total and phosphorylated ERK1/2, AKT and GSK-3 β . No change in AKT and GSK-3 β expression was measured between groups. Additionally, there was no change in total ERK1/2 expression. However, healthy mice treated with DMB had reduced in ERK1/2 phosphorylation. It is important to understand that the RISK pathway is most active during the first 5-15 minutes of reperfusion (180). Hence, changes in protein expression late in reperfusion are not always indicative of the true response to the reintroduction of blood flow. During reperfusion, decreased phosphorylation of ERK1/2 would indicate downregulation of the RISK pathway and changes in associated proteins (205). As only phosphorylated ERK1/2 expression was altered, it can be speculated that this protein was acting independent of other RISK pathway proteins through other involved pathways. Further research involving DMB supplementation and ERK1/2 activation are required to uncover any potential pathways involved. As the changes in the RISK pathway were quite minimal, the SAFE pathway was also assessed to see if other protein pathways could explain the potentially cardioprotective effects associated with DMB treatment.

5.3.3 SAFE Pathway Protein Expression

The current study demonstrated that SAFE pathway signalling via total STAT3 expression was unchanged at baseline. Additionally, there was no change in total or phosphorylated expression of STAT3 after 3 hours of CSS. Like the RISK pathway, SAFE pathway proteins are activated at different time points during reperfusion (262). Considering that expression was only measured at baseline and post-reperfusion, the exact protective role

that the SAFE pathway played was not assessed in detail. In future, measurement of JAK2 protein expression would also be required in describing the outcomes of the SAFE pathway after CSS and reperfusion.

5.3.4 Liver FMO3 Expression

Hepatic FMO3 is the primary enzyme that facilitates the conversion of TMA to TMAO (95). In the present study, DMB and STZ-induced Pre-Db had no effect on liver expression of FMO3. No changes in liver whole homogenate FMO3 expression were measured between groups. Circulating TMAO levels have been found to be correlated with TMA oxidation by hepatic FMO3 in both mice and humans (80). Previously, data revealed that FMO3 expression is increased by chronic disease and CVD risk factors such as Ageing, obesity, insulin resistance (87, 113, 263). Currently, there is conflicting evidence that has been found surrounding FMO3 expression and TMAO levels. One study showed that elevations in circulating TMAO may increase FMO3 expression, with evidence of up to 5-fold increases in male mice (264). Conversely, Wang *et al.* (265) found that choline supplementation (increased plasma TMAO) and vitamin D supplementation (reduced plasma TMAO) had no effect on FMO3 expression (265). Additionally, questions have emerged surrounding difficulty detecting FMO3 mRNA and protein expression in male mice (84, 266). Claims that FMO3 is not a driving factor in TMAO variability have also been made (267). This lack of variability in FMO3 may suggest that the enzyme does not have a role in linking TMAO to disease. It also suggests that DMB may reduce TMAO levels without influencing hepatic FMO3 expression. Interestingly, there is evidence that DMB may reduce pancreatic FMO3 expression (268), with no indications in the literature that hepatic expression is altered by the drug. Furthermore, the mild Pre-Db phenotype could also explain similarities in protein expression between healthy and STZ treated mice. Again, this may further validate the use of Pre-Db donor organs in HTx. As this

is the first study assessing the relationship between hepatic FMO3 expression and Pre-Db, further investigation is required to confirm the findings.

5.4 Limitations and Future Research Directions

A major limitation of the current study is that TMAO levels have not been measured. Without this data, it is unclear if DMB was able to reduce TMA lyase action and prevent downstream formation of TMAO. In future, assessing circulating TMAO via mass spectrometry would allow confirmation of treatment success of DMB through its proposed actions. Furthermore, the administration of DMB can be considered as another limitation of the study as dose volume could not be accurately tracked via average water consumption per cage. To ensure DMB treatment is consistent between groups, the drug should be administered via oral gavage. Optimisation of dose volume and frequency is required. Additionally, lengthening the feeding protocol may ensure that mice are insulin resistant, limiting cage-to-cage variation and skewness of the data. Completion of an insulin tolerance test may also be a helpful tool for confirmation of insulin resistance. Another limitation of the study is that there was no test completed to confirm for specific cellular fractions. In future, western immunoblot analysis should be employed to confirm that the tissue samples have been appropriately fractioned. To confirm, each cellular fraction should be analysed for specific proteins that can only be found in that location within the cell. Finally, there are limitations with the assessment of the RISK and SAFE pathways. This is due to the largest changes in expression occurring during the first 5-10 minutes of reperfusion. To ensure the pathways are accurately represented, expression at different timepoints during reperfusion may provide insight into the impacts of these proteins during CSS. Future quantification of JAK2 and PI3K may also be helpful to paint a complete picture of each pathway.

5.5 Conclusion

In conclusion, the current study has demonstrated that TMA lyase inhibition may be a novel therapeutic target for improved cardiac outcomes during CSS prior to HTx in healthy hearts. This potential target is however ineffective in Pre-Db. Validating the model, Pre-Db was associated with increased weight gain, hyperglycaemia, and glucose intolerance – without changes in fasting insulin. Mice undergoing DMB treatment revealed reduced severity of ischemic contracture during CSS. They also had improved contractile function recovery after CSS, with these cardioprotective effects being lost in Pre-Db mice. Mice with Pre-Db also had reduced expression of key mitochondrial oxidative phosphorylation proteins after CSS, while limited changes in mitochondrial respiration were observed. Interestingly, insignificant changes in both the RISK and SAFE pathways were found between groups.

Although this novel therapy may provide improved cardiac outcomes after CSS for HTx, it is not practicable to provide humans with a pharmacological treatment prior to death for subsequent organ donation. Instead, this study identifies elevations in the gut microbial metabolite, TMAO in donors, as a potential predictor for poor post-transplant outcomes. As DMB prevents the upstream formation of TMAO, it can be proposed that reduced levels of this metabolite in donors may be a predictor of improved HTx success. However, this metabolite may not be reliable predictor of donor heart suitability for Pre-Db or T2D donors.

As untreated Pre-Db and healthy mice presented with similar outcomes during and after CSS – it can be proposed that using extended criteria organs from prediabetic patients is suitable for HTx. Currently, a lack of donor hearts leads to increased waiting time and mortality. Using hearts from unhealthy donors will likely increase the donor pool, ensuring that individuals suffering from end-stage HF are able to receive the critical treatment so urgently required.

Further studies are required to confirm that reduced TMAO levels is a predictor of HTx success. TMAO supplementation and assessment of serum TMAO could be used to map its effects on cardiac outcomes during and after CSS. Additionally, repeating a similar model with overtly T2D mice may uncover how further diabetic progression impacts the viability of donor organs.

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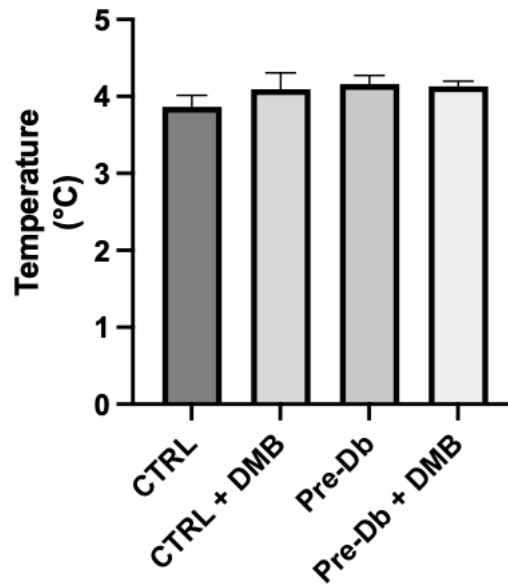
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Appendices

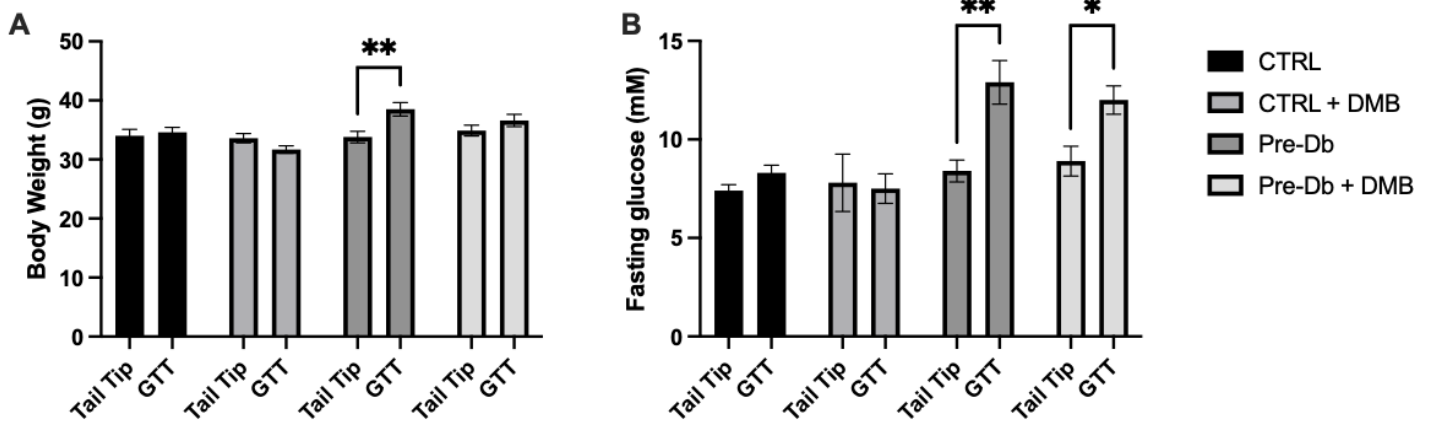
	Control Diet	Pre-Db Diet	Fold-change
	(CTRL)	(Pre-Db)	(CTRL vs. Pre-Db)
Energy (kJ/100g)	1820	2154	1.18
Protein (%)	18.8	12.7	0.68
Carbohydrates (%)	63.4	39.7	0.63
Fat (%)	17.8	43.2	2.43
Sugar (g)	10.0	34.0	3.40
Saturated (g)	0.99	14.3	15.1
MUFA (g)	1.62	6.70	4.14
PUFA (g)	4.05	1.92	0.47
Trans (g)	ND	ND	-
Choline Chloride	0.25	0.31	1.24
Carnitine	NA	NA	-
Betaine	NA	NA	-

Appendix Table 1. Nutrient breakdown and comparison between CTRL and Pre-Db diets. Top: Percentage of total calculated net metabolisable energy from each nutrient. Bottom: Quantity of nutrients per 100 grams of food. NA: not available; ND: not detected.



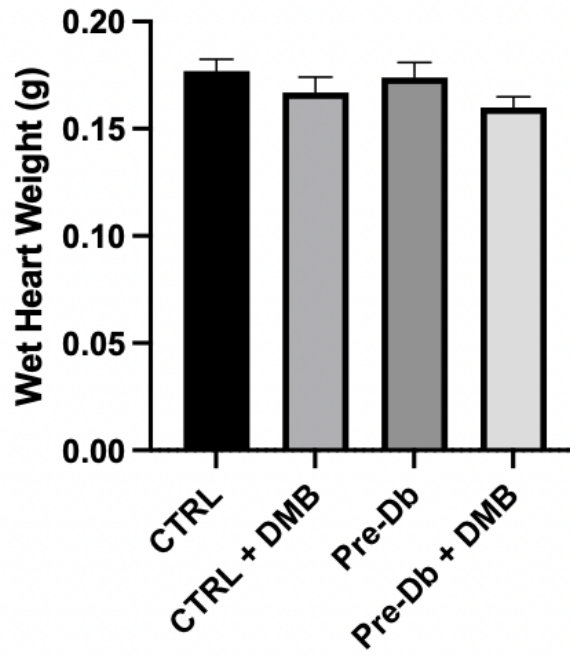
Appendix Figure 1. Average temperature during 3-hour cold ischemic period. * $p < 0.05$. n = 9-10.

Temperature was measured at 15-minute intervals during the 3 hours of CSS and averaged. It was kept consistent between groups over the course of the 3-hour cold ischemia and no significant change was noted (Appendix Figure 2).



Appendix Figure 2. Body weight and fasting blood glucose variation between mice that underwent GTT vs mice that underwent tail tipping. (A) Body weight GTT vs tail tip (B) Fasting glucose GTT vs tail tip. * $p < 0.05$. n = 9-10.

There was a significant increase in body weight between T2D mice that underwent a GTT compared to T2D mice that were tail tipped (Appendix Figure 1A). Both T2D mice and T2D + DMB mice that underwent a GTT also presented with significantly increased fasting blood glucose at week 12 compared to mice that were tail tipped for insulin and TMAO analysis (Appendix Figure 1B). Hence, it seems as there was cage variation in the T2D and T2D + DMB groups.



Appendix Figure 3. Cardiac Mass. Wet Heart Weight. DMB – 3,3-Dimethyl-1-butanol, Pre-Db – Prediabetes, CTRL – Control. n = 9-10.

There was no significant difference in wet heart weight between groups (Figure 4.6).