MITOCHONDRIAL DYSFUNCTION: FROM MOUSE MYOTUBES TO HUMAN CARDIOMYOCYTES

Georges Kanaan

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements for the

Doctorate in Philosophy degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology

Biochemistry Graduate Program

Faculty of Medicine

University of Ottawa

ABSTRACT

Mitochondrial dysfunction is a common feature in a wide range of disorders and diseases from obesity, diabetes, cancer to cardiovascular diseases. The overall goal of my doctoral research has been to investigate mitochondrial metabolic dysfunction in skeletal and cardiac muscles in the context of chronic disease development.

Perinatal nutrition is well known to affect risk for insulin resistance, obesity, and cardiovascular disease during adulthood. The underlying mechanisms however, are poorly understood. Previous research from our lab showed that the *in utero* maternal undernutrition mouse model is one in which skeletal and cardiac muscle physiology and metabolism is impaired. Here we used this model to study the impact of *in utero* undernutrition on offspring skeletal primary muscle cells and to determine if there is a cell autonomous phenotype. Metabolic analyses using extracellular flux technologies revealed a shift from oxidative to glycolytic metabolism in primary myotubes. Gene expression profiling identified significant changes in mRNA expression, including an upregulation of cell stress and OXPHOS genes and a downregulation of cell division genes. However, there were no changes in levels of marker proteins for mitochondrial oxidative phosphorylation (OXPHOS). Findings are consistent with the conclusion that susceptibility to metabolic disease in adulthood can be caused at least in part by muscle defects that are programmed *in utero* and mediated by impaired mitochondrial function.

In my second project, the effects of the absence of glutaredoxin-2 (Grx2) on redox homeostasis and on mitochondrial dynamics and energetics in cardiac muscle from mice

were investigated. Previous work in our lab established that Grx2-deficient mice exhibit fibrotic cardiac hypertrophy, and hypertension, and that complex I of OXPHOS is defective in isolated mitochondria. Here we studied the role of Grx2 in the control of mitochondrial structure and function in intact cells and tissue, as well as the role of GRX2 in human heart disease. We demonstrated that the absence of Grx2 impacts mitochondrial fusion, ultrastructure and energetics in mouse primary cardiomyocytes and cardiac tissue and that provision of the glutathione precursor, N-acetylcysteine (NAC) did not restore glutathione redox or prevent impairments. Furthermore we used data from the human Genotype-Tissue Expression consortium to show that low GRX2 expression is associated with increased fibrosis, hypertrophy, and infarct in the left ventricle. Altogether, our results indicate that GRX2 plays a major role in cardiac mitochondrial structure and function, and protects against left ventricle pathologies in humans.

In my third project, we collaborated with cardiac surgeon, Dr. Calum Redpath, of the Ottawa Heart Institute to study atrial mitochondrial metabolism in atrial fibrillation patients with and without type 2 diabetes (T2DM). T2DM is a major risk factor for atrial fibrillation, but the causes are poorly understood. Atrial appendages from coronary artery bypass graft surgery were collected and analyzed. We showed an impaired complex I respiration in diabetic patients with atrial fibrillation compared to diabetic patients without atrial fibrillation. In addition, and for the first time in atrial fibrillation patients, mitochondrial supercomplexes were studied; results showed no differences in the assembly of the "traditional" complexes but a decrease in the formation of "high oligomeric" complexes. A strong trend for increased protein oxidation was also observed. There were no changes in

markers for OXPHOS protein levels. Overall findings reveal novel aspects of mitochondrial dysfunction in atrial fibrillation and diabetes in humans.

Overall, our results reveal that *in utero* undernutrition affects the programming of skeletal muscle primary cells, thereby increasing susceptibility to metabolic diseases. In addition, we show that GRX2 impacts cardiac mitochondrial dynamics and energetics in both mice and humans. Finally, we show impaired mitochondrial function and supercomplex assembly in humans with atrial fibrillation and T2DM. Ultimately, understanding the mechanisms causing mitochondrial dysfunction in muscle tissues during chronic disease development will increase our capacity to identify effective prevention and treatment strategies.

ACKNOWLEDGMENTS

First and foremost, I want to thank my Lord and my Saviour Jesus Christ for the showers of blessings He bestowed upon me during all these years making the completion of this thesis possible.

This work would not have been accomplishable without the help of many people.

I would like first to thank my supervisor and my mentor Dr. Mary-Ellen Harper for believing in a young Lebanese guy coming from abroad, for providing me with a great environment to conduct research in and for supporting and guiding me through all the obstacles of my doctoral journey. I could not have done it without your leadership and mentorship. You are an exceptional scientist and a real inspiration. I have learned a lot from your experience, wisdom and diplomacy. I finish my PhD a better scientist and even more, a better individual. I am extremely honored and proud to be a member of your team. Dr. Harper's laboratory was not only a great research facility equipped with the state of the art instruments allowing me to conduct various experiments but also where I have met great people and have grown strong friendships. During the years that I spent in this laboratory I met and discussed science with brilliant minds and collaborated with outstanding scientists.

To the members my thesis advisory committee Dr. Kristin Baetz, Dr. Jean-François Couture and Dr. Amanda MacFarlane, I am very grateful for your input and evaluation on my several projects all these years.

To the current and previous members of the Harper Lab, I am thankful that I had the opportunity to meet you and work with you. You were an integral part of my journey, you were there in my cheerful and depressed days, always ready to help and support. It has been a privilege working with all of you.

I want to thank all the previous members of the Harper Lab: Dr. Celine Aguer, Dr. Brianne Thrush Bota, Dr. Ryan Mailloux, Dr. Fiona McMurray, Dr. Brittany Beauchamp, Dr. Ghadi Antoun, Dr. Olivier Charette, Dr. Melissa Pasqua, Cyril Adjeitey, Skye McBride, Ava Liaghati, Michaela Norigen, Bianca Ichim, Megan MacFarlane, Karan Ghandi, Shan Dhaliwal, Cecile Thao Uyen Le, Kijoo Kim, Jessica Quizi, Kristopher komm, Alyssa Peixoto, Pauline Caron, Michael Dysart, William Chen and Dhiya Hassan. My thanks go also to the current lab members: Jian Xuan, Dr. Neoma Tove Boardman, Dr. Chantal Pileggi, Dr. David Patten, Rajaa Sebaa, Abishankari Rajkumar, Daniella Febbraro and Elise Bisset.

To the “lab Musketeers” Elias Abou Samra and Lara Gharibeh, your presence made things a lot easier for me, we ate lunch together, nagged about PhD and about almost everything. We laughed, cried and had a lot of fun together. I am very grateful for your presence beside me my dear friends.

Elias my friend, words cannot explain how thankful I am that I met you 8 years ago. I really appreciate all what you have done to help me come to Canada and study here. Without your help and advice my path would have been way more difficult. Thank you from the bottom of my heart. I wish you the best in everything you do brother.

To all of my other friends near and far who have supported me through the years, I apologize not to thank and mention each and every one of you but for that I need more than one thesis.

To my mother Marcelle, my brother Michel, my aunts Micheline and Antoinette and my entire family, I would have not been here without your continuous support, trust and love. I am thankful and blessed to have you in my life. To my father Nicolas I say: I wish you were here today attending my defense. I hope that from where you are now, I am making you feel happy and proud. To my beautiful fiancée, to you Michelle, there are no words to express how blessed I am to have you in my life. Thank you for all your trust, support and unconditional love. You bared a lot with me during this long journey and you were always there for me when I needed you. My life without you is incomplete.

This thesis is dedicated to your soul father and to all of you.

LIST OF CONTENTS

LIST OF ABBREVIATIONS

γ-GCS	γ-glutamylcysteine synthetase
A1C	Glycated haemoglobin
ADP	Adenosine diphosphate
AF	Atrial fibrillation
AMPK	5' adenosine monophosphate-activated protein kinase
ANGPT2	Angiopoietin-like protein 2
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AV	Atrioventricular node
BMI	Body mass index
BN-PAGE	Blue Native-PAGE
BPA	Bisphenol A
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
CAT	Catalase
CHD	Coronary heart disease
COX	Cytochrome c oxidase
CoA	Acetyl coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
Drp1	Dynamin related protein-1
DTT	Dithiotheitol
E/A	Early filling wave peak/atrial contraction wave peak
ECAR	Extracellular acidification rate
EF	Ejection fraction
ETC	Electron transport chain
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDR	False discovery rate
Fis1	Fission protein 1
GEO	Gene Expression Omnibus
GPx	Glutathione peroxidase
GR	Glutathione reductase

Grx	Glutaredoxin
GS	Glutathione synthetase
GSEA	Gene Set Enrichment Analysis
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTEx	Genotype-Tissue Expression consortium
H_2O_2	Hydrogen peroxide
HIF1α	Hypoxia inducible factor 1α
HPLC	High pressure liquid chromatography
HRR	High resolution respirometry
IL-6	Interleukin 6
IRS	Insulin-receptor substrate
IUGR	*In utero* growth retardation
IVS	Intraventricular septum
LA	Left atrial
LV	Left ventricular
LVID	Left ventricular internal dimension
LVPW	Left ventricular posterior wall
MCT-1	Monocarboxylate transporter 1
Mfn	Mitofusin
MHC	Myosin heavy chain
MIM	Mitochondrial inner membrane
MM-CK	Myofibrillar creatine kinase
MOA	Monoamine oxidase
MOM	Mitochondrial outer membrane
Mon	Monomer
mtDNA	Mitochondrial DNA
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Normalized enrichment score
NOS	Nitric oxide synthase
Nox4	Nicotinamide adenine dinucleotide phosphate oxidase 4
OCR	Oxygen consumption rate
ODR	Obese diet resistant
ODS	Obese diet sensitive
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
$p66^{Shc}$	Growth factor adaptor Shc

PAC	Premature atrial contraction
PBS	Phosphate-buffered saline
PCr	Creatine phosphate
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1-α
PI3K	Phosphatidylinositol 3 kinase
PMF	Proton-motive force
PSVT	Paroxysmal supraventricular tachycardia
PTM	Post-translational modification
PVC	Premature ventricular contraction
PWD	Pulsed-Wave Doppler
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RBP4	Retinol-binding protein 4
RyR2	Type 2 ryanodine receptor
SA	Sinoatrial node
SC	Supercomplex
SEM	Standard error mean
SFHD	Serum-free hormonally defined medium
SFRP5	Secreted frizzled–related protein 5
SOD	Superoxide dismutase
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TIM	Inner membrane translocase
TMPD	N,N,N′,N′-Tetramethyl-p-phenylenediamine
TNF	Tumor necrosis factor
TOM	Outer membrane translocase
Trx	Thioredoxin
UCP	Uncoupling protein
WHO	World Health Organization
WPW	Wolff-Parkinson-White syndrome
WT	Wild type

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 – GENERAL INTRODUCTION

1.1 THE DISEASES

Humankind, since its existence, has tried to ensure its survival, comfort and supremacy. Humans have been able to control planet earth with all its resources, making them available for use almost instantly. In addition to water and energy, food is the most important resource and, in recent generations, the almost effortless access to food has created an imbalance between energy intake and energy expenditure. This imbalance is the root of one of the biggest health challenges facing our modern society: obesity. With obesity comes many health complications that can lead to type 2 diabetes (T2DM) and cardiovascular disease (CVD). My overall goal in this doctoral thesis was to evaluate mitochondrial function in primary cells and tissue of mice and humans affected by these diseases.

1.1.1 OBESITY

As defined by the World Health Organization (WHO), "obesity is an abnormal or excessive fat accumulation that may impair health" (World Health Organization, 2016). Our body, as all isolated systems, obeys the thermodynamics law of conservation of energy, which states that energy can neither be created nor destroyed, rather it transforms from one form to another. Therefore energy intake should be equal to energy expended over a period of time with any remaining energy being stored causing an increase in body mass, of which 60 to 80% is usually body fat (Hill *et al.*, 2012). When this fat accumulation becomes excessive, obesity occurs. To classify different stages of fat accumulation, the body mass

index (BMI) is used, due to its simplicity and non-invasiveness. It is the ratio of a person's weight (in kg) divided by the person's height squared (in m). In adults, a BMI between 25 kg/m^2 and 29.9 kg/m^2 indicates an overweight individual whereas a BMI equal to, or higher than 30 kg/m^2 indicates an obese person (Mendis *et al.*, 2014). Although it is the most widely used index for obesity, BMI has some clinical limitations. Age, sex, ethnicity, distribution of adipose tissue, bone and muscle mass are all factors that are not considered and can affect BMI (Centers for Disease Control and Prevention, 2017). Other relevant measurements in the characterization of obesity include the waist-to-hip ratio and waist circumference.

1.1.1.1 PREVALENCE

Obesity is now considered as one of the most important public health problems facing the world today; its prevalence has more than doubled since 1980 (Ng *et al.*, 2014; Stevens *et al.*, 2012). According to the most recent statistics available from the WHO in 2014, there are around 2 billion overweight adults, 670 million of whom are considered obese and 98 million morbidly obese. These numbers denote 13% of the world's adult population, representing 11% of men and 15% of women. The Americas are the most affected by this epidemic (27% of the population) and South-East Asia the least affected (5% of the population) (Mendis *et al.*, 2014). If the current trends continue it is projected that by 2030 20% of the world population will be obese (Hruby and Hu, 2015; Kelly *et al.*, 2008, Després, 2012).

In Canada, obesity rates increased by 17.5% between 2003 and 2012, reaching almost 25% of the whole population (6.3 million people). Contrary to the global trend, Canadian

men are more obese than women with prevalence rates of 26.1% and 23.4%, respectively (Navaneelan and Janz, 2014).

1.1.1.2 ETIOLOGY

It was only recently that obesity was considered a disease. It was in the early 2000s when health organisations and associations gradually endorsed the notion that obesity is actually a disease (Bray *et al.,* 2017). Obesity is a very complex disease with a multifactorial etiology. Genetic factors are important, and studies show that the estimated heritability of BMI is between 64 and 84% (Stunkard *et al.,* 1986; Bouchard, 2001; Farooqi and O'Rahilly, 2007; O'Rahilly and Farooqi, 2008). Along with genetic factors, other important factors include contextual factors like socioeconomic status, physical and social environment, geography, food preferences, gender, age, culture, and family composition play pivotal roles (González-Muniesa *et al.,* 2017; Williams *et al.,* 2015). Obesity ultimately occurs when a chronic imbalance between energy intake and energy expenditure takes place where excess of energy is stored in the adipose tissue in the form of triglycerides. Epigenetic factors appear to be important as well. Epigenetic mechanisms are DNA modifications resulting in gene expression changes without altering the DNA sequence. Our lab and others have shown that for example *in utero* undernutrition in mice leads to the development of obesity in the offspring later on in their lives (Gluckman *et al.,* 2008a; Beauchamp *et al.,* 2015a). Another great example is the Dutch famine in 1944, in which poor maternal nutrition during pregnancy (often as low as ~700kcal/day) was linked to increased disease susceptibility in the offspring during adulthood, including obesity (Roseboom *et al.,* 2006). Altogether the

development of obesity is complex and is affected by many factors. The major factors are summarised in Table 1.1.

Table 1.1 Different factors contributing to obesity

Factors	Examples	References
Genetic	Mutations in genes like FTO, LEPR, LPL and leptin among others	(Frayling *et al.*, 2007);(Dina *et al.*, 2007);(Mammès *et al.*, 2001); (Bender *et al.*, 2011);(Wang and Eckel, 2009);(Ayyappa *et al.*, 2017)
Lifestyle	Physical inactivity	(Pietiläinen *et al.*, 2008); (ten Hacken, 2009); (Day *et al.*, 2013);(Koutoukidis *et al.*, 2015)
	Diet high in fat, carbohydrates and energy but low in fiber	(Mendis *et al.*, 2014); (Bray *et al.*, 2016, 2017)
	Sleep duration and bedtime	(Jiang *et al.*, 2009);(Liu *et al.*, 2012);(Nedeltcheva and Scheer, 2014); (Wang *et al.*, 2017)
Endocrine disorders	Hypothyroidism, polycystic ovarian syndrome	(Bougnères *et al.*, 2008; Reinehr *et al.*, 2007); (Scerif *et al.*, 2011)
Socioeconomic	Education, income, living location and occupation	(Mendis *et al.*, 2014); (Bray *et al.*, 2016, 2017)
Environmental	*In utero* nutrition	(Beauchamp *et al.*, 2015a; Jimenez-Chillaron *et al.*, 2009); (Reynolds *et al.*, 2015)
	Sugar-sweetened beverages	(Taber *et al.*, 2013)
	Television and video games	(Braithwaite *et al.*, 2013);(Gilbert-Diamond *et al.*, 2014); (Stettler *et al.*, 2004)
	Gut microbiome	(Burcelin, 2012);(Tremaroli and Bäckhed, 2012);(Jess, 2014); (Turta and Rautava, 2016)
	Endocrine-altering toxins (DDT and BPA),	(Mirmira and Evans-Molina, 2014); (Trasande *et al.*, 2012); (Warner *et al.*, 2014); (La Merrill *et al.*, 2014); (Frugé *et al.*, 2016)
	Adenovirus 36	(Atkinson *et al.*, 2005, 2010); (Gabbert *et al.*, 2010);(Esposito *et al.*, 2012); (Ponterio and Gnessi, 2015);

1.1.1.3 COMORBIDITIES

With increasing fat storage, fat cell size also increases resulting in the release of inflammatory cytokines (Bray *et al.,* 2017). Thus obesity negatively impacts human health and can lead ultimately to death, due to the fact that as obesity progresses, the risk for other diseases increases. These diseases include type 2 diabetes mellitus (Menke *et al.,* 2014; DeFronzo *et al.*, 2015), cardiovascular diseases (hypertension, stroke, coronary artery disease and congestive heart failure) (Guh *et al.*, 2009; Schnabel *et al.*, 2013; Yatsuya *et al.*, 2010), gallstone disease (Radmard *et al.,* 2015), osteoarthritis (Reyes *et al.,* 2016), asthma (Guh *et al.,* 2009; Apovian, 2016), cancers (uterine, gallbladder, kidney, cervical, and thyroid, leukemia, liver, colon, ovarian, and postmenopausal breast cancer)(Apovian, 2016; Bray *et al.*, 2016; 2017).

Other conditions related to obesity also include sleep apnea (Li *et al.*, 2007; Foster *et al.,* 2009), chronic back pain (Guh *et al.,* 2009), non-allergic rhinitis, major depressive disorder and stigmatization (Apovian, 2016; Bray *et al.,* 2017). Major diseases and comorbidities associated with obesity are summarized in Figure 1.1.

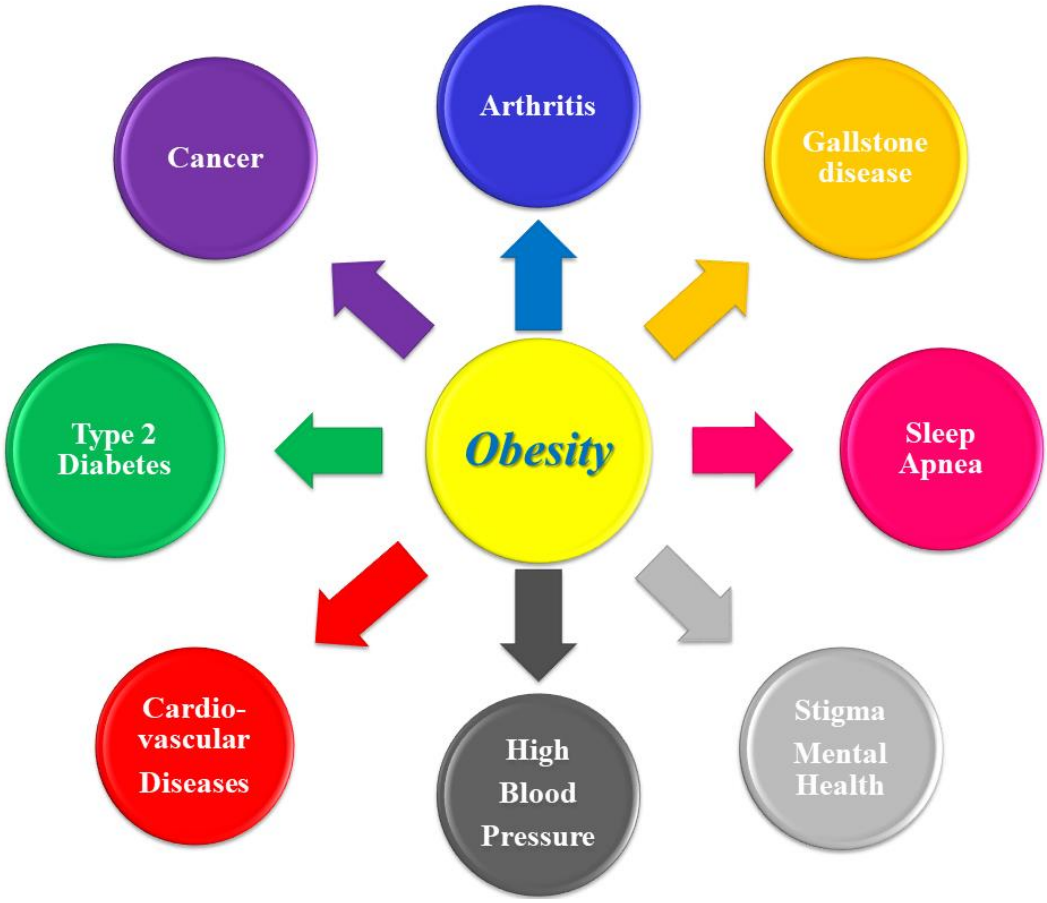


Figure 1.1 Obesity-related diseases and comorbidities.

1.1.1.4 MANAGEMENT

Just as obesity is a complex multifactorial disease, its management should also be diverse and include a multicomponent treatment plan. Fundamentally the goal is to prevent obesity in the first place but whenever it occurs then the goal shifts to weight loss. Losing weight will prevent or reverse obesity related comorbidities and will have a significant effect on mortality (Jensen *et al.,* 2014). For example, using diet/exercise programs to achieve a weight loss of 5.1kg reduces systolic and diastolic blood pressure by 4.44 and 3.47 mm Hg, respectively (Neter *et al.,* 2003). There are three major categories of treatment options for weight loss: lifestyle changes (behavioural training, diet, physical activity), medications and surgical interventions (bariatric surgery) (Bray *et al.,* 2016).

1.1.2 TYPE 2 DIABETES MELLITUS

T2DM is a chronic progressive metabolic disease, in which hyperglycemia develops as a result of insulin resistance and impaired compensatory insulin secretion. Its development is correlated with obesity. Individuals with a BMI of 35kg/m^2 or higher are 20 times more prone to develop T2DM, compared to individuals with a BMI ranging between 18.5 and 24.9 kg/m^2 (Padwal and Sharma, 2010). Chronic hyperglycemia is associated with organ failure and dysfunction, especially the eyes, kidneys, heart, nerves and blood vessels (American Diabetes Association, 2010). T2DM diagnostic criteria are summarized in Table 1.2 (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg and Punthakee, 2013).

Table 1.2 Diagnosis of T2DM

Blood Markers	Diabetic when above
Fasting Plasma Glucose (FPG)*	≥ 7.0 mmol/L
Glycated Hemoglobin (HbA1C)**	≥ 6.5%
2-hour Plasma Glucose (2hPG)***	≥ 11.1 mmol/L
Random Plasma Glucose (PG)	≥ 11.1 mmol/L

Fasting for at least 8hours; ** In adults; * In a 75 g oral glucose tolerance test (OGTT) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013.*

1.1.2.1 PREVALENCE

Recent estimates indicate that diabetes (types 1 and 2) has a global prevalence in adults of 422 million in 2014 (8.5% of the population), which is a substantial increase from 108 million in 1980 (4.7% of the population) (Mathers and Loncar, 2006; World Health Organization, 2017). More than 1.6 million deaths were directly attributed to diabetes in 2015. The WHO is projecting that diabetes will be the seventh cause of death by 2030. In Canada, diabetes affected 9.3% of the population (3.4 million) in 2015 and is estimated to increase and reach 12.1% (5 million) in 2025. With this estimated increase, the cost of treating diabetes will also increase by approximately 25% in 2025 (Diabetes Canada, 2017).

1.1.2.2 ETIOLOGY

While T2DM is a complex chronic disease with many risk factors, obesity is the greatest risk factor. The relative risk to develop T2DM in obese men and women is 10-fold and 11.2-fold respectively (Field *et al.*, 2001; Golay and Ybarra, 2005). Often obesity develops simultaneously with a range of other conditions such as abdominal fat accumulation, glucose intolerance, insulin resistance, dyslipidemia, and hypertension in what

is known as the “metabolic syndrome”. Metabolic syndrome increases 5 times the risk of developing T2DM and doubles the risk of cardiovascular diseases (Grundy *et al.,* 2005; Cornier *et al.,* 2008).

Along with obesity, T2DM has also genetic factors. Studies have shown that race, ethnicity and family history also affect the disease and its severity (Annis *et al.,* 2005; Spanakis and Golden, 2013). Aside from these factors genetic mutations were also linked with the onset of diabetes, genes like FTO, PPARG, IGF2BP2 and others (Das and Elbein, 2006; Billings and Florez, 2010; Fuchsberger *et al.,* 2016). With all the advances in characterizing the genetic factors underlying the development of T2DM, genetic variants explain only 10% of T2DM heritability, and thus are not yet useful in clinical prediction (Billings and Florez, 2010).

Moreover and as in obesity, lifestyle plays a major role in impacting the development of diabetes. Excessive dietary intake and alcohol consumption in addition to physical inactivity and smoking contribute substantially to the onset of T2DM; controlling these factors can reduce these risks (Reis *et al.*, 2011).

It is quite clear that not one factor is responsible for T2DM rather it is the interaction of all these factors combined (Figure 1.2).

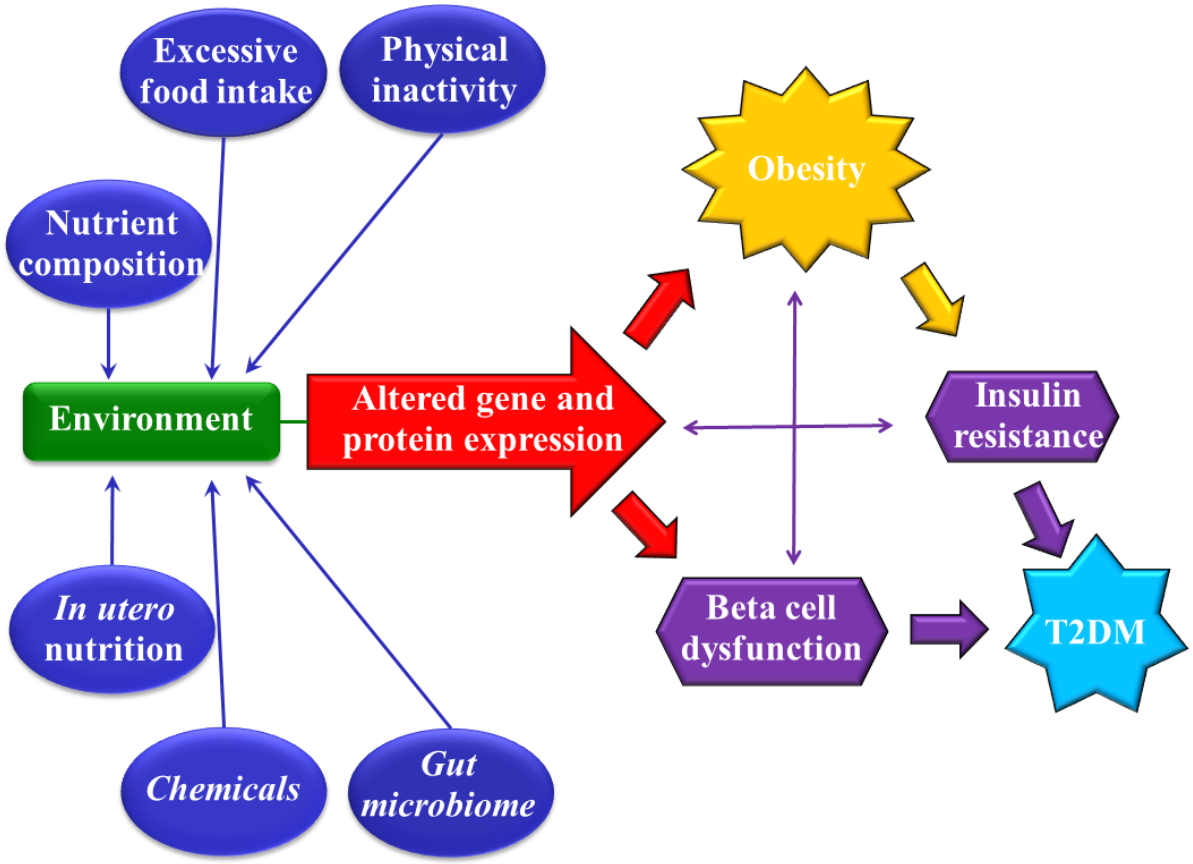


Figure 1.2 Factors involved in T2DM onset

1.1.2.3 NORMAL GLUCOSE HOMEOSTASIS

During fasting glucose homeostasis, the majority of total body glucose metabolism takes place in insulin independent tissues, like the brain (50%) and the splanchnic area (25%). The remaining 25% of glucose metabolism takes place in insulin-dependent tissues, primary in the muscle. Gluconeogenesis occurs mainly in the liver and the remaining amount is de novo glucose production is in the kidney. In the liver glucagon is activated to elevate blood glucose, in which 50% comes from glycogenolysis and the remaining 50% from gluconeogenesis (DeFronzo and Ferrannini, 1987; DeFronzo *et al.,* 1989; Landau *et al.,* 1996).

During postprandial glucose homeostasis the balance between glucose production and tissue uptake is disturbed. This disturbed balance causes blood glucose concentration to increase, which subsequently stimulates insulin release from the pancreatic beta cells and resulting in hyperinsulinemia and a lowering of circulating glucose levels. These conditions will force the body to stimulate glucose uptake by the tissues and to suppress endogenous glucose production. 80 - 85% of glucose that is taken by the peripheral tissues is used in the muscles with only a small amount being metabolized by adipocytes (4-5%). When blood insulin level increases, it inhibits the degradation of lipids. This will decrease plasma free fatty acid, increase muscle glucose uptake and inhibit endogenous glucose production (Boden, 1997; DeFronzo and Tripathy, 2009; McGarry, 2002).

1.1.2.4 PATHOGENESIS OF T2DM

Glucose homeostasis is dependent upon normal insulin secretion by the pancreatic beta cells and the normal tissue sensitivity to the effects of hyperinsulinemia and hyperglycemia. Problems occur when tissues start to resist the effect of insulin in a phenomenon called insulin resistance. This will happen in the early stages of T2DM pathogenesis along with impaired glucose metabolism. Insulin resistance in the liver and kidneys leads to further increases in gluconeogenesis. Insulin resistance in the peripheral tissues such as skeletal muscle and adipose tissue can reduce glucose uptake by 50% in skeletal muscle (DeFronzo *et al.,* 1985; DeFronzo and Tripathy, 2009).

When these insulin-sensitive tissues become resistant, a positive feedback signal is sent to the pancreatic β cells in the islets of Langerhans to increase insulin production and secretion in order to overcome the resistance and maintain normal glycemia. This hyperinsulinemia will manage to keep blood glucose levels under control and it is not until these β-cells become incapable of this adaptive response that hyperglycemia arises (Kahn *et al.*, 2014).

In addition, it is important to mention that individuals with impaired glucose tolerance or impaired fasting glucose have higher than normal blood glucose but lower that the threshold to be considered fully diabetic. So these individuals are considered to have prediabetes with a high risk of developing full T2DM (Perreault *et al.*, 2012, 2014).

1.1.2.5 COMORBIDITIES

Detecting T2DM in the early stages of glucose intolerance and insulin resistance is very important not only to prevent its full-blown development, but also to prevent the development of comorbidities. Unfortunately clinical diagnosis of diabetes is in some cases delayed from its original onset by up to 7 years, which can lead to many serious complications (Fraser *et al.,* 2010). Vascular complications of the disease can be classified into microvascular and macrovascular. Microvascular complications include: retinopathy, peripheral neuropathy, chronic kidney disease and delayed wound healing mostly in the feet causing ulcerations that sometimes require amputation. Macrovascular complications include: coronary artery disease, strokes, heart failure, and erectile dysfunction. These complications are due to the injuries of nerves and organs that happen after chronic hyperglycemia damages small blood vessels and capillary beds (Canadian Diabetes Association Clinical Practice Guideline Expert Committee, 2013).

1.1.2.6 MANAGEMENT

As mentioned before, T2DM is a complex disease and treating it is not a simple task. Health care practitioners start by lifestyle recommendations (a healthy diet and increased physical activity), complemented by pharmacotherapy (*e.g.*, insulin, sulfonylureas and others) depending on the treatment response. These interventions aim to achieve glycemic control. By insulin being a key player in regulating hyperglycemia, exogenous supplementation of this peptide hormone remains the most widely used intervention especially in cases where pancreatic failure occurs. Another aspect of T2DM management is

the fact that as the disease can have multiple complications affecting a wide range of organs and tissues, the ability to refer patients to different specialists is of a major importance.

1.1.3 CARDIOVASCULAR DISEASE (CVD)

Internationally, CVD causes more deaths than any other disease. The majority of these deaths occur in low- and middle-income countries. CVDs are a group of pathologies that affect the heart and the blood vessels and they include a range of disorders summarized in Table 1.3 (World Health Organization, 2017; American Heart Association, 2017).

Table 1.3 List of cardiovascular diseases

Cardiovascular diseases (CVDs) include:
• Coronary/Ischemic heart disease • Cerebrovascular disease (Stroke) • Congestive heart disease • Hypertensive heart disease • Rheumatic heart disease • Myocarditis/Endocarditis/Pericarditis • Cardiomyopathy • Congenital heart disease • Peripheral arterial disease • Arrhythmias • Heart valve problems • Deep vein thrombosis and pulmonary embolism

1.1.3.1 PREVALENCE

According to the WHO, 31% of all global deaths (17.7 million people) were attributed to CVD in 2015 (World Health Organization, 2017). The 2 most common disorders in CVDs are coronary artery disease and cerebrovascular disease, and together they were responsible

for 14.1 million deaths worldwide in 2015. In Canada the situation is not very different; CVDs were the second cause of death with 25.1% of all deaths in 2013, only second to cancer with 29.8%. Data from the Public Health Agency of the Canadian Chronic Disease Surveillance System (CCDSS) in 2012-13 show that 1 in 12 Canadian adults (20 years and over) live with diagnosed heart disease (Statistics Canada, 2017).

1.1.3.2 ETIOLOGY

Over 80% of premature CVD is preventable according to the WHO. This risk factor improvement can help reduce the burden on both patients and healthcare systems (World Health Organization, 2017). One of the most important determinant factors for developing CVD is age. With the increasing lifespan of humans, the percentage of people 65 years and older is also increasing and this percentage will continue to increase in the next 20 years. It is also expected that the percentage of death from CVD in this age group will continue to rise (North and Sinclair, 2012).

In addition to age, other risk factors also contribute in the development of CVD. These include unhealthy diet, physical inactivity, obesity, genetics, tobacco, alcohol, hypertension, diabetes, high blood cholesterol, socioeconomic factors, globalization and urbanization (Bowry *et al.*, 2015).

1.1.3.3 PREVENTION AND MANAGEMENT

It is beyond the scope of this thesis to discuss the prevention and management of CVDs but it is noteworthy to mention that obesity and T2DM are associated with increased

risks for CVD. Prevention of these diseases can help reduce the majority of CVD occurrences. Prevention and treatment approaches include lifestyle interventions (healthy diet, physical activity, balanced alcohol consumption and no smoking), medications and surgical interventions.

1.1.4 ARRYTHMIAS

By definition, an arrhythmia is “a problem with the rate or rhythm of the heartbeat. The heart can beat too fast (tachycardia), too slow (bradycardia), or with an irregular rhythm” (National Heart, Lung and Blood Institute, 2017). Although most arrhythmias are harmless, some can be life threatening as a result of impaired blood flow and damaged organs including the brain, the heart itself and many other organs (National Heart, Lung and Blood Institute, 2017).

Understanding the cardiac conduction system is crucial in understanding arrhythmias. To pump the blood efficiently, the heart relies on its contraction. This process is dependent on the proper generation and conduction of electrical impulses. These electrical signals are generated by a group of cells, located in the right atrium, called the sinus node or the sinoatrial (SA) node. In a healthy adult heart, in 1 minute the SA node fires electrical signals enough for the heart to beat between 60 and 100 times. From the SA node, the electric impulse travels and activates the atria causing them to contract and pump blood towards the ventricles. The signal continues to reach the atrioventricular (AV) node located between the atria and the ventricles. At this stage the electrical signal slows down to allow the blood to finish filling the ventricles. Subsequently, the impulse leaves the AV node and travels rapidly

through the bundle of His and the Purkinje fibre network to stimulate the ventricles to contract and pump the blood to the lungs and to the rest of the body (Boukens and Christoffels, 2012). Any problem at any stage of this highly coordinated process can result in the occurrence of arrhythmias.

1.1.4.1 TYPES

There are four main types of arrhythmias: premature contraction, supraventricular arrhythmias, ventricular arrhythmias, and bradyarrhythmias (Padhi *et al.*, 2014).

- **Premature contraction** consists of extra beats, which are usually harmless and asymptotic. This type usually does not require any treatment. When this occurs in the atria the contractions are called premature atrial contractions (PACs) and when they happen in the ventricles they are called premature ventricular contractions (PVCs). Premature contraction happens naturally in most cases although some heart diseases can cause it too. Stress, excessive exercise, or excessive consumption of caffeine or nicotine can also lead to premature beats (National Heart, Lung and Blood Institute, 2017).
- **Supraventricular arrhythmias** are tachycardias that start in the atria or the AV node. They include atrial fibrillation (AF), atrial flutter, paroxysmal supraventricular tachycardia (PSVT), and Wolff-Parkinson-White (WPW) syndrome.

  Atrial fibrillation (AF) is the most common arrhythmia, and occurs when the heart electrical signals do not initiate at the SA node but instead they begin in another part of the atria or nearby pulmonary veins. Then the signals spread abnormally in a very fast

manner causing the atria walls to fibrillate. As a result, the blood pumping from the atria to the ventricles is affected.

Atrial flutter is much less common than AF but very similar to it. The difference is that the electric signals spread in a fast regular rhythm in atrial flutter, compared to AF.

Paroxysmal supraventricular tachycardia (PSVT) is a very fast heart rate that begins and stops suddenly. It mainly occurs in young individuals and is not life threatening. It occurs when electrical signals coming from the atria and travelling to the ventricles re-enter the atria again, causing extra heartbeats. Vigorous physical activity can cause this type of arrhythmia.

Wolff-Parkinson-White (WPW) syndrome is a special type of PSVT in which the electrical signals travel an extra pathway causing a disruption in the contraction timing that push the ventricles to beat very fast in a life-threatening manner (National Heart, Lung and Blood Institute, 2017).

- **Ventricular arrhythmias** originate in the ventricles and include ventricular tachycardia and ventricular fibrillation. This type of arrhythmia can be caused by a weakened heart muscle, CHD, heart attack and other problems.

Ventricular tachycardia is a fast, regular ventricle beating that can last from few seconds to long periods. When it happens for a long time it can turn to ventricular fibrillation.

Ventricular fibrillation occurs when disorganized electrical signals make the ventricles fibrillate instead of beating normally thereby impairing blood pumping to the body. It is a very dangerous arrhythmia that can lead to sudden cardiac arrest and death in just a few

minutes. To prevent death, defibrillators are used right away treat this condition when it occurs (National Heart, Lung and Blood Institute, 2017).

- **Bradyarrhythmias** occur if the heart is beating slower than normal (less than 60bpm in adults). If the heart is too slow in pumping blood to the brain, it can cause fainting. Bradycardia can happen due to heart attacks/damage, ageing, thyroid hormone disorders, imbalance of substances in the blood like potassium and medicines such as beta-blockers, calcium channel blockers, digoxin and some antiarrhythmia drugs (National Heart, Lung and Blood Institute, 2017).

1.1.4.2 PREVALENCE

Cardiac arrhythmias are common in general population. Prevalence data for arrhythmias are incomplete mainly because individuals suffering from the majority of arrhythmias are asymptomatic (Padhi *et al.,* 2014). The existing data show that arrhythmia prevalence increases with age. In Japan, a study showed that prevalence risk of arrhythmias increases from 1.25% in elementary school students to 2.32% in junior high school students (Niwa *et al.,* 2004). Another study in America showed that a large proportion of healthy individuals aged between 60 and 85 years old have arrhythmias with no symptoms at all; 24% were found to have supraventricular arrhythmia and 49% had ventricular arrhythmia (Fleg and Kennedy, 1982).

1.1.5 ATRIAL FIBRILLATION

As mentioned earlier, atrial fibrillation (AF) is the most common cardiac arrhythmia. Moreover it is a major cause of morbidity and mortality worldwide with an estimated 5 million cases globally in 2010 (Chugh *et al.,* 2014). In addition to the quality of life, it deteriorates myocardial function increasing vulnerability to heart failure, stroke, dementia hospitalization and death (Mirza *et al.,* 2012; Rahman *et al.,* 2016). Furthermore its prevalence is increasing in both developed and developing countries (Conen *et al.*, 2011). It is a leading cause for CVD in the world (Murray *et al.,* 2012). The prevalence in America and European Union was 11 million people in 2010 and is projected to increase to 24-30 million by 2050 (Mozaffarian *et al.,* 2015). It is almost two times more likely to occur in men than in women showing a sex effect (Kodani and Atarashi, 2012). Its occurrence is strongly associated with obesity, T2DM, hypertension, ageing, renal disease and cardiac pathologies (Murphy *et al.,* 2007; Krittayaphong *et al.,* 2016; Odutayo *et al.,* 2016; Rahman *et al.,* 2016).

In Canada, AF affects approximately 350,000 individuals and results in significant morbidity and mortality (Benjamin *et al.*, 1998, 2017; Heart and Stroke, 2017). Individuals with AF have a risk of stroke that is 3 to 5 times greater than those without AF. AF exists in different forms: paroxysmal (24hours), persistent (more than 7 days) or permanent (more than 1 year) (Heart and Stroke, 2017).

1.1.5.1 AF MANAGEMENT

Managing a health problem in the best possible way requires an early detection and diagnosis that leads to an effective treatment strategy. Unfortunately this is rarely the case with AF. Current methods and technologies, although extraordinarily improved from the past, are not always able to detect AF before a major cardiac event occurs (Liew, 2013). Detecting AF early by physicians is very important to prevent cardiogenic stroke and eventually heart failure and death (Kodani, 2015). When AF is detected, treatment strategies are developed according to each individual condition. The choice between rate or rhythm control and the use of anticoagulant drugs or not, are key points to be considered in treating AF (Rahman *et al.,* 2016). Rate control can be achieved by using medicines like β-blockers, calcium-channel blockers and digoxin (inhibits sodium potassium adenosine triphosphatase, mainly in the myocardium) or in rare cases by medical procedures like AV node ablation with ventricular pacing. For rhythm control other interventions are used, including: pharmacological cardioversion (defibrillation) and medical interventions such as electrical cardioversion, and catheter ablation therapy (Le Heuzey *et al.,* 2012; Albert and Stevenson, 2016; Rahman *et al.,* 2016).

Since AF increases the risk of stroke and thromboembolism, anticoagulant drugs like vitamin K antagonists, antiplatelet drugs, heparins, antithrombin, and anti-Xa drugs are prescribed to reduce these risks (Le Heuzey *et al.,* 2012).

1.2 MAJOR MUSCLE TYPES

The human body contains more than 600 muscles. Their function is to produce force and motion thus regulating important processes like maintaining body posture, locomotion and the movement of internal organs like the heart and the digestive system. Muscles are divided into three categories or types: skeletal, smooth and cardiac (Ostrovidov *et al.*, 2014).

1.2.1 SKELETAL MUSCLE

Skeletal muscle is one of the major organ groups in the human body. It is a very dynamic and plastic tissue compromising about 40% of the human adult body weight and containing 50-75% of total body proteins. Its dysfunction is involved in many diseases. It is composed of 75% water, 20% proteins and 5% other substances (minerals, carbohydrates, fat and inorganic salts) (Frontera and Ochala, 2015). Skeletal muscles are attached to bones by tendons, which are fibrous bands of connective tissue. Individual muscle fibrils form muscle fibers (myofibers), the main contractile unit in the muscle, and then muscle fibers are grouped to form fascicles. In turn these fascicles are further organized to form whole muscles contained within a fascia. It is the contraction of these muscles that allow humans to move in a controlled way (Gillies and Lieber, 2011). Furthermore skeletal muscle has the ability to adapt to external conditions and physiological challenges by changing its size, composition and aerobic capacity. These changes are mediated by variations in gene expression and biochemical properties. This is why skeletal muscle can adapt to various metabolic needs, for example exercise increases its size, endurance and respiratory capacity. This is in contrast to

immobilization and a sedentary life, which lead to muscle mass loss (Coyle, 2000; Flück and Hoppeler, 2003; Luquet *et al.,* 2003; Fry, 2004; Hénique *et al.,* 2015)

Myofibers are multinucleated cells that are rich in mitochondria, which produce ATP to support contraction and other energy-demanding processes. Mitochondria in muscle can be found as punctate structures, but are mostly found as a reticular network (Kirkwood *et al.,* 1986). The mitochondrial content in these muscles can vary a lot, depending on many factors such as the physical activity (increases) and age (decreases) (Frontera and Ochala, 2015). In addition, the roles for mitochondria are associated with their subcellular localization. For example, subsarcolemmal mitochondria, located just beneath the sarcolemma surface, produce ATP to feed the different cellular and organelle-related processes like protein synthesis, ion exchange, substrate transport, cell signaling and others. Meanwhile, intermyofibrillar mitochondria, located deep within myofibrils, produce ATP mainly to support muscle contraction (Cogswell *et al.,* 1993).

Human skeletal muscle is heterogeneous with significant variability in the biochemical, mechanical and metabolic phenotypes of muscle fibers. Different muscles have different muscle fibre type mixtures depending on their roles and location in the body. Traditionally fiber types are divided into 2 groups depending on the classification of their myosin heavy chain (MHC) isoforms: type I and type II. Type I fibers are largely more aerobic, more fatigue resistant due to their high mitochondrial content, slow twitch and have higher myoglobin content. Contrary to type I fibers, type II fibers are more glycolytic, fatigue sensitive, fast twitch and have reduced mitochondrial and myoglobin contents. In

human skeletal muscles, type II fibers are further subdivided to IIa (more oxidative) and IIX (more glycolytic). Recent research demonstrates the important role of muscle fiber type heterogeneity in the context of human obesity (Westerblad *et al.,* 2010; Gerrits *et al.,* 2010; Schiaffino and Reggiani, 2011; Galpin *et al.,* 2012; Ciciliot *et al.,* 2013).

1.2.2 SKELETAL MUSCLE AND OBSEITY

In obesity, circulating fatty acids levels are increased and as a result an increase in skeletal muscle fatty acids uptake from circulation leads to ectopic lipid deposition (Goodpaster and Wolf, 2004; Mittendorfer *et al.,* 2009; Akhmedov and Berdeaux, 2013). On the other hand, skeletal muscle is a major energy user; thus it plays an important role in obesity development. As mentioned previously, skeletal muscle represents almost 40% of the human body weight accounting for 20% of total resting metabolic rate of the body. This metabolic rate increases 20 times during exercise in professional athletes largely due to increased energy expenditure by skeletal muscles (Rolfe and Brown, 1997).

Skeletal muscle is a very dynamic organ that adapts with different situations. In obesity, skeletal muscle reacts to the increased weight bearing load by increasing its size and length, leading to greater absolute maximal muscle strength (Lafortuna *et al.,* 2014). Nevertheless in terms of strength per body mass, obese individuals are weaker (Blimkie *et al.,* 1990; Hilton *et al.,* 2008; Lafortuna *et al.,* 2014). This may be due to obesity-related muscle performance functional limitations and disabilities like mobility, strength, postural and dynamic balance limitations (Tomlinson *et al.,* 2016).

1.2.3 SKELETAL MUSCLE AND T2DM

Insulin resistance occurs in the early stages of T2DM pathogenesis. In these stages, insulin-dependent tissues like skeletal muscle have impaired insulin-stimulated glucose uptake. This is extremely important since skeletal muscle accounts for 80-90% of postprandial glucose uptake making it a major player in T2DM pathogenesis (Petersen and Shulman, 2006).

Even after years of research and an enormous number of studies, the exact role of skeletal muscle in insulin resistance and T2DM is not fully understood. What is known is that decreased insulin-stimulated glucose uptake in skeletal muscle is due to impaired insulin signaling in addition to multiple intracellular defects such as impaired translocation of glucose transporter-4 (GLUT4) to the plasma membrane, impaired glucose phosphorylation, reduced glucose oxidation and glycogen synthesis and decreases in TCA cycle enzymes (Lithell *et al.,* 1981; Cusi *et al.,* 2000; Ducluzeau *et al.,* 2001; Bajaj and Defronzo, 2003; Bouzakri *et al.,* 2005; Karlsson and Zierath, 2007; Abdul-Ghani and DeFronzo, 2010). Increased intramyocellular fat and fatty acid metabolites are shown to play an important role in inducing insulin resistance in muscles of different fiber types where the severity of insulin resistance was correlated with the amount of accumulated lipids (Jacob *et al.,* 1999; Malenfant *et al.,* 2001; Bays *et al.,* 2004; Peterson *et al.,* 2009; Lara-Castro and Garvey, 2008; Lettner and Roden, 2008). Furthermore, lipid accumulation inhibits insulin-receptor substrate (IRS)-1 tyrosine phosphorylation, reducing the activity of IRS-1-associated phosphatidylinositol 3 kinase (PI3K). This step is critical in the development of insulin

resistance since PI3K activates protein kinase B/Akt, which is a key player in insulin metabolic and growth actions (Abdul-Ghani and DeFronzo, 2010). In addition, the accumulation of fat in the muscle was shown to be linked with the inability to optimally use various fuel sources by muscle cells in what is known as “metabolic inflexibility”. Metabolic inflexibility occurs due to the decreased glucose and increased fatty acid metabolism, when most needed, during post-prandial periods when glucose levels in the blood are elevated (Phielix and Mensink, 2008). The mechanisms underlying these phenomena are not yet fully understood but it is thought that mitochondrial dysfunction plays a major role (Rieusset, 2015).

1.3 CARDIAC MUSCLE

At structural and organisational levels, cardiac muscle is similar to skeletal muscle; however, cardiac fibers are usually shorter and contain only one nucleus located in the central region of the cell. There are two major types of cardiomyocytes: myocardial contractile cells and myocardial electrical cells. The former type constitutes 99% of cells in atria and ventricles and the latter constitutes the remaining 1%. These contractile cells conduct electrical impulses causing the heart to contract and to pump the blood through the body (Downey and Heusch, 2001). These cardiac muscle cells are by far the most physiologically energetic cells in the body, contracting continuously, allowing the heart to achieve its proper function as a pump. To do so, the heart beats about 100,000 times daily pumping approximately 10 tons of blood delivering oxygen and nutrients to the entire body (Severs, 2000; Neubauer, 2007a). This intense workload requires extremely high energy

demand reaching approximately 6 kg of ATP daily, which is 20 to 30 times the weight of the heart itself (Neubauer, 2007a). To sustain such high rates of ATP daily, the heart is packed with mitochondria which account for almost 35% of the volume of cardiac tissue (Stride *et al.*, 2013).

1.3.1 CARDIAC MUSCLE AND OBESITY

As discussed in previous sections, obesity is associated with the development of major risk factors for CVD such as atherosclerosis, hypertension, hyperlipidemia, and diabetes. Furthermore there is a distinct effect of obesity on the heart independently from atherosclerosis (Vasan, 2003).

Adipose tissue is mainly present in subcutaneous and visceral depots. However, other important sites of adipose deposition include epicardial, perivascular, and pulmonary adipose tissue, and these deposits can have important implications for CVD. These fat tissues also function as endocrine organs producing adipocyte-derived cytokines known as adipokines. In a healthy individual there is a balance between pro-inflammatory adipokines and anti-inflammatory adipokines. Obesity induces an imbalance in adipokines by increasing pro-inflammatory adipokine expression, causing the development of a chronic low-grade inflammatory state (Nakamura *et al.*, 2014). Pro-inflammatory cytokines include leptin, resistin, retinol-binding protein 4 (RBP4), angiopoietin-like protein 2 (ANGPT2), tumor necrosis factor (TNF), chemirin and interleukin 6 (IL-6). Anti-inflammatory adipokines include adiponectin, secreted frizzled–related protein 5 (SFRP5), adipolin, vaspin, apelin and omentin (Wang and Nakayama, 2010; Nakamura *et al.*, 2014).

Obesity-associated inflammation causes immune cell infiltration, impaired vascular structure and function, adipose tissue fibrosis and cardiac remodeling. Many structural and functional changes occur at the heart level including left ventricular (LV) hypertrophy, left atrial (LA) enlargement, increased cardiac adiposity and subclinical impairment of LV systolic and diastolic function leading eventually to heart failure (Vasan, 2003; Abel *et al.*, 2008; Fuster *et al.*, 2006). Atrial enlargement was also shown to be correlated with high risk factor for new-onset of atrial fibrillation (Wang *et al.*, 2004).

1.3.2 CARDIAC MUSCLE AND T2DM

In North America, 80% of all diabetic deaths are due to CVD making it a leading cause of mortality. Myocardial infarction and heart failure are common events in patients with T2DM (Glass *et al.*, 2010; Flink *et al.*, 2013). Cardiac metabolic rearrangements caused by T2DM including fatty acid metabolism and suppressed glucose oxidation contribute to the cardiac problems along with increased in visceral fat tissue. In addition, diabetes was shown to impact intercellular signaling, causing impairments in excitation-contraction coupling, insufficient energy production and increased vulnerability to ischemia/reperfusion injuries. Remodeling of extracellular matrix and loss of normal blood vessels in diabetic hearts are also involved in diabetic cardiomyopathy pathogenesis. All of these effects are independent from the presence of ischemic or hypertensive or valvular heart disease (Cai and Kang, 2003; Miki *et al.*, 2013; Cai and Keller, 2014; Noyes *et al.*, 2014; Isfort *et al.*, 2014; Bugger and Abel, 2014; Huynh *et al.*, 2014). Many challenges remain in detecting diabetic cardiomyopathies in asymptotic diabetic patients requiring

improved strategy and guidelines in preventing and treating this type of heart disease (Trachanas *et al.,* 2014).

1.4 THE ORGANELLE: MITOCHONDRIA

Human life depends on the energy that is required to maintain ordered and functional states in cells and tissues. The energy required is transduced during cellular fuel oxidation processes to support the energy demands for building and maintaining cellular and organismic structures and their function (Schafer and Buettner, 2001). These processes depend on the "powerhouses" of the cells: the mitochondria.

1.4.1 BACKGROUND

The word, mitochondria originates from the Greek, "mitos", which means thread, and "chondrion", which means granule. Mitochondria were discovered in mid-1800s and named by Carl Benda in 1898 (Ernster and Schatz, 1981). They were given this name due to their filamentous, interconnected reticulum, or independent granule shapes that they can take. Their size and shape can vary depending on their context and localization. They are abundant organelles, accounting for 10 to 20% of most cell type's mass and reaching 30% in more energetic cells like cardiomyocytes (Schaper *et al.,* 1985). Like all cellular organelles, mitochondria contribute to cellular function by performing many specific roles with the most important task being the conversion of extracted free energy from nutrients to that of the β-γ pyrophosphate bond of adenosine triphosphate (ATP). ATP is the universal cellular energy currency in all cell types. Mitochondrial oxidative phosphorylation (OXPHOS) provides,

under normal conditions, more than 80% of the ATP produced in most cell types. Glycolysis usually covers the remaining ATP production. Mitochondria have also other essential roles in metabolism and cell signaling like ketogenesis, amino acid catabolism, calcium homeostasis, urea cycle, production of Fe/S clusters, and steroidogenesis (Patti and Corvera, 2010; Papa *et al.,* 2012). Furthermore they are crucial in controlling apoptotic and necrotic cell death (Duchen and Szabadkai, 2010).

Mitochondria are double-membrane organelles, composed of a mitochondrial outer membrane (MOM) and a mitochondrial inner membrane (MIM). The MOM encloses mitochondria and is in direct contact with the cytoplasm. It is very rich in phospholipids and permeable to a number of small molecules. On the other hand, the MIM separates the mitochondrial matrix from the intermembrane space. The MIM is rich in proteins and cardiolipin and is highly selectively impermeable (Simbeni *et al.,* 1991; Hatch, 2004; Mileykovskaya *et al.,* 2005; Paradies *et al.,* 2014). The MIM has a much larger surface area than the MOM due to invaginations termed cristae and is less fluid than the MOM. These invaginations harbor the heavy machinery of OXPHOS. Furthermore their formation and dynamics are controlled by specific proteins such as optic atrophy 1 (OPA1) (Vogel *et al.,* 2006; Patten *et al.,* 2014).

Mitochondria, by nature are dynamic organelles, and this structural plasticity provides additional functional attributes. Mitochondria are constantly undergoing fusion and fission processes. These processes are essential for quality control (removal of damaged mitochondria through autophagy “mitophagy” or apoptosis) and for mitochondrial

biogenesis. They impact mitochondrial functions by affecting the distribution of metabolites across the mitochondrial network and by affecting calcium homeostasis, reactive oxygen species (ROS) production and respiration. In fusion, the major proteins involved are three large GTPases of the dynamin superfamily: mitofusin -1 and -2 (Mfn1, Mfn2) and optic atrophy 1 (Opa1). Since mitochondria are double membrane organelles, fusion occurs in two highly coordinated steps: the fusion of the MOM mediated by Mfn1 and Mfn2 followed by the fusion of the MIM mediated by OPA1 (Detmer and Chan, 2007; Parone *et al.,* 2008; Soubannier and McBride, 2009; Wada and Nakatsuka, 2016). Any error at any of the above-mentioned steps or any genetic mutation in any of the involved genes can result in mitochondrial fragmentation and severely impact mitochondrial function and ATP production (Friedman and Nunnari, 2014; Mishra and Chan, 2016). Fission on the other hand is mediated by the outer membrane GTPase dynamin related protein-1 (Drp1) with the participation of receptor proteins like fission protein 1 (Fis1). Mitochondrial morphology, transport, mitophagy and apoptosis are all affected by fission. Low ROS emission and efficient OXPHOS were correlated with fused mitochondria whereas fission is associated with mitochondrial degradation (Westermann, 2012; Archer, 2013; Ong *et al.*, 2015).

While studying models for ATP production, Peter Mitchell proposed in 1961 in his chemi-osmotic hypothesis that protons are pumped across the MIM, in an electrogenic process, driving ATP synthesis (Mitchell, 1961). In 1978, he earned a Nobel Prize for his protonmotive force (PMF) driven ATP-synthesis hypothesis. What helped in confirming Mitchell's hypothesis was the vast amount of discoveries and characterizations of the four electron transport chain (ETC) multi-protein complexes (complexes I-IV) and associated

factors, and ATP synthase (complex V). The ETC is located in the MIM and pump protons from the matrix to the intermembrane space forming PMF. This occurs when electrons from NADH or $FADH_2$ (generated by glycolysis and TCA cycle) are shuttled through the different complexes of the ETC, complex I, II, III and IV, reaching the final acceptor molecular oxygen and producing enough energy to pump protons out of the matrix. PMF, which is usually between 140 and 200 mV, then is used by ATP synthase to phosphorylate ADP to ATP as protons return to the matrix (Nicholls and Ferguson, 2002).

The majority of mitochondrial proteins are nuclear encoded and imported through the outer and inner membrane translocases, TOM and TIM complexes, respectively; however some members of the ETC are encoded by mitochondrial DNA (mtDNA). For example out of the 80 human ETC proteins, 13 are encoded by mtDNA (Neupert, 1997; Wiedemann *et al.,* 2004; Chen and Butow, 2005). Human mtDNA is circular and tiny (16.5Kb) compared to nuclear DNA but is also highly polyploid (more than 1000 copies/cell) forming almost 1% of total DNA. In addition to the 13 components of the ETC complexes, mtDNA also encodes 2 rRNAs and 22 tRNAs (Takamatsu *et al.,* 2002; Holt *et al.,* 2007; Pagliarini *et al.,* 2008).

Many factors can determine the efficiency of OXPHOS in ATP production. One of the factors is the process of protons bypassing complex V by returning to the matrix through a process known as proton leak. Proton leak decreases the efficiency of ATP production since energy substrates can be oxidized without the coincident synthesis of ATP. Its purpose is thought to be related to the lowering of mitochondrial membrane potential when ATP demand is low so that ROS emission from the ETC is minimized (Jastroch *et al.,* 2010;

Mailloux *et al.*, 2013a). Mechanisms controlling proton leak are as yet poorly understood, but it is clear that there are various possible MIM proteins involved. However, proton leak is quantitatively important; for example in hepatocytes and skeletal muscle proton leak is responsible for 30 and 50% of resting respiration respectively (Rolfe and Brown, 1997). A source of proton leak is via a protein family of 5 members numbered from 1 to 5 and named uncoupling proteins (UCPs) (Nicholls and Ferguson, 2002; Mailloux and Harper, 2012). The expression of these proteins varies from tissue to tissue; however, UCP1 is exclusively expressed in brown (or beige) adipocytes. In skeletal muscle, the only UCP expressed at the protein level is UCP3.

During OXPHOS, the ETC can be a major source of reactive species. At several sites of the ETC, mainly complex I and III, variable amounts of superoxide ($O2^{\cdot-}$), and peroxynitrite are formed, and these specific molecules can then be metabolized to form other types of ROS and reactive nitrogen species RNS like hydroxyl radical, hydrogen peroxide, carbonate radical , hypochlorite, and nitrogen dioxide. Although it is estimated that the bulk majority of the mitochondrial ROS is produced by the ETC, other sources also exist like nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 (Nox4), monoamine oxidase (MAO), the growth factor adaptor Shc ($p66^{Shc}$), glycerol-3-phosphate dehydrogenase cytochrome b5 reductase, pyruvate dehydrogenase, dihydroorotate dehydrogenase, succinate dehydrogenase, and α-ketoglutarate dehydrogenase (Nemoto *et al.,* 2000; Balaban *et al.*, 2005; Sas *et al.*, 2007; Valko *et al.*, 2007; Murphy, 2009; Zorov *et al.*, 2014; Holmström and Finkel, 2014; Bartz *et al.*, 2015). In the past, free radical production from OXPHOS was explained as “electron slippage”. Later on it was shown that that low or moderate

(physiological) concentrations of ROS can have beneficial effects such as participating in cellular signaling and protecting against infectious agents. An overproduction of these radicals that overwhelms antioxidant systems causes oxidative damage (Kovacic *et al.*, 2005). Uncoupling proteins are considered important sensors of ROS production intervening when ROS levels increase (Mailloux *et al.*, 2012). UCP2 and UCP3 (but not UCP1) can undergo a form of post-translational modification (PTM), called glutathionylation. Glutathionylation is a reversible redox-sensitive PTM of protein thiols by the formation of mixed disulphides with glutathione. It has been shown that when ROS levels increase, UCP2 and UCP3 are deglutathionylated and activated mitigating oxidative stress by decreasing membrane potential and ROS production (Mailloux *et al.*, 2011).

Furthermore, ETC complexes under certain conditions can bind together to form "supercomplexes", which affect mitochondrial respiration. Chance and Williams were the first to propose this concept in 1955 (Chance and Williams, 1955). The idea of supercomplex assembly to enhance mitochondrial respiration was debated for a long time until till recently when advanced biochemical techniques (*i.e.* Blue-Native PAGE) allowed scientists to visualize and measure these supercomplexes (Schägger and Pfeiffer, 2000; Cruciat *et al.*, 2000; Acín-Pérez *et al.*, 2008). Furthermore, recent discoveries unveiled many supercomplex assembly factors affecting mitochondrial respiration and ROS production (Lapuente-Brun *et al.*, 2013; Maranzana *et al.*, 2013).

To ensure maximum efficiency and protection from ROS damage, mitochondria use antioxidant systems to protect cells from oxidative stress. The antioxidant defence

mechanisms can be divided into two types: enzymatic and non-enzymatic (McMurray *et al.*, 2016). Many enzymes are part of the enzymatic defense systems, including glutathione peroxidase (GPx), thioredoxin (Trx), superoxide dismutase (SOD) and catalase (CAT). Non-enzymatic antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids and others (Cadenas, 1997; Valko *et al.*, 2007; Mailloux *et al.*, 2013a). Glutathione, a tripeptide (γ-L-glutamyl-L-cysteinylglycine), is thought to be the major cellular redox buffer and thiol antioxidant (Schafer and Buettner, 2001; Marí *et al.*, 2009, 2013). It is very abundant in cells (1-10mM) and its synthesis occurs in the cytosol by two ATP-requiring enzymes: γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS). Once synthesized, GSH is distributed in the endoplasmic reticulum, nucleus, and mitochondria. The majority of synthesized GSH is present in the cytosol (almost 90%), ~ 10% in the mitochondria and a very small percentage in the endoplasmic reticulum and the nucleus. GSH cellular concentration is regulated by GSH itself with a negative feedback mechanism. 95-99% of glutathione is in the reduced form (GSH) and the oxidized form (GSSG) content is usually between 1-5% of the total amount (Marí *et al.*, 2009, 2013; Murphy, 2012). When GSH is oxidized to GSSG, its reduction is assured by the NADPH-dependent enzyme glutathione reductase (GR). During oxidative stress, many antioxidant defences collaborate to protect mitochondria from the harmful effects of ROS. Superoxide radical anion is converted into hydrogen peroxide (H_2O_2) by MnSOD in the mitochondrial matrix. H_2O_2 can diffuse to the cytosol to participate in cell signaling processes, or if not well controlled, it can generate more free radicals such as hydroxyl radical, which leads to protein, lipid and DNA oxidation. Thus this delicate control

of H_2O_2 levels in mitochondria is provided by the balance in activity between MnSOD and GSH redox cycle (Fernandez-Checa and Kaplowitz, 2005). In order to achieve this goal, many players come into action, including GPx, peroxiredoxin and GR. Moreover, GSH supports the glutaredoxin system in which the GSH-dependent glutaredoxins reduce GSH-protein mixed disulfides (Herrero and Ros, 2002; Yant *et al.*, 2003; Hayes *et al.*, 2005; Orrenius *et al.*, 2007).

The glutaredoxin (glutathione dependent reductase and oxidase; *Grx*) system was first discovered in 1976 and exists in most organisms. Belonging to the oxidoreductase family, these small proteins catalyze the exchange of thiol-disulfide groups from oxidized protein disulfides and mixed disulfides. This exchange is due to electrons shuttling from NADPH via GR and GSH, reducing the targeted proteins. Thus glutaredoxins remain oxidized until they are again reduced by GSH (Holmgren, 1976; Padilla *et al.*, 1995; Lundberg *et al.*, 2001). Four mammalian isoforms of glutaredoxins exist: glutaredoxin-(1, 2, 3, and 5) (*Grx1, 2, 3, 5*), all of which play crucial roles in maintaining cellular redox homeostasis. They are divided into two categories depending on the cysteine residues in their active sites: the dithiol *Grx1* and *Grx2* with a CXXC motif and GSH binding site and the monothiol *Grx3* and *Grx5* with a CXXS active site motif (Höög *et al.*, 1983; Hanschmann *et al.*, 2013). Grx2 will be discussed below.

Due to alternative splicing of *Grx2* (encoded by 1 gene), there are two isoforms: one that targets the nucleus and the other the mitochondria. Grx2 is highly expressed in many tissues with high levels in the brain, heart, testis, liver and skeletal muscle. It is an 18kDa

protein that shares 34% homology with *Grx1* but harbors a dithiol active site (Cys-Ser-Try-Cys) that differs from *Grx1* by the serine residue (Lundberg *et al.,* 2001; Jurado *et al.,* 2003; Johansson *et al.,* 2004; Fernando *et al.,* 2006). *Grx2* catalyses glutathionylation and de-glutathionylation reactions. As defined previously, glutathionylation is a reversible redox-sensitive PTM of protein thiols by the formation of mixed disulphides with glutathione, and protects proteins from oxidative stress. Glutathionylation is affected by the GSH:GSSG ratio whereby an increase in oxidation increases glutathionylation reactions. Glutathionylation of vulnerable cysteine residues can activate or deactivate proteins, making it very important in redox homeostasis, protection from oxidative damage and cellular signaling (Mailloux *et al.,* 2014; Mailloux and Willmore, 2014; Mailloux and Treberg, 2016). ETC proteins are rich with thiol groups making them a perfect target for *Grx2* and indeed it was shown that complex I (specifically Ndufs1 and Ndufv1 subunits) is an important target (Taylor *et al.,* 2003; Dalle-Donne *et al.,* 2009; Kang *et al.,* 2012; Ribas *et al.,* 2014; Mailloux and Willmore, 2014). *Grx2* was also shown to control OXPHOS in cardiac muscle by mediating deglutathionylation reactions and depletion of *Grx2* in mice also led to fibrotic cardiac hypertrophy, hypertension, impaired redox homeostasis and disordered mitochondrial structure and function (Mailloux *et al.,* 2014).

1.4.2 MITOCHONDRIA IN OBESITY

With obesity being the result of energy imbalance between dietary intake and energy expenditure, the role of mitochondria in the pathogenesis of this disease is paramount. This

pivotal role is due to the fact that mitochondria are responsible for transducing energy from energy-containing nutrients into ATP.

In 1967, the work of Alton and Bray was of the first to link obesity and mitochondrial dysfunction by identifying a deficiency in α-glycerol phosphate metabolism in adipose tissue (Alton and Bray, 1967). In 1995, first evidence linking impaired mitochondria with obesity and T2DM in skeletal muscle was shown, in which reductions in key mitochondrial enzymes like cytochrome C oxidase (COX), carnitine palmitoyltransferase 1 (CPT1), malate dehydrogenase, and citrate synthase were observed during the fasting state. These reductions were also associated with increases in enzymes related to glycolysis (Colberg *et al.,* 1995; Simoneau and Bouchard, 1995; Simoneau *et al.,* 1995; Simoneau and Kelley, 1997). Furthermore, they were associated with a decrease in the general activity of the ETC and a reduced intermyofibrillar mitochondrial size (Simoneau *et al.,* 1999; Kim *et al.,* 2000; Kelley *et al.,* 2002). ETC activity was found to be lower in both intermyofibrillar and subsarcolemmal mitochondria in skeletal muscle of obese individuals and that the total mitochondrial content was also decreased showing an impaired mitochondrial function in obese and diabetic individuals (Kelley *et al.,* 2002; Morino *et al.,* 2005; Ritov *et al.,* 2005, 2010). Moreover, reductions in mitochondrial OXPHOS transcripts in skeletal muscle were reported in obese subjects and linked to increases in fat accumulation in skeletal muscle, high fat diet and lipid infusion (Sparks *et al.,* 2005; Richardson *et al.,* 2005; Crunkhorn *et al.,* 2007; Loos, 2012).

Furthermore and as mentioned earlier, mitochondrial proton leak mediated by UCPs has a significant impact on mitochondrial function. This effect is thought to be an important player in obesity pathogenesis. Our group has shown that obese diet sensitive (ODS) individuals, compared to obese diet resistant (ODR) individuals, have 25% increase in UCP3 mRNA expression in addition to double the proton leak-dependent respiration in mitochondria from the *rectus femoris* muscle (Harper *et al.,* 2002). As a response to a 900KCal/day regimen, ODS and ODR individuals were defined as those ranking in the highest and lowest quintile for weight loss rate, respectively. Furthermore, gene expression analysis of skeletal muscles from ODS individuals showed upregulation of gene sets involved in oxidative phosphorylation and glucose and fatty acid metabolism (Gerrits *et al.,* 2010). Moreover myotubes derived from ODS vs ODR individuals exhibited increased proton leak (Thrush *et al.,* 2014). Altogether these data show the importance of mitochondrial proton leak in diet resistance, rate of weight loss and obesity (Thrush *et al.,* 2013).Thus proton leak could be used as therapeutic target to treat obesity since in addition to the studies mentioned above, studies overexpressing UCP1 and UCP3 in skeletal muscles show to cause fat-specific weight loss and to provide resistance to diet induced obesity(Clapham *et al.,* 2000; Li *et al.,* 2000; Cadenas *et al.,* 2002).

Many theories have tried to explain the relationship between obesity and mitochondrial dysfunction. Deficiencies in fatty acid metabolism were reported as a debated cause for obesity. In skeletal muscle of obese individuals many impaired phenomena were observed such as lipid accumulation, increased fatty acid uptake, reduced capacity for lipid oxidations and oxidative stress (Colberg *et al.,* 1995; Simoneau *et al.,* 1999; Kim *et al.,*

2000; Kelley *et al.,* 2002; Furukawa *et al.,* 2004; Marseglia *et al.,* 2014). Incomplete fatty acid oxidation was shown to release acylcarnitines, which cause mitochondrial dysfunction and insulin resistance and are implicated in the pathogenesis of obesity and T2DM (Bell *et al.,* 2005; Thyfault *et al.,* 2007; Xia and Grant, 2013; Aguer *et al.,* 2015).

Besides mitochondrial dysfunction, mitochondrial morphology and dynamics were also linked with obesity in skeletal muscle. Shortening in mitochondrial length, impaired fission and a fragmented mitochondrial network due to reduced Mfn2 expression occurred in muscle of obese mice and cultured C2C12 cells treated with palmitate (DeLany *et al.,* 2014).

1.4.3 MITOCHONDRIA IN T2DM

As mentioned above, mitochondrial dysfunctions are thought to cause an increase in lipid accumulation and eventually insulin resistance in skeletal muscle. Although some studies contradict this conclusion with no evidence of mitochondrial dysfunctions in T2DM or no correlation between the two (Boushel *et al.,* 2007), it is important to note that other studies showed that impaired mitochondrial respiration is present even before T2DM onset (DeLany *et al.,* 2014). Our lab group showed impaired OXPHOS and supercomplexes assembly in *rectus abdominis* muscle from diabetic obese individuals vs obese individuals (Antoun *et al.,* 2015).

One possible explanation linking mitochondrial dysfunctions, insulin resistance and T2DM implicates impaired fatty acid oxidation. The products of incomplete β-oxidation and other species such as ceramides, diacylglycerols, fatty acyl coenzyme A (CoA) have been

linked to insulin resistance development (Holland *et al.*, 2007; Savage *et al.*, 2007; Koves *et al.*, 2008; Chibalin *et al.*, 2008).

Additionally impaired mitochondrial dynamics were also observed in muscle of diabetic patients impacting directly mitochondrial function. In skeletal muscle cells of individuals with T2DM, there is lower expression levels of Mfn2 (Bach *et al.*, 2005). Disruptions in calcium homeostasis have also been detected, potentially impacting glucose uptake and fuel oxidation processes after exercise (Lanner *et al.*, 2006, 2008; Park *et al.*, 2009). Oxidative stress in skeletal muscle was also linked to the development and progress of T2DM. Overproduction of ROS by many mitochondrial sources like xanthine oxidase for example, helped in developing insulin resistance and later on T2DM. Targeting xanthine oxidase thought did not ameliorate the T2DM phenotype in animals, contrary to the effects of overexpressing antioxidant enzymes (e.g., catalase and MnSOD) which decreased insulin resistance (Anderson *et al.*, 2009; Lee *et al.*, 2010; Bravard *et al.*, 2011). Thus targeting mitochondrial ROS can improve insulin resistance and glucose homeostasis (Rieusset, 2015; Hesselink *et al.*, 2016).

1.4.4 MITOCHONDRIA IN ATRIAL FIBRILLATION

As mentioned earlier, mitochondrial function in the heart is of great metabolic importance; it is thus not surprising that mitochondrial dysfunctions are associated with many cardiac pathologies, including hypertrophy, arrhythmias, myocardial ischemia/reperfusion injury, and heart failure. Mitochondrial and cellular redox dysfunctions

contribute extensively in the development of cardiovascular diseases (Kanaan and Harper, 2017) . Such dysfunctions in atrial fibrillation are discussed below.

Factors associated with the development of AF, as mentioned earlier, include age, obesity and diabetes (Watanabe *et al.,* 2008; Nyström *et al.,* 2015). Decades of research have helped to expand our knowledge about the causes and consequences of this complicated disease. Currently more is known about the cellular electrophysiology and structural remodelling influencing AF than ever before. Whereas our understanding of the role of atrial metabolism in the pathogenesis of AF is still preliminary (Opacic *et al.,* 2016). During AF, the frequency of electrical activity increases significantly challenging atrial metabolism.

Ventricular metabolism is more widely studied than atrial metabolism due to its involvement in many cardiac diseases. Nevertheless, it is known that during AF rapid electrical activity increase 4 to 6-fold, thus requiring more ATP (Konings *et al.,* 1994; Wijffels *et al.,* 1995; Li *et al.,* 1999). This increase in energy demand should be matched by increase in ATP production with the required oxygen and nutrients supplied by atrial vasculature (Opacic *et al.,* 2016). In this regard, it was shown that during acute AF the atrial blood flow increased 2- to 3-fold in dogs and pigs (White *et al.,* 1982; McHale and Greenfield, 1986). An increase in venous oxygen extraction was also reported but only in the atria and not the ventricles. Even though with all these adaptations ensuring oxygen supply to the atria, supply–demand ischemia was detected in the left atria of pigs as evidenced by increased atrial lactate production (van Bragt *et al.,* 2014).

In normal conditions, in the ventricles 60-70% of total ATP produced is used to support contractility, and 30-40% of the ATP is used for ion transport processes (Gibbs, 1978). No such data are reported for the atria but it may be similar. To maintain ATP levels, a balance between production and consumption must exist. In the early weeks of AF, this balance is interrupted along with supply demand ischemia. It was shown that after 2 hours of inducing AF in sheep ATP synthase activity was increased (Barbey *et al.,* 2000). After a week of induced AF in rabbits, a decrease in ATP synthase activity, down-regulation of ETC subunits in addition to a decrease in atrial total ATP were detected (Dong *et al.,* 2016). In another study in goats, a week of AF caused no changes in atrial ATP levels but decreased creatine phosphate (PCr) levels (indication of an imbalance in ATP supply a demand), and increased hypoxia inducible factor 1α (HIF1α) (Weiss *et al.,* 1999; Ausma *et al.,* 2000; Thijssen *et al.,* 2002).

On the other hand, a study in human patients with permanent AF undergoing cardiac surgery showed a decrease in myofibrillar creatine kinase (MM-CK) coincident with increases in protein carbonyls and 3-nitrotyrosine. Decreases in MM-CK are associated with impaired ATP generation (Mihm *et al.,* 2001). A metabolomic and proteomic analysis in AF patients undergoing coronary artery bypass surgery (CABG) or valve surgery showed increases in metabolites and enzymes involved in ketone body metabolism (Mayr *et al.,* 2008). Furthermore, phosphorylation levels of ion channels and of proteins involved in calcium handling were altered in patients with paroxysmal AF (Chiang *et al.,* 2015). In 2005, while studying AF patients, Seppet and colleagues reported an increase in succinate dependent respiration along with an increase in mitochondrial proton leak (Seppet *et al.,*

2005). Moreover other studies showed accumulation of mtDNA mutations affecting ATP synthesis in patients suffering from AF (Tsuboi *et al.,* 2001; Lai *et al.,* 2003). Xu *et al.* proposed that increased intracellular lactate signaling via mitochondrial monocarboxylate transporter 1 (MCT-1) along with increased ROS production can lead to increased mitochondrial control of apoptosis (Xu *et al.,* 2013).

The ventricles get the majority of their remaining ATP from fatty acid oxidation (60-70%), the remaining is provided by glucose and lactate oxidation (Wisneski *et al.,* 1985; Doenst *et al.,* 2013). During heart failure and hypoxia, ventricles shift from lipid oxidation to glycolysis (Horowitz *et al.,* 2010). Such studies in healthy atria have not been conducted, but substrate preference may not differ greatly from that of the ventricles. However, it has been suggested that glycolysis could be higher in atria (Savabi and Kirsch, 1991).

AF development has been linked to oxidative stress in experimental animals and in humans (Mihm *et al.,* 2001; Carnes *et al.,* 2001; Korantzopoulos *et al.,* 2007; Huang *et al.,* 2009). Cellular sources of ROS vary depending on AF duration. In the first weeks, atrial ROS production is increased and some studies found that it is associated with increased activity of cytoplasmic NADPH oxidase (NOX), whereas at later stages of the disease it is thought that ROS production is more associated with mitochondrial sources and with uncoupled nitric oxide synthase (NOS) (Dudley *et al.,* 2005; Reilly *et al.,* 2011). Oxidative stress in AF resulted in altering cellular proteins affecting: OXPHOS by reducing ETC activity, myofibrillar proteins, ion channels, mitochondrial dynamics and calcium handling by targeting type 2 ryanodine receptor (RyR2) (Mihm *et al.,* 2001; Redpath *et al.,* 2013;

Yang *et al.,* 2014; Emelyanova *et al.,* 2016). Although our understanding of AF pathophysiology has improved over the last decades, more work is needed to understand the exact role of mitochondrial dysfunction in this complex disease.

1.5 PROJECT OBJECTIVES AND HYPOTHESES

The overall goal of my doctoral studies was to investigate mitochondrial function in muscle cells and tissues from mice and humans. In order to achieve this goal, studies detailed below were conducted in various models using the most advanced techniques available.

Objective 1: To investigate if primary skeletal muscle cells derived from low birth mice exhibit impaired bioenergetics. To study gene expression changes due to *in utero* undernutrition and nutrient restriction in these cells.

Hypothesis: *In utero* undernutrition impacts oxidative and glycolytic functions in addition to fatty acid oxidation. Gene expression shows strong changes in genes related to OXPHOS and fatty acid oxidation in undernourished cells.

Objective 2: To study the roles of Grx2 in maintaining mitochondrial structure and cardiac cellular energetics in mice. To investigate the effect of N-acetylcysteine (NAC) supplementation. To assess the role of GRX2 expression in the human heart in association with cardiac pathologies.

Hypothesis: Grx2 controls cardiac mitochondrial dynamics and function in cellular and mouse models, and low expression levels are associated with human heart disease. *In vitro* and *in vivo* provision of the glutathione precursor NAC restores cardiac energetics in neonatal cardiomyocytes and in *Grx2-/-* mice.

Objective 3: To examine mitochondrial energetics in atrial myocardium in AF patients with diabetes undergoing coronary artery bypass graft surgery. To assess supercomplex assembly, oxidative damage and fibrosis in atrial tissue.

Hypothesis: Pre-existing fibrillation or T2DM impacts atrial mitochondrial energetics and electron transport chain (ETC) supercomplexes. These effects are associated with oxidative damage and fibrosis.

1.6 REFERENCES

Abdul-Ghani, M.A., and DeFronzo, R.A. (2010). Pathogenesis of insulin resistance in skeletal muscle. J. Biomed. Biotechnol. *2010*, 476279.

Abel, E.D., Litwin, S.E., and Sweeney, G. (2008). Cardiac Remodeling in Obesity. Physiol. Rev. *88*, 389–419.

Acín-Pérez, R., Fernández-Silva, P., Peleato, M.L., Pérez-Martos, A., and Enriquez, J.A. (2008). Respiratory active mitochondrial supercomplexes. Mol. Cell *32*, 529–539.

Adamy, C., Mulder, P., Khouzami, L., Andrieu-abadie, N., Defer, N., Candiani, G., Pavoine, C., Caramelle, P., Souktani, R., Le Corvoisier, P., et al. (2007). Neutral sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats. J. Mol. Cell. Cardiol. *43*, 344–353.

Aguer, C., Pasqua, M., Thrush, A.B., Moffat, C., McBurney, M., Jardine, K., Zhang, R., Beauchamp, B., Dent, R., McPherson, R., et al. (2013). Increased proton leak and SOD2 expression in myotubes from obese non-diabetic subjects with a family history of type 2 diabetes. Biochim. Biophys. Acta *1832*, 1624–1633.

Aguer, C., McCoin, C.S., Knotts, T.A., Thrush, A.B., Ono-Moore, K., McPherson, R., Dent, R., Hwang, D.H., Adams, S.H., and Harper, M.-E. (2015). Acylcarnitines: potential implications for skeletal muscle insulin resistance. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *29*, 336–345.

Akhmedov, D., and Berdeaux, R. (2013). The effects of obesity on skeletal muscle regeneration. Front. Physiol. *4*, 371.

Albert, C.M., and Stevenson, W.G. (2016). The Future of Cardiovascular Biomedicine: Arrhythmias and Electrophysiology. Circulation *133*, 2687–2696.

Alton, D.J., and Bray, G.A. (1967). Metabolism of alpha-glycerol phosphate in human adipose tissue in obesity. J. Clin. Endocrinol. Metab. *27*, 1573–1580.

Anderson, E.J., Lustig, M.E., Boyle, K.E., Woodlief, T.L., Kane, D.A., Lin, C.-T., Price, J.W., Kang, L., Rabinovitch, P.S., Szeto, H.H., et al. (2009). Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J. Clin. Invest. *119*, 573–581.

Andrade, J., Khairy, P., Dobrev, D., and Nattel, S. (2014). The clinical profile and pathophysiology of atrial fibrillation: relationships among clinical features, epidemiology, and mechanisms. Circ. Res. *114*, 1453–1468.

Annis, A.M., Caulder, M.S., Cook, M.L., and Duquette, D. (2005). Family History, Diabetes, and Other Demographic and Risk Factors Among Participants of the National Health and Nutrition Examination Survey 1999–2002. Prev. Chronic. Dis. *2*.

Antoun, G., McMurray, F., Thrush, A.B., Patten, D.A., Peixoto, A.C., Slack, R.S., McPherson, R., Dent, R., and Harper, M.-E. (2015). Impaired mitochondrial oxidative phosphorylation and supercomplex assembly in rectus abdominis muscle of diabetic obese individuals. Diabetologia *58*, 2861–2866.

Aon, M.A., Tocchetti, C.G., Bhatt, N., Paolocci, N., and Cortassa, S. (2015). Protective mechanisms of mitochondria and heart function in diabetes. Antioxid. Redox Signal. *22*, 1563–1586.

Apovian, C.M. (2016). Obesity: definition, comorbidities, causes, and burden. Am. J. Manag. Care *22*, s176-185.

Archer, S.L. (2013). Mitochondrial dynamics--mitochondrial fission and fusion in human diseases. N. Engl. J. Med. *369*, 2236–2251.

Atkinson, R.L., Dhurandhar, N.V., Allison, D.B., Bowen, R.L., Israel, B.A., Albu, J.B., and Augustus, A.S. (2005). Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. Int. J. Obes. 2005 *29*, 281–286.

Atkinson, R.L., Lee, I., Shin, H.-J., and He, J. (2010). Human adenovirus-36 antibody status is associated with obesity in children. Int. J. Pediatr. Obes. IJPO Off. J. Int. Assoc. Study Obes. *5*, 157–160.

Ausma, J., Coumans, W.A., Duimel, H., Van der Vusse, G.J., Allessie, M.A., and Borgers, M. (2000). Atrial high energy phosphate content and mitochondrial enzyme activity during chronic atrial fibrillation. Cardiovasc. Res. *47*, 788–796.

Ayyappa, K.A., Shatwan, I., Bodhini, D., Bramwell, L.R., Ramya, K., Sudha, V., Anjana, R.M., Lovegrove, J.A., Mohan, V., Radha, V., et al. (2017). High fat diet modifies the association of lipoprotein lipase gene polymorphism with high density lipoprotein cholesterol in an Asian Indian population. Nutr. Metab. *14*.

Bach, D., Naon, D., Pich, S., Soriano, F.X., Vega, N., Rieusset, J., Laville, M., Guillet, C., Boirie, Y., Wallberg-Henriksson, H., et al. (2005). Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. Diabetes *54*, 2685–2693.

Bajaj, M., and Defronzo, R.A. (2003). Metabolic and molecular basis of insulin resistance. J. Nucl. Cardiol. Off. Publ. Am. Soc. Nucl. Cardiol. *10*, 311–323.

Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell *120*, 483–495.

Barbey, O., Pierre, S., Duran, M.J., Sennoune, S., Lévy, S., and Maixent, J.M. (2000). Specific up-regulation of mitochondrial F0F1-ATPase activity after short episodes of atrial fibrillation in sheep. J. Cardiovasc. Electrophysiol. *11*, 432–438.

Barker, D.J.P. (2004). The developmental origins of adult disease. J. Am. Coll. Nutr. *23*, 588S–595S.

Barker, D.J.P. (2007). The origins of the developmental origins theory. J. Intern. Med. *261*, 412–417.

Barker, D.J., Osmond, C., Golding, J., Kuh, D., and Wadsworth, M.E. (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ *298*, 564–567.

Barker, D.J., Hales, C.N., Fall, C.H., Osmond, C., Phipps, K., and Clark, P.M. (1993a). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia *36*, 62–67.

Barker, D.J., Gluckman, P.D., Godfrey, K.M., Harding, J.E., Owens, J.A., and Robinson, J.S. (1993b). Fetal nutrition and cardiovascular disease in adult life. Lancet Lond. Engl. *341*, 938–941.

Bartz, R.R., Suliman, H.B., and Piantadosi, C.A. (2015). Redox mechanisms of cardiomyocyte mitochondrial protection. Front. Physiol. *6*, 291.

Bays, H., Mandarino, L., and DeFronzo, R.A. (2004). Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. J. Clin. Endocrinol. Metab. *89*, 463–478.

Beauchamp, B., and Harper, M.-E. (2015). In utero Undernutrition Programs Skeletal and Cardiac Muscle Metabolism. Front. Physiol. *6*, 401.

Beauchamp, B., Ghosh, S., Dysart, M.W., Kanaan, G.N., Chu, A., Blais, A., Rajamanickam, K., Tsai, E.C., Patti, M.E., and Harper, M.E. (2014). Low birth weight is associated with adiposity, impaired skeletal muscle energetics and weight loss resistance in mice. Int. J. Obes. 2005.

Beauchamp, B., Ghosh, S., Dysart, M.W., Kanaan, G.N., Chu, A., Blais, A., Rajamanickam, K., Tsai, E.C., Patti, M.-E., and Harper, M.-E. (2015a). Low birth weight is associated with

adiposity, impaired skeletal muscle energetics and weight loss resistance in mice. Int. J. Obes. 2005 *39*, 702–711.

Beauchamp, B., Thrush, A.B., Quizi, J., Antoun, G., McIntosh, N., Al-Dirbashi, O.Y., Patti, M.-E., and Harper, M.-E. (2015b). Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring. Biosci. Rep. *35*.

Bell, C.G., Walley, A.J., and Froguel, P. (2005). The genetics of human obesity. Nat. Rev. Genet. *6*, 221–234.

Benjamin, E.J., Blaha, M.J., Chiuve, S.E., Cushman, M., Das, S.R., Deo, R., de Ferranti, S.D., Floyd, J., Fornage, M., Gillespie, C., et al. (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation *135*, e146–e603.

Billings, L.K., and Florez, J.C. (2010). The genetics of type 2 diabetes: what have we learned from GWAS? Ann. N. Y. Acad. Sci. *1212*, 59–77.

Blimkie, C.J., Sale, D.G., and Bar-Or, O. (1990). Voluntary strength, evoked twitch contractile properties and motor unit activation of knee extensors in obese and non-obese adolescent males. Eur. J. Appl. Physiol. *61*, 313–318.

Boden, G. (1997). Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes *46*, 3–10.

Bouchard, C. (2001). The genetics of human obesity: recent progress. Bull. Mem. Acad. R. Med. Belg. *156*, 455-462; discussion 463-464.

Bougnères, P., Pantalone, L., Linglart, A., Rothenbühler, A., and Le Stunff, C. (2008). Endocrine manifestations of the rapid-onset obesity with hypoventilation, hypothalamic, autonomic dysregulation, and neural tumor syndrome in childhood. J. Clin. Endocrinol. Metab. *93*, 3971–3980.

Boukens, B.J., and Christoffels, V.M. (2012). Electrophysiological patterning of the heart. Pediatr. Cardiol. *33*, 900–906.

Boushel, R., Gnaiger, E., Schjerling, P., Skovbro, M., Kraunsøe, R., and Dela, F. (2007). Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. Diabetologia *50*, 790–796.

Bouzakri, K., Koistinen, H.A., and Zierath, J.R. (2005). Molecular mechanisms of skeletal muscle insulin resistance in type 2 diabetes. Curr. Diabetes Rev. *1*, 167–174.

van Bragt, K.A., Nasrallah, H.M., Kuiper, M., Luiken, J.J., Schotten, U., and Verheule, S. (2014). Atrial supply-demand balance in healthy adult pigs: coronary blood flow, oxygen extraction, and lactate production during acute atrial fibrillation. Cardiovasc. Res. *101*, 9–19.

Braithwaite, I., Stewart, A.W., Hancox, R.J., Beasley, R., Murphy, R., and Mitchell, E.A. (2013). The Worldwide Association between Television Viewing and Obesity in Children and Adolescents: Cross Sectional Study. PLoS ONE *8*.

Bravard, A., Bonnard, C., Durand, A., Chauvin, M.-A., Favier, R., Vidal, H., and Rieusset, J. (2011). Inhibition of xanthine oxidase reduces hyperglycemia-induced oxidative stress and improves mitochondrial alterations in skeletal muscle of diabetic mice. Am. J. Physiol. Endocrinol. Metab. *300*, E581-591.

Bray, G.A., Frühbeck, G., Ryan, D.H., and Wilding, J.P.H. (2016). Management of obesity. Lancet Lond. Engl. *387*, 1947–1956.

Bray, G.A., Kim, K.K., Wilding, J.P.H., and World Obesity Federation (2017). Obesity: a chronic relapsing progressive disease process. A position statement of the World Obesity Federation. Obes. Rev. Off. J. Int. Assoc. Study Obes. *18*, 715–723.

Brown, L.D., and Hay, W.W. (2016). Impact of placental insufficiency on fetal skeletal muscle growth. Mol. Cell. Endocrinol. *435*, 69–77.

Bugger, H., and Abel, E.D. (2014). Molecular mechanisms of diabetic cardiomyopathy. Diabetologia *57*, 660–671.

Burcelin, R. (2012). Regulation of metabolism: a cross talk between gut microbiota and its human host. Physiol. Bethesda Md *27*, 300–307.

Cadenas, E. (1997). Basic mechanisms of antioxidant activity. BioFactors Oxf. Engl. *6*, 391–397.

Cadenas, S., Echtay, K.S., Harper, J.A., Jekabsons, M.B., Buckingham, J.A., Grau, E., Abuin, A., Chapman, H., Clapham, J.C., and Brand, M.D. (2002). The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. J. Biol. Chem. *277*, 2773–2778.

Cai, L., and Kang, Y.J. (2003). Cell death and diabetic cardiomyopathy. Cardiovasc. Toxicol. *3*, 219–228.

Cai, L., and Keller, B.B. (2014). Cardiac regeneration and diabetes. Regen. Med. Res. *2*.

Carnes, C.A., Chung, M.K., Nakayama, T., Nakayama, H., Baliga, R.S., Piao, S., Kanderian, A., Pavia, S., Hamlin, R.L., McCarthy, P.M., et al. (2001). Ascorbate attenuates atrial

pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation. Circ. Res. *89*, E32-38.

Chaban, Y., Boekema, E.J., and Dudkina, N.V. (2014). Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. Biochim. Biophys. Acta *1837*, 418–426.

Chance, B., and Williams, G.R. (1955). A method for the localization of sites for oxidative phosphorylation. Nature *176*, 250–254.

Chen, X.J., and Butow, R.A. (2005). The organization and inheritance of the mitochondrial genome. Nat. Rev. Genet. *6*, 815–825.

Chiang, D.Y., Lebesgue, N., Beavers, D.L., Alsina, K.M., Damen, J.M.A., Voigt, N., Dobrev, D., Wehrens, X.H.T., and Scholten, A. (2015). Alterations in the interactome of serine/threonine protein phosphatase type-1 in atrial fibrillation patients. J. Am. Coll. Cardiol. *65*, 163–173.

Chibalin, A.V., Leng, Y., Vieira, E., Krook, A., Björnholm, M., Long, Y.C., Kotova, O., Zhong, Z., Sakane, F., Steiler, T., et al. (2008). Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell *132*, 375–386.

Chugh, S.S., Havmoeller, R., Narayanan, K., Singh, D., Rienstra, M., Benjamin, E.J., Gillum, R.F., Kim, Y.-H., McAnulty, J.H., Zheng, Z.-J., et al. (2014). Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. Circulation *129*, 837–847.

Ciciliot, S., Rossi, A.C., Dyar, K.A., Blaauw, B., and Schiaffino, S. (2013). Muscle type and fiber type specificity in muscle wasting. Int. J. Biochem. Cell Biol. *45*, 2191–2199.

Clapham, J.C., Arch, J.R., Chapman, H., Haynes, A., Lister, C., Moore, G.B., Piercy, V., Carter, S.A., Lehner, I., Smith, S.A., et al. (2000). Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature *406*, 415–418.

Cogswell, A.M., Stevens, R.J., and Hood, D.A. (1993). Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions. Am. J. Physiol. *264*, C383-389.

Colberg, S.R., Simoneau, J.A., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle utilization of free fatty acids in women with visceral obesity. J. Clin. Invest. *95*, 1846–1853.

Conen, D., Chae, C.U., Glynn, R.J., Tedrow, U.B., Everett, B.M., Buring, J.E., and Albert, C.M. (2011). Risk of death and cardiovascular events in initially healthy women with new-onset atrial fibrillation. JAMA *305*, 2080–2087.

Coyle, E.F. (2000). Physical activity as a metabolic stressor. Am. J. Clin. Nutr. *72*, 512S–20S.

Cruciat, C.M., Brunner, S., Baumann, F., Neupert, W., and Stuart, R.A. (2000). The cytochrome bc1 and cytochrome c oxidase complexes associate to form a single supracomplex in yeast mitochondria. J. Biol. Chem. *275*, 18093–18098.

Crunkhorn, S., Dearie, F., Mantzoros, C., Gami, H., da Silva, W.S., Espinoza, D., Faucette, R., Barry, K., Bianco, A.C., and Patti, M.E. (2007). Peroxisome proliferator activator receptor gamma coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. J. Biol. Chem. *282*, 15439–15450.

Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M.E., Pratipanawatr, T., DeFronzo, R.A., Kahn, C.R., and Mandarino, L.J. (2000). Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J. Clin. Invest. *105*, 311–320.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. (2009). Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends Biochem. Sci. *34*, 85–96.

Das, S.K., and Elbein, S.C. (2006). The Genetic Basis of Type 2 Diabetes. Cellscience *2*, 100–131.

Day, K., Alfonzo, M., Chen, Y., Guo, Z., and Lee, K.K. (2013). Overweight, obesity, and inactivity and urban design in rapidly growing Chinese cities. Health Place *21*, 29–38.

DeFronzo, R.A., and Ferrannini, E. (1987). Regulation of hepatic glucose metabolism in humans. Diabetes. Metab. Rev. *3*, 415–459.

DeFronzo, R.A., and Tripathy, D. (2009). Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. Diabetes Care *32*, S157–S163.

DeFronzo, R.A., Gunnarsson, R., Björkman, O., Olsson, M., and Wahren, J. (1985). Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J. Clin. Invest. *76*, 149–155.

DeFronzo, R.A., Ferrannini, E., and Simonson, D.C. (1989). Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metabolism. *38*, 387–395.

DeFronzo, R.A., Ferrannini, E., Groop, L., Henry, R.R., Herman, W.H., Holst, J.J., Hu, F.B., Kahn, C.R., Raz, I., Shulman, G.I., et al. (2015). Type 2 diabetes mellitus. Nat. Rev. Dis. Primer *1*, 15019.

DeLany, J.P., Dubé, J.J., Standley, R.A., Distefano, G., Goodpaster, B.H., Stefanovic-Racic, M., Coen, P.M., and Toledo, F.G.S. (2014). Racial differences in peripheral insulin sensitivity and mitochondrial capacity in the absence of obesity. J. Clin. Endocrinol. Metab. *99*, 4307–4314.

Després, J.-P. (2012). Body fat distribution and risk of cardiovascular disease: an update. Circulation *126*, 1301–1313.

Detmer, S.A., and Chan, D.C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nat. Rev. Mol. Cell Biol. *8*, 870–879.

Diabetes Canada (2017). Diabetes Statistics in Canada.

Dina, C., Meyre, D., Gallina, S., Durand, E., Körner, A., Jacobson, P., Carlsson, L.M.S., Kiess, W., Vatin, V., Lecoeur, C., et al. (2007). Variation in FTO contributes to childhood obesity and severe adult obesity. Nat. Genet. *39*, 724–726.

Doenst, T., Nguyen, T.D., and Abel, E.D. (2013). Cardiac metabolism in heart failure: implications beyond ATP production. Circ. Res. *113*, 709–724.

Dong, J., Zhao, J., Zhang, M., Liu, G., Wang, X., Liu, Y., Yang, N., Liu, Y., Zhao, G., Sun, J., et al. (2016). β3-Adrenoceptor Impairs Mitochondrial Biogenesis and Energy Metabolism During Rapid Atrial Pacing-Induced Atrial Fibrillation. J. Cardiovasc. Pharmacol. Ther. *21*, 114–126.

Downey, J.M., and Heusch, G. (2001). CHAPTER 1 - Sequence of Cardiac Activation and Ventricular Mechanics. In Heart Physiology and Pathophysiology (Fourth Edition), N. Sperelakis, Y. Kurachi, A. Terzic, and M.V. Cohen, eds. (San Diego: Academic Press), pp. 3–18.

Dröge, W. (2002). Free radicals in the physiological control of cell function. Physiol. Rev. *82*, 47–95.

Duchen, M.R., and Szabadkai, G. (2010). Roles of mitochondria in human disease. Essays Biochem. *47*, 115–137.

Ducluzeau, P.H., Perretti, N., Laville, M., Andreelli, F., Vega, N., Riou, J.P., and Vidal, H. (2001). Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. Diabetes *50*, 1134–1142.

Dudkina, N.V., Kouril, R., Peters, K., Braun, H.-P., and Boekema, E.J. (2010). Structure and function of mitochondrial supercomplexes. Biochim. Biophys. Acta *1797*, 664–670.

Dudkina, N.V., Folea, I.M., and Boekema, E.J. (2015). Towards structural and functional characterization of photosynthetic and mitochondrial supercomplexes. Micron Oxf. Engl. 1993 *72*, 39–51.

Dudley, S.C., Hoch, N.E., McCann, L.A., Honeycutt, C., Diamandopoulos, L., Fukai, T., Harrison, D.G., Dikalov, S.I., and Langberg, J. (2005). Atrial fibrillation increases production of superoxide by the left atrium and left atrial appendage: role of the NADPH and xanthine oxidases. Circulation *112*, 1266–1273.

Emelyanova, L., Ashary, Z., Cosic, M., Negmadjanov, U., Ross, G., Rizvi, F., Olet, S., Kress, D., Sra, J., Tajik, A.J., et al. (2016). Selective downregulation of mitochondrial electron transport chain activity and increased oxidative stress in human atrial fibrillation. Am. J. Physiol. Heart Circ. Physiol. *311*, H54-63.

Ernster, L., and Schatz, G. (1981). Mitochondria: a historical review. J. Cell Biol. *91*, 227s–255s.

Esposito, S., Preti, V., Consolo, S., Nazzari, E., and Principi, N. (2012). Adenovirus 36 infection and obesity. J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol. *55*, 95–100.

Farooqi, I.S., and O'Rahilly, S. (2007). Genetic factors in human obesity. Obes. Rev. Off. J. Int. Assoc. Study Obes. *8 Suppl 1*, 37–40.

Fernandez-Checa, J.C., and Kaplowitz, N. (2005). Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicol. Appl. Pharmacol. *204*, 263–273.

Fernando, M.R., Lechner, J.M., Löfgren, S., Gladyshev, V.N., and Lou, M.F. (2006). Mitochondrial thioltransferase (glutaredoxin 2) has GSH-dependent and thioredoxin reductase-dependent peroxidase activities in vitro and in lens epithelial cells. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *20*, 2645–2647.

Field, A.E., Coakley, E.H., Must, A., Spadano, J.L., Laird, N., Dietz, W.H., Rimm, E., and Colditz, G.A. (2001). Impact of overweight on the risk of developing common chronic diseases during a 10-year period. Arch. Intern. Med. *161*, 1581–1586.

Fleg, J.L., and Kennedy, H.L. (1982). Cardiac arrhythmias in a healthy elderly population: detection by 24-hour ambulatory electrocardiography. Chest *81*, 302–307.

Flink, L., Mochari-Greenberger, H., and Mosca, L. (2013). Gender Differences in Clinical Outcomes Among Diabetics Hospitalized for Cardiovascular Disease (CVD). Am. Heart J. *165*, 972–978.

Flück, M., and Hoppeler, H. (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. Rev. Physiol. Biochem. Pharmacol. *146*, 159–216.

Foster, G.D., Sanders, M.H., Millman, R., Zammit, G., Borradaile, K.E., Newman, A.B., Wadden, T.A., Kelley, D., Wing, R.R., Sunyer, F.X.P., et al. (2009). Obstructive sleep apnea among obese patients with type 2 diabetes. Diabetes Care *32*, 1017–1019.

Fraser, L.-A., Twombly, J., Zhu, M., Long, Q., Hanfelt, J.J., Narayan, K.M.V., Wilson, P.W.F., and Phillips, L.S. (2010). Delay in Diagnosis of Diabetes Is Not the Patient's Fault. Diabetes Care *33*, e10.

Frayling, T.M., Timpson, N.J., Weedon, M.N., Zeggini, E., Freathy, R.M., Lindgren, C.M., Perry, J.R.B., Elliott, K.S., Lango, H., Rayner, N.W., et al. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science *316*, 889–894.

Friedman, J.R., and Nunnari, J. (2014). Mitochondrial form and function. Nature *505*, 335–343.

Frontera, W.R., and Ochala, J. (2015). Skeletal muscle: a brief review of structure and function. Calcif. Tissue Int. *96*, 183–195.

Fry, A.C. (2004). The role of resistance exercise intensity on muscle fibre adaptations. Sports Med. Auckl. NZ *34*, 663–679.

Fuchsberger, C., Flannick, J., Teslovich, T.M., Mahajan, A., Agarwala, V., Gaulton, K.J., Ma, C., Fontanillas, P., Moutsianas, L., McCarthy, D.J., et al. (2016). The genetic architecture of type 2 diabetes. Nature *536*, 41–47.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Invest. *114*, 1752–1761.

Fuster, V., Rydén, L.E., Cannom, D.S., Crijns, H.J., Curtis, A.B., Ellenbogen, K.A., Halperin, J.L., Heuzey, J.-Y.L., Kay, G.N., Lowe, J.E., et al. (2006). ACC/AHA/ESC 2006 Guidelines for the Management of Patients With Atrial Fibrillation. Circulation *114*, e257–e354.

Gabbert, C., Donohue, M., Arnold, J., and Schwimmer, J.B. (2010). Adenovirus 36 and obesity in children and adolescents. Pediatrics *126*, 721–726.

Galpin, A.J., Raue, U., Jemiolo, B., Trappe, T.A., Harber, M.P., Minchev, K., and Trappe, S. (2012). Human skeletal muscle fiber type specific protein content. Anal. Biochem. *425*, 175–182.

Gao, X.-H., Qanungo, S., Pai, H.V., Starke, D.W., Steller, K.M., Fujioka, H., Lesnefsky, E.J., Kerner, J., Rosca, M.G., Hoppel, C.L., et al. (2013). Aging-dependent changes in rat heart mitochondrial glutaredoxins—Implications for redox regulation. Redox Biol. *1*, 586–598.

Gerrits, M.F., Ghosh, S., Kavaslar, N., Hill, B., Tour, A., Seifert, E.L., Beauchamp, B., Gorman, S., Stuart, J., Dent, R., et al. (2010). Distinct skeletal muscle fiber characteristics and gene expression in diet-sensitive versus diet-resistant obesity. J. Lipid Res. *51*, 2394–2404.

Giam, B., Chu, P.-Y., Kuruppu, S., Smith, A.I., Horlock, D., Kiriazis, H., Du, X.-J., Kaye, D.M., and Rajapakse, N.W. (2016). N-acetylcysteine attenuates the development of cardiac fibrosis and remodeling in a mouse model of heart failure. Physiol. Rep. *4*.

Gibbs, C.L. (1978). Cardiac energetics. Physiol. Rev. *58*, 174–254.

Gilbert-Diamond, D., Li, Z., Adachi-Mejia, A.M., McClure, A.C., and Sargent, J.D. (2014). Association of a Television in the Bedroom With Increased Adiposity Gain in a Nationally Representative Sample of Children and Adolescents. JAMA Pediatr. *168*, 427–434.

Gillies, A.R., and Lieber, R.L. (2011). Structure and function of the skeletal muscle extracellular matrix. Muscle Nerve *44*, 318–331.

Glass, C.E., Singal, P.K., and Singla, D.K. (2010). Stem cells in the diabetic infarcted heart. Heart Fail. Rev. *15*, 581–588.

Gluckman, P.D., and Hanson, M.A. (2004). Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr. Res. *56*, 311–317.

Gluckman, P.D., Hanson, M.A., Cooper, C., and Thornburg, K.L. (2008a). Effect of In Utero and Early-Life Conditions on Adult Health and Disease. N. Engl. J. Med. *359*, 61–73.

Gluckman, P.D., Hanson, M.A., Cooper, C., and Thornburg, K.L. (2008b). Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med. *359*, 61–73.

Golay, A., and Ybarra, J. (2005). Link between obesity and type 2 diabetes. Best Pract. Res. Clin. Endocrinol. Metab. *19*, 649–663.

Goldenberg, R., and Punthakee, Z. (2013). Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. Can. J. Diabetes *37*, S8–S11.

González-Muniesa, P., Mártinez-González, M.-A., Hu, F.B., Després, J.-P., Matsuzawa, Y., Loos, R.J.F., Moreno, L.A., Bray, G.A., and Martinez, J.A. (2017). Obesity. Nat. Rev. Dis. Primer *3*, 17034.

Goodpaster, B.H., and Wolf, D. (2004). Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes. Pediatr. Diabetes *5*, 219–226.

Greggio, C., Jha, P., Kulkarni, S.S., Lagarrigue, S., Broskey, N.T., Boutant, M., Wang, X., Conde Alonso, S., Ofori, E., Auwerx, J., et al. (2017). Enhanced Respiratory Chain Supercomplex Formation in Response to Exercise in Human Skeletal Muscle. Cell Metab. *25*, 301–311.

Guh, D.P., Zhang, W., Bansback, N., Amarsi, Z., Birmingham, C.L., and Anis, A.H. (2009). The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health *9*, 88.

ten Hacken, N.H.T. (2009). Physical inactivity and obesity: relation to asthma and chronic obstructive pulmonary disease? Proc. Am. Thorac. Soc. *6*, 663–667.

Hales, C.N., Barker, D.J., Clark, P.M., Cox, L.J., Fall, C., Osmond, C., and Winter, P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. BMJ *303*, 1019–1022.

Hanschmann, E.-M., Lönn, M.E., Schütte, L.D., Funke, M., Godoy, J.R., Eitner, S., Hudemann, C., and Lillig, C.H. (2010). Both thioredoxin 2 and glutaredoxin 2 contribute to the reduction of the mitochondrial 2-Cys peroxiredoxin Prx3. J. Biol. Chem. *285*, 40699–40705.

Hanschmann, E.-M., Godoy, J.R., Berndt, C., Hudemann, C., and Lillig, C.H. (2013). Thioredoxins, glutaredoxins, and peroxiredoxins--molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxid. Redox Signal. *19*, 1539–1605.

Harper, M.-E., Dent, R., Monemdjou, S., Bézaire, V., Van Wyck, L., Wells, G., Kavaslar, G.N., Gauthier, A., Tesson, F., and McPherson, R. (2002). Decreased mitochondrial proton leak and reduced expression of uncoupling protein 3 in skeletal muscle of obese diet-resistant women. Diabetes *51*, 2459–2466.

Hatch, G.M. (2004). Cell biology of cardiac mitochondrial phospholipids. Biochem. Cell Biol. Biochim. Biol. Cell. *82*, 99–112.

Hayes, J.D., Flanagan, J.U., and Jowsey, I.R. (2005). Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. *45*, 51–88.

Heather, L.C., and Clarke, K. (2011). Metabolism, hypoxia and the diabetic heart. J. Mol. Cell. Cardiol. *50*, 598–605.

Hénique, C., Mansouri, A., Vavrova, E., Lenoir, V., Ferry, A., Esnous, C., Ramond, E., Girard, J., Bouillaud, F., Prip-Buus, C., et al. (2015). Increasing mitochondrial muscle fatty acid oxidation induces skeletal muscle remodeling toward an oxidative phenotype. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *29*, 2473–2483.

Herrero, E., and Ros, J. (2002). [14] Glutaredoxins and oxidative stress defense in yeast. Methods Enzymol. *348*, 136–146.

Hesselink, M.K.C., Schrauwen-Hinderling, V., and Schrauwen, P. (2016). Skeletal muscle mitochondria as a target to prevent or treat type 2 diabetes mellitus. Nat. Rev. Endocrinol. *12*, 633–645.

Hill, J.O., Wyatt, H.R., and Peters, J.C. (2012). Energy Balance and Obesity. Circulation *126*, 126–132.

Hilton, T.N., Tuttle, L.J., Bohnert, K.L., Mueller, M.J., and Sinacore, D.R. (2008). Excessive adipose tissue infiltration in skeletal muscle in individuals with obesity, diabetes mellitus, and peripheral neuropathy: association with performance and function. Phys. Ther. *88*, 1336–1344.

Holland, W.L., Brozinick, J.T., Wang, L.-P., Hawkins, E.D., Sargent, K.M., Liu, Y., Narra, K., Hoehn, K.L., Knotts, T.A., Siesky, A., et al. (2007). Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. Cell Metab. *5*, 167–179.

Holmgren, A. (1976). Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. U. S. A. *73*, 2275–2279.

Holmström, K.M., and Finkel, T. (2014). Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat. Rev. Mol. Cell Biol. *15*, 411–421.

Holt, I.J., He, J., Mao, C.-C., Boyd-Kirkup, J.D., Martinsson, P., Sembongi, H., Reyes, A., and Spelbrink, J.N. (2007). Mammalian mitochondrial nucleoids: organizing an independently minded genome. Mitochondrion *7*, 311–321.

Höög, J.O., Jörnvall, H., Holmgren, A., Carlquist, M., and Persson, M. (1983). The primary structure of Escherichia coli glutaredoxin. Distant homology with thioredoxins in a superfamily of small proteins with a redox-active cystine disulfide/cysteine dithiol. Eur. J. Biochem. *136*, 223–232.

Horowitz, J.D., Chirkov, Y.Y., Kennedy, J.A., and Sverdlov, A.L. (2010). Modulation of myocardial metabolism: an emerging therapeutic principle. Curr. Opin. Cardiol. *25*, 329–334.

Hruby, A., and Hu, F.B. (2015). The Epidemiology of Obesity: A Big Picture. PharmacoEconomics *33*, 673–689.

Huang, C.-X., Liu, Y., Xia, W.-F., Tang, Y.-H., and Huang, H. (2009). Oxidative stress: a possible pathogenesis of atrial fibrillation. Med. Hypotheses *72*, 466–467.

Huynh, K., Bernardo, B.C., McMullen, J.R., and Ritchie, R.H. (2014). Diabetic cardiomyopathy: mechanisms and new treatment strategies targeting antioxidant signaling pathways. Pharmacol. Ther. *142*, 375–415.

Iguchi, Y., Kimura, K., Aoki, J., Kobayashi, K., Terasawa, Y., Sakai, K., and Shibazaki, K. (2008). Prevalence of atrial fibrillation in community-dwelling Japanese aged 40 years or older in Japan: analysis of 41,436 non-employee residents in Kurashiki-city. Circ. J. Off. J. Jpn. Circ. Soc. *72*, 909–913.

Isfort, M., Stevens, S.C.W., Schaffer, S., Jong, C.J., and Wold, L.E. (2014). Metabolic dysfunction in diabetic cardiomyopathy. Heart Fail. Rev. *19*, 35–48.

Jacob, C., Knight, I., and Winyard, P.G. (2006). Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. Biol. Chem. *387*, 1385–1397.

Jacob, S., Machann, J., Rett, K., Brechtel, K., Volk, A., Renn, W., Maerker, E., Matthaei, S., Schick, F., Claussen, C.D., et al. (1999). Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes *48*, 1113–1119.

Jastroch, M., Divakaruni, A.S., Mookerjee, S., Treberg, J.R., and Brand, M.D. (2010). Mitochondrial proton and electron leaks. Essays Biochem. *47*, 53–67.

Jeltsch, A., and Jurkowska, R.Z. (2014). New concepts in DNA methylation. Trends Biochem. Sci. *39*, 310–318.

Jensen, M.D., Ryan, D.H., Apovian, C.M., Ard, J.D., Comuzzie, A.G., Donato, K.A., Hu, F.B., Hubbard, V.S., Jakicic, J.M., Kushner, R.F., et al. (2014). 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. Circulation *129*, S102-138.

Jess, T. (2014). Microbiota, antibiotics, and obesity. N. Engl. J. Med. *371*, 2526–2528.

Jiang, F., Zhu, S., Yan, C., Jin, X., Bandla, H., and Shen, X. (2009). Sleep and obesity in preschool children. J. Pediatr. *154*, 814–818.

Jimenez-Chillaron, J.C., Isganaitis, E., Charalambous, M., Gesta, S., Pentinat-Pelegrin, T., Faucette, R.R., Otis, J.P., Chow, A., Diaz, R., Ferguson-Smith, A., et al. (2009). Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. Diabetes *58*, 460–468.

Jimenez-Chillaron, J.C., Ramon-Krauel, M., Ribo, S., and Diaz, R. (2016). Transgenerational epigenetic inheritance of diabetes risk as a consequence of early nutritional imbalances. Proc. Nutr. Soc. *75*, 78–89.

Johansson, C., Lillig, C.H., and Holmgren, A. (2004). Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. J. Biol. Chem. *279*, 7537–7543.

Jurado, J., Prieto-Alamo, M.-J., Madrid-Rísquez, J., and Pueyo, C. (2003). Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse. J. Biol. Chem. *278*, 45546–45554.

Kahn, S.E., Cooper, M.E., and Del Prato, S. (2014). PATHOPHYSIOLOGY AND TREATMENT OF TYPE 2 DIABETES: PERSPECTIVES ON THE PAST, PRESENT AND FUTURE. Lancet *383*, 1068–1083.

Kanaan, G.N., and Harper, M.-E. (2017). Cellular redox dysfunction in the development of cardiovascular diseases. Biochim. Biophys. Acta.

Kang, P.T., Zhang, L., Chen, C.-L., Chen, J., Green, K.B., and Chen, Y.-R. (2012). Protein thiyl radical mediates S-glutathionylation of complex I. Free Radic. Biol. Med. *53*, 962–973.

Karlsson, H.K.R., and Zierath, J.R. (2007). Insulin signaling and glucose transport in insulin resistant human skeletal muscle. Cell Biochem. Biophys. *48*, 103–113.

Kelley, D.E., Goodpaster, B., Wing, R.R., and Simoneau, J.A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. Am. J. Physiol. *277*, E1130-1141.

Kelley, D.E., He, J., Menshikova, E.V., and Ritov, V.B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes *51*, 2944–2950.

Kelly, T., Yang, W., Chen, C.-S., Reynolds, K., and He, J. (2008). Global burden of obesity in 2005 and projections to 2030. Int. J. Obes. 2005 *32*, 1431–1437.

Khacho, M., Tarabay, M., Patten, D., Khacho, P., MacLaurin, J.G., Guadagno, J., Bergeron, R., Cregan, S.P., Harper, M.-E., Park, D.S., et al. (2014). Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival. Nat. Commun. *5*, 3550.

Kim, J.Y., Hickner, R.C., Cortright, R.L., Dohm, G.L., and Houmard, J.A. (2000). Lipid oxidation is reduced in obese human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *279*, E1039-1044.

Kirkwood, S.P., Munn, E.A., and Brooks, G.A. (1986). Mitochondrial reticulum in limb skeletal muscle. Am. J. Physiol. *251*, C395-402.

Kodani, E. (2015). Early detection of atrial fibrillation is the first step to prevent cardiogenic stroke: Usefulness of B-type natriuretic peptide. J. Cardiol. *65*, 451–452.

Kodani, E., and Atarashi, H. (2012). Prevalence of atrial fibrillation in Asia and the world. J. Arrhythmia *28*, 330–337.

Koehler, C.M., Beverly, K.N., and Leverich, E.P. (2006). Redox pathways of the mitochondrion. Antioxid. Redox Signal. *8*, 813–822.

Konings, K.T., Kirchhof, C.J., Smeets, J.R., Wellens, H.J., Penn, O.C., and Allessie, M.A. (1994). High-density mapping of electrically induced atrial fibrillation in humans. Circulation *89*, 1665–1680.

Korantzopoulos, P., Kolettis, T.M., Galaris, D., and Goudevenos, J.A. (2007). The role of oxidative stress in the pathogenesis and perpetuation of atrial fibrillation. Int. J. Cardiol. *115*, 135–143.

Koutoukidis, D.A., Knobf, M.T., and Lanceley, A. (2015). Obesity, diet, physical activity, and health-related quality of life in endometrial cancer survivors. Nutr. Rev. *73*, 399–408.

Kovacic, P., Pozos, R.S., Somanathan, R., Shangari, N., and O'Brien, P.J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. Curr. Med. Chem. *12*, 2601–2623.

Koves, T.R., Ussher, J.R., Noland, R.C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J.R.B., Newgard, C.B., et al. (2008). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. *7*, 45–56.

La Merrill, M., Karey, E., Moshier, E., Lindtner, C., La Frano, M.R., Newman, J.W., and Buettner, C. (2014). Perinatal Exposure of Mice to the Pesticide DDT Impairs Energy Expenditure and Metabolism in Adult Female Offspring. PLoS ONE *9*.

Lafortuna, C.L., Tresoldi, D., and Rizzo, G. (2014). Influence of body adiposity on structural characteristics of skeletal muscle in men and women. Clin. Physiol. Funct. Imaging *34*, 47–55.

Lai, L.-P., Tsai, C.-C., Su, M.-J., Lin, J.-L., Chen, Y.-S., Tseng, Y.-Z., and Huang, S.K.S. (2003). Atrial fibrillation is associated with accumulation of aging-related common type mitochondrial DNA deletion mutation in human atrial tissue. Chest *123*, 539–544.

Landau, B.R., Wahren, J., Chandramouli, V., Schumann, W.C., Ekberg, K., and Kalhan, S.C. (1996). Contributions of gluconeogenesis to glucose production in the fasted state. J. Clin. Invest. *98*, 378–385.

Lanner, J.T., Katz, A., Tavi, P., Sandström, M.E., Zhang, S.-J., Wretman, C., James, S., Fauconnier, J., Lännergren, J., Bruton, J.D., et al. (2006). The role of Ca2+ influx for insulin-mediated glucose uptake in skeletal muscle. Diabetes *55*, 2077–2083.

Lanner, J.T., Bruton, J.D., Katz, A., and Westerblad, H. (2008). Ca(2+) and insulin-mediated glucose uptake. Curr. Opin. Pharmacol. *8*, 339–345.

Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellicer, A., Colás, C., Balsa, E., Perales-Clemente, E., Quirós, P.M., Calvo, E., Rodríguez-Hernández, M.A., et al. (2013). Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science *340*, 1567–1570.

Lara-Castro, C., and Garvey, W.T. (2008). Intracellular Lipid Accumulation in Liver and Muscle and the Insulin Resistance Syndrome. Endocrinol. Metab. Clin. North Am. *37*, 841–856.

Le Heuzey, J.-Y., Marijon, E., Lavergne, T., and Otmani, A. (2012). Management of ventricular and atrial arrhythmias in humans: towards a patient-specific approach. Eur. Eur. Pacing Arrhythm. Card. Electrophysiol. J. Work. Groups Card. Pacing Arrhythm. Card. Cell. Electrophysiol. Eur. Soc. Cardiol. *14 Suppl 5*, v125–v128.

Lee, H.-Y., Choi, C.S., Birkenfeld, A.L., Alves, T.C., Jornayvaz, F.R., Jurczak, M.J., Zhang, D., Woo, D.K., Shadel, G.S., Ladiges, W., et al. (2010). Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. Cell Metab. *12*, 668–674.

Lettner, A., and Roden, M. (2008). Ectopic fat and insulin resistance. Curr. Diab. Rep. *8*, 185–191.

Li, B., Nolte, L.A., Ju, J.S., Han, D.H., Coleman, T., Holloszy, J.O., and Semenkovich, C.F. (2000). Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. Nat. Med. *6*, 1115–1120.

Li, D., Fareh, S., Leung, T.K., and Nattel, S. (1999). Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. Circulation *100*, 87–95.

Li, J., Webster, M., Furuya, M., and Gilmartin, P.M. (2007). Identification and characterization of pin and thrum alleles of two genes that co-segregate with the Primula S locus. Plant J. Cell Mol. Biol. *51*, 18–31.

Liew, R. (2013). Almanac 2013: cardiac arrhythmias and pacing. Heart Br. Card. Soc. *99*, 1398–1407.

Liguori, A., Puglianiello, A., Germani, D., Deodati, A., Peschiaroli, E., and Cianfarani, S. (2010). Epigenetic Changes Predisposing to Type 2 Diabetes in Intrauterine Growth Retardation. Front. Endocrinol. *1*.

Lithell, H., Lindgärde, F., Hellsing, K., Lundqvist, G., Nygaard, E., Vessby, B., and Saltin, B. (1981). Body weight, skeletal muscle morphology, and enzyme activities in relation to fasting serum insulin concentration and glucose tolerance in 48-year-old men. Diabetes *30*, 19–25.

Liu, J., Zhang, A., and Li, L. (2012). Sleep duration and overweight/obesity in children: implication for pediatric nursing. J. Spec. Pediatr. Nurs. *17*, 193–204.

Loos, R.J.F. (2012). Genetic determinants of common obesity and their value in prediction. Best Pract. Res. Clin. Endocrinol. Metab. *26*, 211–226.

Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001). Cloning and Expression of a Novel Human Glutaredoxin (Grx2) with Mitochondrial and Nuclear Isoforms. J. Biol. Chem. *276*, 26269–26275.

Luquet, S., Lopez-Soriano, J., Holst, D., Fredenrich, A., Melki, J., Rassoulzadegan, M., and Grimaldi, P.A. (2003). Peroxisome proliferator-activated receptor delta controls muscle development and oxidative capability. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *17*, 2299–2301.

Mailloux, R.J., and Harper, M.-E. (2012). Mitochondrial proticity and ROS signaling: lessons from the uncoupling proteins. Trends Endocrinol. Metab. TEM *23*, 451–458.

Mailloux, R.J., and Treberg, J.R. (2016). Protein S-glutathionlyation links energy metabolism to redox signaling in mitochondria. Redox Biol. *8*, 110–118.

Mailloux, R.J., and Willmore, W.G. (2014). S-glutathionylation reactions in mitochondrial function and disease. Front. Cell Dev. Biol. *2*, 68.

Mailloux, R.J., Seifert, E.L., Bouillaud, F., Aguer, C., Collins, S., and Harper, M.E. (2011). Glutathionylation acts as a control switch for uncoupling proteins UCP2 and UCP3. J. Biol. Chem. *286*, 21865–21875.

Mailloux, R.J., Fu, A., Robson-Doucette, C., Allister, E.M., Wheeler, M.B., Screaton, R., and Harper, M.-E. (2012). Glutathionylation state of uncoupling protein-2 and the control of glucose-stimulated insulin secretion. J. Biol. Chem. *287*, 39673–39685.

Mailloux, R.J., McBride, S.L., and Harper, M.-E. (2013a). Unearthing the secrets of mitochondrial ROS and glutathione in bioenergetics. Trends Biochem. Sci. *38*, 592–602.

Mailloux, R.J., Xuan, J.Y., Beauchamp, B., Jui, L., Lou, M., and Harper, M.E. (2013b). Glutaredoxin-2 is required to control proton leak through uncoupling protein-3. J. Biol. Chem. *288*, 8365–8379.

Mailloux, R.J., Xuan, J.Y., McBride, S., Maharsy, W., Thorn, S., Holterman, C.E., Kennedy, C.R., Rippstein, P., deKemp, R., da Silva, J., et al. (2014). Glutaredoxin-2 is required to control oxidative phosphorylation in cardiac muscle by mediating deglutathionylation reactions. J. Biol. Chem. *289*, 14812–14828.

Malenfant, P., Joanisse, D.R., Thériault, R., Goodpaster, B.H., Kelley, D.E., and Simoneau, J.A. (2001). Fat content in individual muscle fibers of lean and obese subjects. Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes. *25*, 1316–1321.

Maranzana, E., Barbero, G., Falasca, A.I., Lenaz, G., and Genova, M.L. (2013). Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. Antioxid. Redox Signal. *19*, 1469–1480.

Marí, M., Morales, A., Colell, A., García-Ruiz, C., and Fernández-Checa, J.C. (2009). Mitochondrial glutathione, a key survival antioxidant. Antioxid. Redox Signal. *11*, 2685–2700.

Marí, M., Morales, A., Colell, A., García-Ruiz, C., Kaplowitz, N., and Fernández-Checa, J.C. (2013). Mitochondrial glutathione: features, regulation and role in disease. Biochim. Biophys. Acta *1830*, 3317–3328.

Marseglia, L., Manti, S., D'Angelo, G., Nicotera, A., Parisi, E., Di Rosa, G., Gitto, E., and Arrigo, T. (2014). Oxidative Stress in Obesity: A Critical Component in Human Diseases. Int. J. Mol. Sci. *16*, 378–400.

Mayr, M., Yusuf, S., Weir, G., Chung, Y.-L., Mayr, U., Yin, X., Ladroue, C., Madhu, B., Roberts, N., De Souza, A., et al. (2008). Combined metabolomic and proteomic analysis of human atrial fibrillation. J. Am. Coll. Cardiol. *51*, 585–594.

McGarry, J.D. (2002). Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes *51*, 7–18.

McHale, P.A., and Greenfield, J.C. (1986). Origin of atrial coving in canine phasic coronary artery blood flow. Am. J. Physiol. *251*, H774-778.

McMurray, F., Patten, D.A., and Harper, M.-E. (2016). Reactive Oxygen Species and Oxidative Stress in Obesity-Recent Findings and Empirical Approaches. Obes. Silver Spring Md *24*, 2301–2310.

Menke, A., Rust, K.F., Fradkin, J., Cheng, Y.J., and Cowie, C.C. (2014). Associations between trends in race/ethnicity, aging, and body mass index with diabetes prevalence in the United States: a series of cross-sectional studies. Ann. Intern. Med. *161*, 328–335.

Mihm, M.J., Yu, F., Carnes, C.A., Reiser, P.J., McCarthy, P.M., Van Wagoner, D.R., and Bauer, J.A. (2001). Impaired myofibrillar energetics and oxidative injury during human atrial fibrillation. Circulation *104*, 174–180.

Miki, T., Yuda, S., Kouzu, H., and Miura, T. (2013). Diabetic cardiomyopathy: pathophysiology and clinical features. Heart Fail. Rev. *18*, 149–166.

Mileykovskaya, E., Zhang, M., and Dowhan, W. (2005). Cardiolipin in energy transducing membranes. Biochem. Biokhimiia *70*, 154–158.

Mirmira, P., and Evans-Molina, C. (2014). Bisphenol A, obesity, and type 2 diabetes mellitus: genuine concern or unnecessary preoccupation? Transl. Res. J. Lab. Clin. Med. *164*, 13–21.

Mirza, M., Strunets, A., Shen, W.-K., and Jahangir, A. (2012). Mechanisms of Arrhythmias and Conduction Disorders in Older Adults. Clin. Geriatr. Med. *28*, 555–573.

Mishra, P., and Chan, D.C. (2016). Metabolic regulation of mitochondrial dynamics. J. Cell Biol. *212*, 379–387.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature *191*, 144–148.

Mittendorfer, B., Magkos, F., Fabbrini, E., Mohammed, B.S., and Klein, S. (2009). Relationship between body fat mass and free fatty acid kinetics in men and women. Obes. Silver Spring Md *17*, 1872–1877.

Mogensen, M., Sahlin, K., Fernström, M., Glintborg, D., Vind, B.F., Beck-Nielsen, H., and Højlund, K. (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes *56*, 1592–1599.

Morino, K., Petersen, K.F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., Neschen, S., White, M.F., Bilz, S., Sono, S., et al. (2005). Reduced mitochondrial density and increased

IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J. Clin. Invest. *115*, 3587–3593.

Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., de Ferranti, S., Després, J.-P., Fullerton, H.J., Howard, V.J., et al. (2015). Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation *131*, e29-322.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1–13.

Murphy, M.P. (2012). Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. Antioxid. Redox Signal. *16*, 476–495.

Murray, C.J.L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., et al. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet Lond. Engl. *380*, 2197–2223.

Nakamura, K., Fuster, J.J., and Walsh, K. (2014). Adipokines: a link between obesity and cardiovascular disease. J. Cardiol. *63*, 250–259.

NCD Risk Factor Collaboration (NCD-RisC) (2016). Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet Lond. Engl. *387*, 1513–1530.

Nedeltcheva, A.V., and Scheer, F.A.J.L. (2014). Metabolic effects of sleep disruption, links to obesity and diabetes. Curr. Opin. Endocrinol. Diabetes Obes. *21*, 293–298.

Nemoto, S., Takeda, K., Yu, Z.X., Ferrans, V.J., and Finkel, T. (2000). Role for mitochondrial oxidants as regulators of cellular metabolism. Mol. Cell. Biol. *20*, 7311–7318.

Neter, J.E., Stam, B.E., Kok, F.J., Grobbee, D.E., and Geleijnse, J.M. (2003). Influence of weight reduction on blood pressure: a meta-analysis of randomized controlled trials. Hypertens. Dallas Tex 1979 *42*, 878–884.

Neubauer, S. (2007a). The failing heart--an engine out of fuel. N. Engl. J. Med. *356*, 1140–1151.

Neubauer, S. (2007b). The failing heart--an engine out of fuel. N. Engl. J. Med. *356*, 1140–1151.

Neupert, W. (1997). Protein import into mitochondria. Annu. Rev. Biochem. *66*, 863–917.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F., et al. (2014). Global, regional and national prevalence of overweight and obesity in children and adults 1980-2013: A systematic analysis. Lancet Lond. Engl. *384*, 766–781.

Nicholls, D.G., and Ferguson, S.J. (2002). Bioenergetics (Gulf Professional Publishing).

Niwa, K., Warita, N., Sunami, Y., Shimura, A., Tateno, S., and Sugita, K. (2004). Prevalence of arrhythmias and conduction disturbances in large population-based samples of children. Cardiol. Young *14*, 68–74.

North, B.J., and Sinclair, D.A. (2012). The Intersection Between Aging and Cardiovascular Disease. Circ. Res. *110*, 1097–1108.

Noyes, A.M., Dua, K., Devadoss, R., and Chhabra, L. (2014). Cardiac adipose tissue and its relationship to diabetes mellitus and cardiovascular disease. World J. Diabetes *5*, 868–876.

Nyström, P.K., Carlsson, A.C., Leander, K., de Faire, U., Hellenius, M.-L., and Gigante, B. (2015). Obesity, metabolic syndrome and risk of atrial fibrillation: a Swedish, prospective cohort study. PloS One *10*, e0127111.

Odutayo, A., Wong, C.X., Hsiao, A.J., Hopewell, S., Altman, D.G., and Emdin, C.A. (2016). Atrial fibrillation and risks of cardiovascular disease, renal disease, and death: systematic review and meta-analysis. BMJ *354*, i4482.

Ong, S.-B., Kalkhoran, S.B., Cabrera-Fuentes, H.A., and Hausenloy, D.J. (2015). Mitochondrial fusion and fission proteins as novel therapeutic targets for treating cardiovascular disease. Eur. J. Pharmacol. *763*, 104–114.

Opacic, D., van Bragt, K.A., Nasrallah, H.M., Schotten, U., and Verheule, S. (2016). Atrial metabolism and tissue perfusion as determinants of electrical and structural remodelling in atrial fibrillation. Cardiovasc. Res. *109*, 527–541.

O'Rahilly, S., and Farooqi, I.S. (2008). Human obesity as a heritable disorder of the central control of energy balance. Int. J. Obes. 2005 *32 Suppl 7*, S55-61.

Orrenius, S., Gogvadze, V., and Zhivotovsky, B. (2007). Mitochondrial Oxidative Stress: Implications for Cell Death. Annu. Rev. Pharmacol. Toxicol. *47*, 143–183.

Ostrovidov, S., Hosseini, V., Ahadian, S., Fujie, T., Parthiban, S.P., Ramalingam, M., Bae, H., Kaji, H., and Khademhosseini, A. (2014). Skeletal Muscle Tissue Engineering: Methods to Form Skeletal Myotubes and Their Applications. Tissue Eng. Part B Rev. *20*, 403–436.

Padhi, S., Patel, N., Driscoll, D., and Budgell, B. (2014). Prevalence of cardiac arrhythmias in a community based chiropractic practice. J. Can. Chiropr. Assoc. *58*, 238–245.

Padilla, C.A., Martínez-Galisteo, E., Bárcena, J.A., Spyrou, G., and Holmgren, A. (1995). Purification from placenta, amino acid sequence, structure comparisons and cDNA cloning of human glutaredoxin. Eur. J. Biochem. *227*, 27–34.

Padwal, R.S., and Sharma, A.M. (2010). Prevention of cardiovascular disease: Obesity, diabetes and the metabolic syndrome. Can. J. Cardiol. *26*, 18C–20C.

Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.-E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K., et al. (2008). A mitochondrial protein compendium elucidates complex I disease biology. Cell *134*, 112–123.

Papa, S., Martino, P.L., Capitanio, G., Gaballo, A., Rasmo, D.D., Signorile, A., and Petruzzella, V. (2012). The Oxidative Phosphorylation System in Mammalian Mitochondria. In Advances in Mitochondrial Medicine, (Springer, Dordrecht), pp. 3–37.

Paradies, G., Paradies, V., Ruggiero, F.M., and Petrosillo, G. (2014). Cardiolipin and mitochondrial function in health and disease. Antioxid. Redox Signal. *20*, 1925–1953.

Park, S., Scheffler, T.L., Gunawan, A.M., Shi, H., Zeng, C., Hannon, K.M., Grant, A.L., and Gerrard, D.E. (2009). Chronic elevated calcium blocks AMPK-induced GLUT-4 expression in skeletal muscle. Am. J. Physiol. Cell Physiol. *296*, C106-115.

Parone, P.A., Da Cruz, S., Tondera, D., Mattenberger, Y., James, D.I., Maechler, P., Barja, F., and Martinou, J.-C. (2008). Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PloS One *3*, e3257.

Passarelli, C., Tozzi, G., Pastore, A., Bertini, E., and Piemonte, F. (2010). GSSG-mediated Complex I defect in isolated cardiac mitochondria. Int. J. Mol. Med. *26*, 95–99.

Patten, D.A., Wong, J., Khacho, M., Soubannier, V., Mailloux, R.J., Pilon-Larose, K., MacLaurin, J.G., Park, D.S., McBride, H.M., Trinkle-Mulcahy, L., et al. (2014). OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. EMBO J. *33*, 2676–2691.

Patti, M.-E., and Corvera, S. (2010). The role of mitochondria in the pathogenesis of type 2 diabetes. Endocr. Rev. *31*, 364–395.

Peake, J., and Suzuki, K. (2004). Neutrophil activation, antioxidant supplements and exercise-induced oxidative stress. Exerc. Immunol. Rev. *10*, 129–141.

Perreault, L., Pan, Q., Mather, K.J., Watson, K.E., Hamman, R.F., Kahn, S.E., and Diabetes Prevention Program Research Group (2012). Effect of regression from prediabetes to normal glucose regulation on long-term reduction in diabetes risk: results from the Diabetes Prevention Program Outcomes Study. Lancet Lond. Engl. *379*, 2243–2251.

Perreault, L., Temprosa, M., Mather, K.J., Horton, E., Kitabchi, A., Larkin, M., Montez, M.G., Thayer, D., Orchard, T.J., Hamman, R.F., et al. (2014). Regression From Prediabetes to Normal Glucose Regulation Is Associated With Reduction in Cardiovascular Risk: Results From the Diabetes Prevention Program Outcomes Study. Diabetes Care *37*, 2622–2631.

Petersen, K.F., and Shulman, G.I. (2006). Etiology of insulin resistance. Am. J. Med. *119*, S10-16.

Peterson, J.R., Burmeister, M.A., Tian, X., Zhou, Y., Guruju, M.R., Stupinski, J.A., Sharma, R.V., and Davisson, R.L. (2009). Genetic silencing of Nox2 and Nox4 reveals differential roles of these NADPH oxidase homologues in the vasopressor and dipsogenic effects of brain angiotensin II. Hypertension *54*, 1106–1114.

Phielix, E., and Mensink, M. (2008). Type 2 diabetes mellitus and skeletal muscle metabolic function. Physiol. Behav. *94*, 252–258.

Pietiläinen, K.H., Kaprio, J., Borg, P., Plasqui, G., Yki-Järvinen, H., Kujala, U.M., Rose, R.J., Westerterp, K.R., and Rissanen, A. (2008). Physical inactivity and obesity: A vicious circle. Obes. Silver Spring Md *16*, 409–414.

Ponterio, E., and Gnessi, L. (2015). Adenovirus 36 and Obesity: An Overview. Viruses *7*, 3719–3740.

Radmard, A.R., Merat, S., Kooraki, S., Ashraf, M., Keshtkar, A., Sharafkhah, M., Jafari, E., Malekzadeh, R., and Poustchi, H. (2015). Gallstone disease and obesity: a population-based study on abdominal fat distribution and gender differences. Ann. Hepatol. *14*, 702–709.

Rahman, F., Kwan, G.F., and Benjamin, E.J. (2016). Global epidemiology of atrial fibrillation. Nat. Rev. Cardiol. *13*, 501.

Ravelli, A.C., van Der Meulen, J.H., Osmond, C., Barker, D.J., and Bleker, O.P. (1999). Obesity at the age of 50 y in men and women exposed to famine prenatally. Am. J. Clin. Nutr. *70*, 811–816.

Redpath, C.J., Bou Khalil, M., Drozdzal, G., Radisic, M., and McBride, H.M. (2013). Mitochondrial hyperfusion during oxidative stress is coupled to a dysregulation in calcium handling within a C2C12 cell model. PloS One *8*, e69165.

Reilly, S.N., Jayaram, R., Nahar, K., Antoniades, C., Verheule, S., Channon, K.M., Alp, N.J., Schotten, U., and Casadei, B. (2011). Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. Circulation *124*, 1107–1117.

Reinehr, T., Hinney, A., de Sousa, G., Austrup, F., Hebebrand, J., and Andler, W. (2007). Definable somatic disorders in overweight children and adolescents. J. Pediatr. *150*, 618–622, 622.e1-5.

Reis, J.P., Loria, C.M., Sorlie, P.D., Park, Y., Hollenbeck, A., and Schatzkin, A. (2011). Lifestyle factors and risk for new-onset diabetes: a population-based cohort study. Ann. Intern. Med. *155*, 292–299.

Reyes, C., Leyland, K.M., Peat, G., Cooper, C., Arden, N.K., and Prieto-Alhambra, D. (2016). Association Between Overweight and Obesity and Risk of Clinically Diagnosed Knee, Hip, and Hand Osteoarthritis: A Population-Based Cohort Study. Arthritis Rheumatol. Hoboken NJ *68*, 1869–1875.

Reynolds, C.M., Gray, C., Li, M., Segovia, S.A., and Vickers, M.H. (2015). Early Life Nutrition and Energy Balance Disorders in Offspring in Later Life. Nutrients *7*, 8090–8111.

Ribas, V., García-Ruiz, C., and Fernández-Checa, J.C. (2014). Glutathione and mitochondria. Front. Pharmacol. *5*, 151.

Richardson, D.K., Kashyap, S., Bajaj, M., Cusi, K., Mandarino, S.J., Finlayson, J., DeFronzo, R.A., Jenkinson, C.P., and Mandarino, L.J. (2005). Lipid infusion decreases the expression of nuclear encoded mitochondrial genes and increases the expression of extracellular matrix genes in human skeletal muscle. J. Biol. Chem. *280*, 10290–10297.

Rieusset, J. (2015). Contribution of mitochondria and endoplasmic reticulum dysfunction in insulin resistance: Distinct or interrelated roles? Diabetes Metab. *41*, 358–368.

Ritov, V.B., Menshikova, E.V., He, J., Ferrell, R.E., Goodpaster, B.H., and Kelley, D.E. (2005). Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes *54*, 8–14.

Ritov, V.B., Menshikova, E.V., Azuma, K., Wood, R., Toledo, F.G.S., Goodpaster, B.H., Ruderman, N.B., and Kelley, D.E. (2010). Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. Am. J. Physiol. Endocrinol. Metab. *298*, E49-58.

Rolfe, D.F., and Brown, G.C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. *77*, 731–758.

Roseboom, T., de Rooij, S., and Painter, R. (2006). The Dutch famine and its long-term consequences for adult health. Early Hum. Dev. *82*, 485–491.

Rushworth, G.F., and Megson, I.L. (2014). Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. Pharmacol. Ther. *141*, 150–159.

Sas, K., Robotka, H., Toldi, J., and Vécsei, L. (2007). Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. J. Neurol. Sci. *257*, 221–239.

Savabi, F., and Kirsch, A. (1991). Alteration of the phosphocreatine energy shuttle components in diabetic rat heart. J. Mol. Cell. Cardiol. *23*, 1323–1333.

Savage, D.B., Petersen, K.F., and Shulman, G.I. (2007). Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol. Rev. *87*, 507–520.

Scerif, M., Goldstone, A.P., and Korbonits, M. (2011). Ghrelin in obesity and endocrine diseases. Mol. Cell. Endocrinol. *340*, 15–25.

Schafer, F.Q., and Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. *30*, 1191–1212.

Schägger, H., and Pfeiffer, K. (2000). Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J. *19*, 1777–1783.

Schaper, J., Meiser, E., and Stämmler, G. (1985). Ultrastructural morphometric analysis of myocardium from dogs, rats, hamsters, mice, and from human hearts. Circ. Res. *56*, 377–391.

Schiaffino, S., and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. Physiol. Rev. *91*, 1447–1531.

Schnabel, R.B., Yin, X., Larson, M.G., Yamamoto, J.F., Fontes, J.D., Kathiresan, S., Rong, J., Levy, D., Keaney, J.F., Wang, T.J., et al. (2013). Multiple inflammatory biomarkers in relation to cardiovascular events and mortality in the community. Arterioscler. Thromb. Vasc. Biol. *33*, 1728–1733.

Schütte, L.D., Baumeister, S., Weis, B., Hudemann, C., Hanschmann, E.-M., and Lillig, C.H. (2013). Identification of potential protein dithiol-disulfide substrates of mammalian Grx2. Biochim. Biophys. Acta *1830*, 4999–5005.

Seppet, E., Eimre, M., Peet, N., Paju, K., Orlova, E., Ress, M., Kõvask, S., Piirsoo, A., Saks, V.A., Gellerich, F.N., et al. (2005). Compartmentation of energy metabolism in atrial myocardium of patients undergoing cardiac surgery. Mol. Cell. Biochem. *270*, 49–61.

Severs, N.J. (2000). The cardiac muscle cell. BioEssays News Rev. Mol. Cell. Dev. Biol. *22*, 188–199.

Shan, J., Xie, W., Betzenhauser, M., Reiken, S., Chen, B.-X., Wronska, A., and Marks, A.R. (2012). Calcium leak through ryanodine receptors leads to atrial fibrillation in 3 mouse models of catecholaminergic polymorphic ventricular tachycardia. Circ. Res. *111*, 708–717.

Shutt, T., Geoffrion, M., Milne, R., and McBride, H.M. (2012). The intracellular redox state is a core determinant of mitochondrial fusion. EMBO Rep. *13*, 909–915.

Simbeni, R., Pon, L., Zinser, E., Paltauf, F., and Daum, G. (1991). Mitochondrial membrane contact sites of yeast. Characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids. J. Biol. Chem. *266*, 10047–10049.

Simoneau, J.A., and Bouchard, C. (1995). Skeletal muscle metabolism and body fat content in men and women. Obes. Res. *3*, 23–29.

Simoneau, J.A., and Kelley, D.E. (1997). Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. J. Appl. Physiol. Bethesda Md 1985 *83*, 166–171.

Simoneau, J.A., Colberg, S.R., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *9*, 273–278.

Simoneau, J.A., Veerkamp, J.H., Turcotte, L.P., and Kelley, D.E. (1999). Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *13*, 2051–2060.

Soubannier, V., and McBride, H.M. (2009). Positioning mitochondrial plasticity within cellular signaling cascades. Biochim. Biophys. Acta *1793*, 154–170.

Spanakis, E.K., and Golden, S.H. (2013). Race/Ethnic Difference in Diabetes and Diabetic Complications. Curr. Diab. Rep. *13*.

Sparks, L.M., Xie, H., Koza, R.A., Mynatt, R., Hulver, M.W., Bray, G.A., and Smith, S.R. (2005). A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes *54*, 1926–1933.

Stanley, W.C., Recchia, F.A., and Lopaschuk, G.D. (2005). Myocardial substrate metabolism in the normal and failing heart. Physiol. Rev. *85*, 1093–1129.

Stettler, N., Signer, T.M., and Suter, P.M. (2004). Electronic games and environmental factors associated with childhood obesity in Switzerland. Obes. Res. *12*, 896–903.

Stevens, G.A., Singh, G.M., Lu, Y., Danaei, G., Lin, J.K., Finucane, M.M., Bahalim, A.N., McIntire, R.K., Gutierrez, H.R., Cowan, M., et al. (2012). National, regional, and global trends in adult overweight and obesity prevalences. Popul. Health Metr. *10*, 22.

Stride, N., Larsen, S., Hey-Mogensen, M., Sander, K., Lund, J.T., Gustafsson, F., Køber, L., and Dela, F. (2013). Decreased mitochondrial oxidative phosphorylation capacity in the human heart with left ventricular systolic dysfunction. Eur. J. Heart Fail. *15*, 150–157.

Stunkard, A.J., Sørensen, T.I., Hanis, C., Teasdale, T.W., Chakraborty, R., Schull, W.J., and Schulsinger, F. (1986). An adoption study of human obesity. N. Engl. J. Med. *314*, 193–198.

Sun, Y., and Hu, D. (2010). The link between diabetes and atrial fibrillation: cause or correlation? J. Cardiovasc. Dis. Res. *1*, 10–11.

Taber, D.R., Chriqui, J.F., Powell, L., and Chaloupka, F.J. (2013). Association between state laws governing school meal nutrition content and student weight status: implications for new USDA school meal standards. JAMA Pediatr. *167*, 513–519.

Tadic, M., and Cuspidi, C. (2015). Type 2 diabetes mellitus and atrial fibrillation: From mechanisms to clinical practice. Arch. Cardiovasc. Dis. *108*, 269–276.

Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., Shinagawa, H., Hamasaki, N., and Kang, D. (2002). Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. EMBO Rep. *3*, 451–456.

Taylor, E.R., Hurrell, F., Shannon, R.J., Lin, T.-K., Hirst, J., and Murphy, M.P. (2003). Reversible glutathionylation of complex I increases mitochondrial superoxide formation. J. Biol. Chem. *278*, 19603–19610.

Thijssen, V.L.J.L., van der Velden, H.M.W., van Ankeren, E.P., Ausma, J., Allessie, M.A., Borgers, M., van Eys, G.J.J.M., and Jongsma, H.J. (2002). Analysis of altered gene expression during sustained atrial fibrillation in the goat. Cardiovasc. Res. *54*, 427–437.

Thrush, A.B., Dent, R., McPherson, R., and Harper, M.-E. (2013). Implications of mitochondrial uncoupling in skeletal muscle in the development and treatment of obesity. FEBS J. *280*, 5015–5029.

Thrush, A.B., Zhang, R., Chen, W., Seifert, E.L., Quizi, J.K., McPherson, R., Dent, R., and Harper, M.-E. (2014). Lower mitochondrial proton leak and decreased glutathione redox in primary muscle cells of obese diet-resistant versus diet-sensitive humans. J. Clin. Endocrinol. Metab. *99*, 4223–4230.

Thyfault, J.P., Cree, M.G., Zheng, D., Zwetsloot, J.J., Tapscott, E.B., Koves, T.R., Ilkayeva, O., Wolfe, R.R., Muoio, D.M., and Dohm, G.L. (2007). Contraction of insulin-resistant muscle normalizes insulin action in association with increased mitochondrial activity and fatty acid catabolism. Am. J. Physiol. Cell Physiol. *292*, C729-739.

Tomlinson, D.J., Erskine, R.M., Morse, C.I., Winwood, K., and Onambélé-Pearson, G. (2016). The impact of obesity on skeletal muscle strength and structure through adolescence to old age. Biogerontology *17*, 467–483.

Trachanas, K., Sideris, S., Aggeli, C., Poulidakis, E., Gatzoulis, K., Tousoulis, D., and Kallikazaros, I. (2014). Diabetic cardiomyopathy: from pathophysiology to treatment. Hell. J. Cardiol. HJC Hell. Kardiologike Epitheorese *55*, 411–421.

Trasande, L., Attina, T.M., and Blustein, J. (2012). Association between urinary bisphenol A concentration and obesity prevalence in children and adolescents. JAMA *308*, 1113–1121.

Tremaroli, V., and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. Nature *489*, 242–249.

Treweeke, A.T., Winterburn, T.J., Mackenzie, I., Barrett, F., Barr, C., Rushworth, G.F., Dransfield, I., MacRury, S.M., and Megson, I.L. (2012). N-Acetylcysteine inhibits platelet-monocyte conjugation in patients with type 2 diabetes with depleted intraplatelet glutathione: a randomised controlled trial. Diabetologia *55*, 2920–2928.

Tsuboi, M., Hisatome, I., Morisaki, T., Tanaka, M., Tomikura, Y., Takeda, S., Shimoyama, M., Ohtahara, A., Ogino, K., Igawa, O., et al. (2001). Mitochondrial DNA deletion associated with the reduction of adenine nucleotides in human atrium and atrial fibrillation. Eur. J. Clin. Invest. *31*, 489–496.

Turta, O., and Rautava, S. (2016). Antibiotics, obesity and the link to microbes - what are we doing to our children? BMC Med. *14*.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. *39*, 44–84.

Vartak, R., Porras, C.A.-M., and Bai, Y. (2013). Respiratory supercomplexes: structure, function and assembly. Protein Cell *4*, 582–590.

Vasan, R.S. (2003). Cardiac function and obesity. Heart *89*, 1127–1129.

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2004). Energy metabolism in heart failure. J. Physiol. *555*, 1–13.

Vogel, F., Bornhövd, C., Neupert, W., and Reichert, A.S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. J. Cell Biol. *175*, 237–247.

Wada, J., and Nakatsuka, A. (2016). Mitochondrial Dynamics and Mitochondrial Dysfunction in Diabetes. Acta Med. Okayama *70*, 151–158.

Wang, H., and Eckel, R.H. (2009). Lipoprotein lipase: from gene to obesity. Am. J. Physiol. Endocrinol. Metab. *297*, E271-288.

Wang, Z., and Nakayama, T. (2010). Inflammation, a link between obesity and cardiovascular disease. Mediators Inflamm. *2010*, 535918.

Wang, J., Adab, P., Liu, W., Chen, Y., Li, B., Lin, R., Liu, W., Cheng, K.K., and Pallan, M. (2017). Prevalence of adiposity and its association with sleep duration, quality, and timing among 9-12-year-old children in Guangzhou, China. J. Epidemiol.

Wang, S.-B., Foster, D.B., Rucker, J., O'Rourke, B., Kass, D.A., and Van Eyk, J.E. (2011). Redox regulation of mitochondrial ATP synthase: implications for cardiac resynchronization therapy. Circ. Res. *109*, 750–757.

Wang, T.J., Parise, H., Levy, D., D'Agostino, R.B., Wolf, P.A., Vasan, R.S., and Benjamin, E.J. (2004). Obesity and the risk of new-onset atrial fibrillation. JAMA *292*, 2471–2477.

Warner, M., Wesselink, A., Harley, K.G., Bradman, A., Kogut, K., and Eskenazi, B. (2014). Prenatal exposure to dichlorodiphenyltrichloroethane and obesity at 9 years of age in the CHAMACOS study cohort. Am. J. Epidemiol. *179*, 1312–1322.

Watanabe, H., Tanabe, N., Watanabe, T., Darbar, D., Roden, D.M., Sasaki, S., and Aizawa, Y. (2008). Metabolic Syndrome and Risk of Development of Atrial Fibrillation. Circulation *117*, 1255–1260.

Weiss, R.G., Mejia, M.A., Kass, D.A., DiPaula, A.F., Becker, L.C., Gerstenblith, G., and Chacko, V.P. (1999). Preservation of canine myocardial high-energy phosphates during low-flow ischemia with modification of hemoglobin-oxygen affinity. J. Clin. Invest. *103*, 739–746.

Westerblad, H., Bruton, J.D., and Katz, A. (2010). Skeletal muscle: energy metabolism, fiber types, fatigue and adaptability. Exp. Cell Res. *316*, 3093–3099.

Westermann, B. (2012). Bioenergetic role of mitochondrial fusion and fission. Biochim. Biophys. Acta *1817*, 1833–1838.

White, C.W., Kerber, R.E., Weiss, H.R., and Marcus, M.L. (1982). The effects of atrial fibrillation on atrial pressure-volume and flow relationships. Circ. Res. *51*, 205–215.

Wiedemann, N., Frazier, A.E., and Pfanner, N. (2004). The protein import machinery of mitochondria. J. Biol. Chem. *279*, 14473–14476.

Wijffels, M.C., Kirchhof, C.J., Dorland, R., and Allessie, M.A. (1995). Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation *92*, 1954–1968.

Wilder, T., Ryba, D.M., Wieczorek, D.F., Wolska, B.M., and Solaro, R.J. (2015). N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy. Am. J. Physiol. Heart Circ. Physiol. *309*, H1720-1730.

Williams, E.P., Mesidor, M., Winters, K., Dubbert, P.M., and Wyatt, S.B. (2015). Overweight and Obesity: Prevalence, Consequences, and Causes of a Growing Public Health Problem. Curr. Obes. Rep. *4*, 363–370.

Wisneski, J.A., Gertz, E.W., Neese, R.A., Gruenke, L.D., and Craig, J.C. (1985). Dual carbon-labeled isotope experiments using D-[6-14C] glucose and L-[1,2,3-13C3] lactate: a new approach for investigating human myocardial metabolism during ischemia. J. Am. Coll. Cardiol. *5*, 1138–1146.

Wittig, I., Carrozzo, R., Santorelli, F.M., and Schägger, H. (2006). Supercomplexes and subcomplexes of mitochondrial oxidative phosphorylation. Biochim. Biophys. Acta *1757*, 1066–1072.

World Health Organization (2016). WHO Global Report on Diabetes (World Health Organization).

World Health Organization (2017). The challenge of cardiovascular disease - quick statistics.

Wu, H., Lin, L., Giblin, F., Ho, Y.-S., and Lou, M.F. (2011). Glutaredoxin 2 knockout increases sensitivity to oxidative stress in mouse lens epithelial cells. Free Radic. Biol. Med. *51*, 2108–2117.

Wu, H., Yu, Y., David, L., Ho, Y.-S., and Lou, M.F. (2014). Glutaredoxin 2 (Grx2) gene deletion induces early onset of age-dependent cataracts in mice. J. Biol. Chem. *289*, 36125–36139.

Xia, Q., and Grant, S.F.A. (2013). The genetics of human obesity. Ann. N. Y. Acad. Sci. *1281*, 178–190.

Xie, W., Santulli, G., Guo, X., Gao, M., Chen, B.-X., and Marks, A.R. (2013). Imaging atrial arrhythmic intracellular calcium in intact heart. J. Mol. Cell. Cardiol. *64*, 120–123.

Xie, W., Santulli, G., Reiken, S.R., Yuan, Q., Osborne, B.W., Chen, B.-X., and Marks, A.R. (2015a). Mitochondrial oxidative stress promotes atrial fibrillation. Sci. Rep. *5*, 11427.

Xie, X., Lin, T., Zhang, M., Liao, L., Yuan, G., Gao, H., Ning, Q., and Luo, X. (2015b). IUGR with infantile overnutrition programs an insulin-resistant phenotype through DNA methylation of peroxisome proliferator-activated receptor-γ coactivator-1α in rats. Pediatr. Res. *77*, 625–632.

Xu, J., Xu, X., Si, L., Xue, L., Zhang, S., Qin, J., Wu, Y., Shao, Y., Chen, Y., and Wang, X. (2013). Intracellular lactate signaling cascade in atrial remodeling of mitral valvular patients with atrial fibrillation. J. Cardiothorac. Surg. *8*, 34.

Yang, K.-C., Bonini, M.G., and Dudley, S.C. (2014). Mitochondria and arrhythmias. Free Radic. Biol. Med. *71*, 351–361.

Yant, L.J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J.G., Motta, L., Richardson, A., and Prolla, T.A. (2003). The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. Free Radic. Biol. Med. *34*, 496–502.

Yates, D.T., Macko, A.R., Nearing, M., Chen, X., Rhoads, R.P., and Limesand, S.W. (2012). Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. J. Pregnancy *2012*, 631038.

Yates, D.T., Clarke, D.S., Macko, A.R., Anderson, M.J., Shelton, L.A., Nearing, M., Allen, R.E., Rhoads, R.P., and Limesand, S.W. (2014). Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. J. Physiol. *592*, 3113–3125.

Yates, D.T., Cadaret, C.N., Beede, K.A., Riley, H.E., Macko, A.R., Anderson, M.J., Camacho, L.E., and Limesand, S.W. (2016). Intrauterine growth-restricted sheep fetuses exhibit smaller hindlimb muscle fibers and lower proportions of insulin-sensitive Type I fibers near term. Am. J. Physiol. - Regul. Integr. Comp. Physiol. *310*, R1020–R1029.

Yatsuya, H., Folsom, A.R., Yamagishi, K., North, K.E., Brancati, F.L., Stevens, J., and Atherosclerosis Risk in Communities Study Investigators (2010). Race- and sex-specific associations of obesity measures with ischemic stroke incidence in the Atherosclerosis Risk in Communities (ARIC) study. Stroke *41*, 417–425.

Zhang, H., Du, Y., Zhang, X., Lu, J., and Holmgren, A. (2014). Glutaredoxin 2 reduces both thioredoxin 2 and thioredoxin 1 and protects cells from apoptosis induced by auranofin and 4-hydroxynonenal. Antioxid. Redox Signal. *21*, 669–681.

Zhang, Y., Fraser, J.A., Jeevaratnam, K., Hao, X., Hothi, S.S., Grace, A.A., Lei, M., and Huang, C.L.-H. (2011). Acute atrial arrhythmogenicity and altered Ca(2+) homeostasis in murine RyR2-P2328S hearts. Cardiovasc. Res. *89*, 794–804.

Zorov, D.B., Juhaszova, M., and Sollott, S.J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol. Rev. *94*, 909–950.

CHAPTER 2

In utero undernutrition impacts skeletal muscle gene expression and energy metabolism of primary muscle cells

Brittany Beauchamp[1#], **Georges N. Kanaan[1#],** Olivier Charette[1], Kijoo Kim[1], Grace Daher[2], Mary-Elizabeth Patti[2], Alexandre Blais[1] and Mary-Ellen Harper[1*].

[1]Department of Biochemistry, Microbiology and Immunology, and Ottawa Institute of Systems Biology, Faculty of Medicine, 451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5;
[2]Division of Integrative Physiology and Metabolism, Joslin Diabetes Center, Boston, MA, USA, 02215.

[#] **Co-first authors**

[*] **To whom correspondence should be addressed:**

Dr. Mary-Ellen Harper, PhD
Professor
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine, University of Ottawa
451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5
Email: mharper@uottawa.ca
Tel: +1-613-562-5800 Ext 8235

2.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

2.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript "*In utero* undernutrition impacts skeletal muscle gene expression and energy metabolism of primary muscle cells" has been submitted for publication in the journal *Metabolism- Clinical and Experimental (METABOLISM-S-17-01781)* on 20 December, 2017.

2.1.2 CONTRIBUTION STATEMENT

BB, GNK and MEH designed and interpreted all experiments. BB, OC and KK performed and analyzed bioenergetic determinations. GNK performed and analyzed western blot determinations. GNK, GD, MEP, AB and MEH interpreted and analyzed gene expression results. BB, GNK and MEH wrote the manuscript and MEP and AB edited it. All authors reviewed and approved the manuscript.

2.1.3 ACKNOWLEDGMENTS AND FUNDING

We would like to thank Jian Xuan for technical assistance with animal work and Mahmoud Salkhordeh for technical assistance with cell isolations. This research was supported through a grant from Canadian Institutes of Health Research (CIHR; MOP57810 and FDN143278, MEH and MOP119458, AB). MEP acknowledges support from R01 DK106193 and P30 DK 036836. Georges N. Kanaan was supported by the University of Ottawa PhD full admission scholarship. Brittany Beauchamp received a scholarship from

Natural Sciences and Engineering Research Council of Canada (Canada Graduate Scholarship -Doctoral, BB).

2.1.4 CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

2.2 ABSTRACT

Objective: *In utero* undernutrition is associated with an increased risk for obesity and insulin resistance in adulthood. Low birth weight offspring have been shown to have alterations in skeletal muscle mass and function. Here we hypothesized that primary muscle cells of low birth weight offspring exhibit cell autonomous metabolic defects and respond differently to nutrient restriction *in vitro* compared to cells from control mice.

Methods: In differentiated myotubes from *in utero* undernourished mice (U) and control mice (C) we conducted gene expression profiling under control and nutrient restriction conditions. Mitochondrial bioenergetics, oxidative and glycolytic capacities, and mitochondrial content markers were measured under control and nutrient restriction conditions.

Results: Gene set enrichment analysis on the gene expression data indicated a number of gene groups being affected by undernourishment *in utero* and/or glucose deprivation *in vitro*. Myotubes from U have decreased resting respiration and ATP turnover under standard incubation conditions. In response to nutrient restriction, myotubes from U have impaired fatty acid oxidation characteristics, and increased glycolysis compared to myotubes from C. There was no difference in myotube mitochondrial content.

Conclusions: We provide the first evidence that myotubes established from satellite cells of *in utero* undernourished mice have altered gene expression associated with energy production and energy storage. Moreover, myotubes have marked impairments in oxidative metabolism and enhanced glycolytic characteristics. Altogether these cell autonomous results are consistent with the conclusion that susceptibility to metabolic disease in adulthood can be

caused by muscle defects, including aberrant mitochondrial energetics, that are programmed *in utero*.

Keywords: fetal programming, satellite cells, muscle development, mitochondria, obesity

2.3 INTRODUCTION

Suboptimal early life nutrition can increase risk for the development of disease in adulthood. Human epidemiological studies and research in animal models have shown that low birth weight, as a result of maternal undernutrition, is associated with increased risk of metabolic diseases, such as obesity and type 2 diabetes mellitus (Reviewed in [1]). It has been hypothesized that permanent alterations in tissue structure and function can be induced by perinatal events and thereby increase disease risk [2, 3]. However, how a suboptimal *in utero* environment leads to adult disease has not been fully elucidated.

Skeletal muscle is a remarkably adaptable tissue that modifies its structure and function in response to needs [4, 5]. Skeletal muscle is a key determinant of systemic metabolism and insulin sensitivity and therefore alterations in its function contribute to metabolic disease risk [6-8]. Common physiological phenotypes in low birth weight offspring include alterations in skeletal muscle mass and function. Low birth weight has been associated with reduced muscle mass and reduced oxidative capacity [9-14]. Skeletal muscle dysfunction is also strongly associated with obesity and insulin resistance [14-18]. We have recently used a mouse model of low birth weight generated through 50% food restriction during the third week of pregnancy, originally described in [12]. Similar to human phenotypes, it has been shown that these low birth weight mice develop glucose intolerance and increased adiposity in adulthood, similar to findings in human populations [12, 19]. We recently showed that the low birth weight mice have decreased skeletal muscle mitochondrial content in mixed fiber muscles, decreased respiration in both isolated skeletal muscle mitochondria and permeabilized muscle fibers, and intriguingly, a blunted weight loss response to a

hypocaloric diet in adulthood [19]. Furthermore, prenatally undernourished mice have been shown to have reduced myogenic stem cell frequency and reduced regenerative capacity [20].

Therefore, in the current study we aimed to determine if there are primary, cell autonomous, defects in gene transcription and metabolic functions in cells derived from muscle progenitors (*i.e.,* satellite cells). We hypothesized that the previously documented increased susceptibility to obesity and glucose intolerance is due in part to primary defects in muscle that are programmed *in utero*.

2.4 MATERIALS AND METHODS

2.4.1 ANIMALS

All procedures involving the use of animals were performed according to the principles and guidelines of the Canadian Council of Animal Care and the study was approved by the Animal Care Committee of the University of Ottawa. Mice were housed in a facility with controlled temperature (23^{o}C), humidity, and light-dark cycle (0600h – 1800h). Virgin female ICR mice (Harlan, Indianapolis, IN, USA; age 6-8 weeks) were paired with male ICR mice (Harlan; age 6-8 weeks). Pregnancies were dated by vaginal plug (day 0.5) and pregnant mice were housed individually with *ad libitum* access to standard rodent chow (T.2018, Harlan Teklad, Indianapolis, IN, USA). On day 12.5 of pregnancy, dams were randomly assigned to either a control or undernutrition group. In the undernutrition group, food was restricted to 50% that of gestational day -matched controls for the remainder of pregnancy. After delivery, mothers were given *ad libitum* access to chow and 24 hours after

birth, litters were equalized to eight. We studied two groups of mice, *in utero* undernourished offspring (U) and control offspring (C).

2.4.2 ISOLATION OF MOUSE PRIMARY CELLS

Primary muscle cells were isolated from the quadriceps of U and C at 3 weeks old. Cells were isolated as previously described [21].

2.4.3 CELL CULTURE

Isolated primary muscle cells were grown on Matrigel-coated flasks in low glucose (5.5 mmol/L) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% FBS, 1X antibiotic-antimycotic, 2.5 μg/ml gentamycin and 2.5 ng/ml basic fibroblast growth factor. For experiments, when cells reached approximately 90% confluency, they were differentiated in low glucose DMEM supplemented with 2% FBS, 1X antibiotic-antimycotic, and 2.5 μg/ml gentamycin. Differentiation was verified by immunofluorescence staining (Desmin and DAPI) as described previously [22].

2.4.4 BIOENERGETIC ANALYSIS OF CELLULAR METABOLIC CHARACTERISTICS

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at 37°C using a Seahorse XF24 Extracellular Flux Analyzer. Cells were plated at 20,000 cells per well on Matrigel-coated Seahorse plates.

2.4.5 INITIAL METABOLIC CHARACTERIZATION

Myotubes were changed to unbuffered assay medium (HCO_3-free DMEM with 5 mM glucose, 4 mM glutamine, 1 mM pyruvate, pH 7.4) and incubated at 37°C in a non CO_2 incubator for 30 min before being placed in the machine for OCR measurement. Resting measurements were taken before sequential additions of oligomycin (1 µg/ml), FCCP (1 µM), and antimycin A (1 µM) and rotenone (40 uM) to determine state 4, maximal, and non-mitochondrial OCR respectively. All measurements were obtained in quintuplicate over a 2 min measurement with a 2 min mix and 2 min incubation between measurements. Values were corrected to protein content determined using a Bradford assay.

2.4.6 FATTY ACID OXIDATION ASSAY

Myotubes were incubated overnight in a substrate limited media (glucose- and glutamine-free DMEM supplemented with 0.5 mM glucose, 1 mM glutamine, 0.5 mM carnitine and 1% (v/v) FBS; pH 7.4). 45 minutes prior to the experiment, cells were changed to a fatty acid oxidation assay medium (111 mM NaCl, 4.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM $Na3PO_4$, 2.5 mM glucose, 0.5 mM carnitine and 5 mM HEPES; pH 7.4) and incubated at 37˚C at ambient CO_2. 40 µM etomoxir or vehicle control was added 15 minutes prior to the experiment. 75 µM Palmitate:BSA or BSA control was added immediately prior to the experiment. OCR measurements were taken at rest before sequential additions of oligomycin (2.5 µg/ml), FCCP (1 µM), and antimycin A (1 µM) and rotenone (40 uM) to determine state 4, maximal, and non-mitochondrial OCR respectively. All measurements were obtained in quintuplicate over a 2 min measurement with a 2 min mix and 2 min incubation between measurements. Values were corrected to protein content determined using a Bradford assay.

2.4.7 GLYCOLYSIS ASSAY

60 minutes prior to the experiment, myotubes were incubated at 37˚C at ambient CO_2 in a glycolysis stress assay media (DMEM without glucose and glutamine supplemented with 143 mM NaCl, 0.5% phenol Red and 2 mM glutamine; pH 7.4). ECAR was measured at baseline followed by sequential injections of glucose (10 mM), oligomycin (2.5 μg/mL), and 2-deoxy-D-glucose (100 mM) to measure glycolysis, maximal glycolytic capacity, and non-glycolytic acidification respectively. Measurements were obtained in quintuplicate over a 2 min measurement with a 2 min mix and 2 min incubation between measurements with the exception of basal rates that were measured for 4 min. Values were corrected to protein content determined using a Bradford assay.

2.4.8 WESTERN BLOTTING

Myotubes were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM NaF, 0.2 mM Na_3VO_4, protease inhibitor cocktail (Roche, Mississauga, ON; pH 7.6)). Protein content was measured using a bicinchoninic acid assay or Bradford assay and samples were stored at -80°C. Samples were subjected to reducing SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes. After blocking for 1 h at room temperature in 5% BSA in TBS + 0.1% Tween-20 (TBST), incubation in primary antibody was overnight at 4°C. The following primary antibodies were used at the indicated dilutions: MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam; 1:800), AMPK alpha (2532, Cell Signaling; 1:1000) and pAMPK alpha (T172) (2531, Cell Signaling, 1:1000). Following 3 x 10 min washes with TBST, membranes were incubated in the appropriate horseradish peroxidase-conjugated

secondary antibody diluted in 5% BSA in TBST at room temperature for 2h. Bands were visualized using enhanced chemiluminescence. Band intensity was quantified by density analysis using Image J (NIH) and normalized to vinculin.

2.4.9 RNA EXTRACTION AND MICROARRAY PROTOCOL

Trizol extraction of total RNA from primary muscle cells was performed according to the manufacturer's instructions. Samples were processed using the WT Plus kit (Affymetrix). A hybridization cocktail containing 3.5 ug of fragmented, biotin labeled cDNA was hybridized to GeneChip Mouse Gene 2.0 ST arrays for 16 hours at 45°C. Arrays were washed, stained, and scanned using standard protocols with the Affymetrix Fluidics Station 450 and GeneChip Scanner 3000. The data were uploaded onto GEO with accession number GSE103689. Data were imported in R 3.3.0, and analyzed using Bioconductor 3.4 with the *oligo* package [23] and RMA background correction, quantile normalization and summarization. One of 24 samples was removed from further analyses as it was identified as an outlier compared to others in its group, as evidenced by principal component analysis. Probes with no gene annotation were removed, and the expression data table was analysed using the Broad Institute program for Gene Set Enrichment Analysis (GSEA) [24, 25]. GSEA was run with Molecular Signatures Database v6.1 [25, 26] and using default parameters, except that permutations were performed on the gene sets instead of the phenotype labels.

2.4.10 STATISTICAL ANALYSES

All measures were analyzed using GraphPad Prism, version 7.0 (La Jolla, CA, USA). Values are reported as mean ± SEM. $p<0.05$ was considered significant. In GSEA, a FDR q-value of less than 5% was judged significant.

2.5 RESULTS

2.5.1 INITIAL METABOLIC CHARACTERIZATION OF MYOTUBES

To assess overall bioenergetics characteristics of myotubes from U and C, mitochondrial OCR was measured under resting, oligomycin-induced state 4, and maximal uncoupled (FCCP-induced) conditions. Respiration under resting conditions was decreased in U (Figure 2.1A). There was no difference in respiration under state 4 or maximal uncoupled conditions (Figure 2.1B, 2.1C). To further assess metabolic characteristics of the cells, we measured fatty acid oxidation and glycolysis.

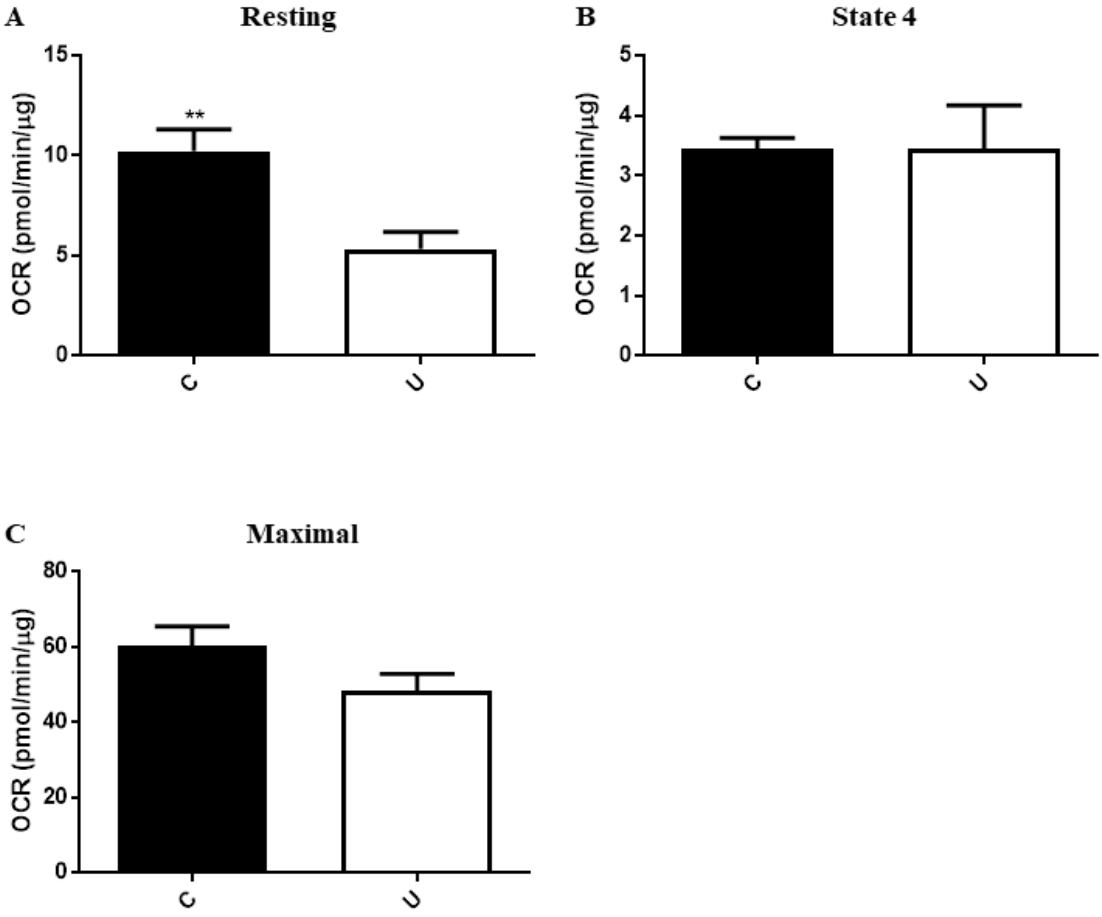


Figure 2.1: Metabolic characterization of myotubes from U and C mice.

Data are shown for resting (A), state 4 (B), and maximal (C) oxygen consumption rate (OCR) in myotubes from in utero undernourished mice (U; white bars) and control mice (C; black bars). Data are presented as mean ± SEM, n=5-6. Student's t-test, **p<0.01.

2.5.2 DYSFUNCTIONAL FATTY ACID OXIDATION IN MYOTUBES FROM IN UTERO UNDERNOURISHED MICE

To measure fatty acid oxidation, cells were first incubated overnight in a substrate-limited medium, which was then changed to a fatty acid oxidation assay medium and treated with 75 μM palmitate. OCR in the presence of palmitate was measured under resting, state 4, and maximal uncoupled conditions. Respiration under resting (Figure 2.2A) and maximal uncoupled conditions (Figure 2.2C) was decreased in U compared to C, with a trend for a decrease in state 4 respiration (Figure 2.2B). Cells were also treated with etomoxir to allow for determination of fatty acid oxidation due to endogenous or exogenous fatty acid (palmitate) use. Etomoxir inhibits carnitine palmitoyltransferase I, an enzyme required for the formation and transport of long chain acyl carnitines from the cytosol into the mitochondria thus inhibiting exogenous fatty acid oxidation [27]. Under resting conditions, there is no difference in the rates of endogenous fatty acid oxidation between U and C (Figure 2.2D) but exogenous fatty acid oxidation is decreased in U (roughly 15% of C; Figure 2.2E). Under maximal respiration conditions (*i.e.*, in the presence of FCCP), endogenous fatty acid oxidation is decreased to about half in U compared to C and there is no difference in exogenous fatty acid oxidation (Figure 2.2F, 2.2G).

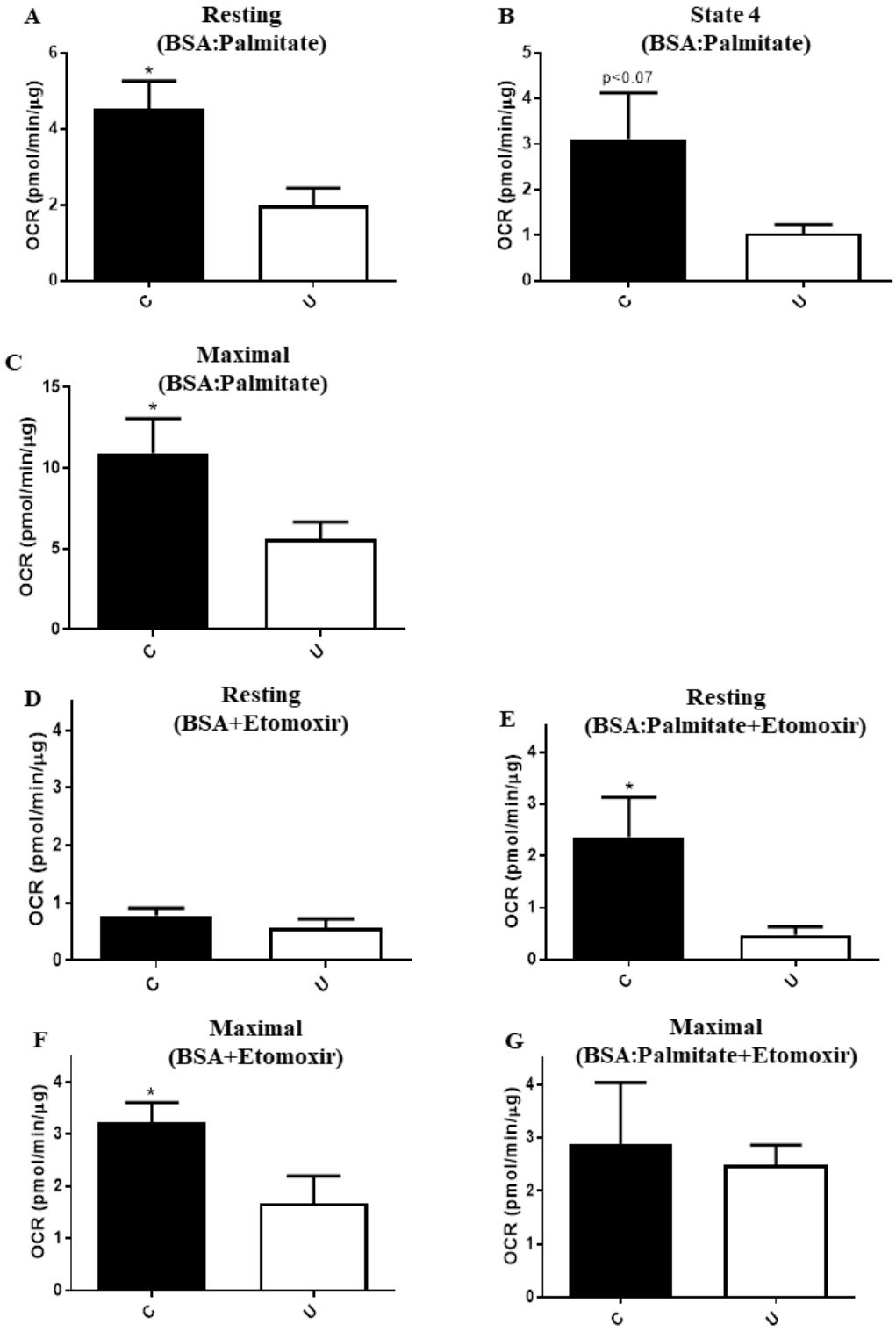

A
Resting
(BSA:Palmitate)
OCR (pmol/min/µg)
*
C
U
B
State 4
(BSA:Palmitate)
p<0.07
C
Maximal
(BSA:Palmitate)
D
Resting
(BSA+Etomoxir)
E
Resting
(BSA:Palmitate+Etomoxir)
F
Maximal
(BSA+Etomoxir)
G
Maximal
(BSA:Palmitate+Etomoxir)

Figure 2.2: Decreased fatty acid oxidation in myotubes from *in utero* undernourished mice.

Oxygen consumption rate (OCR) was measured in myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars) in the presence of 75 uM palmitate. Resting (A), state 4 (B), and maximal (C) OCR were measured. Resting respiration due to utilization of endogenous fatty acids (D), resting respiration due to utilization of exogenous fatty acids (E), maximal respiration due to utilization of endogenous fatty acids (F), and maximal respiration due to utilization of exogenous fatty acids (G) were determined. Data are presented as mean ± SEM, n=7. Student's *t*-test, *$p<0.05$.

2.5.3 ENHANCED GLYCOLYSIS IN MYOTUBES FROM IN UTERO UNDERNOURISHED MICE

For the glycolysis assay, cells were incubated in glucose-free media for 1 hour and ECAR was measured after the sequential addition of glucose, oligomycin, and 2-deoxy-D-glucose to assess glycolysis, maximal glycolytic capacity, and non-glycolytic acidification, respectively. Myotubes from U had an approximately doubled glycolytic activity in response to the addition of glucose and had a similarly increased maximal glycolytic capacity compared to C (Figure 2.3A, 2.3B). Non-glycolytic acidification was not significantly different between U and C (Figure 2.3C).

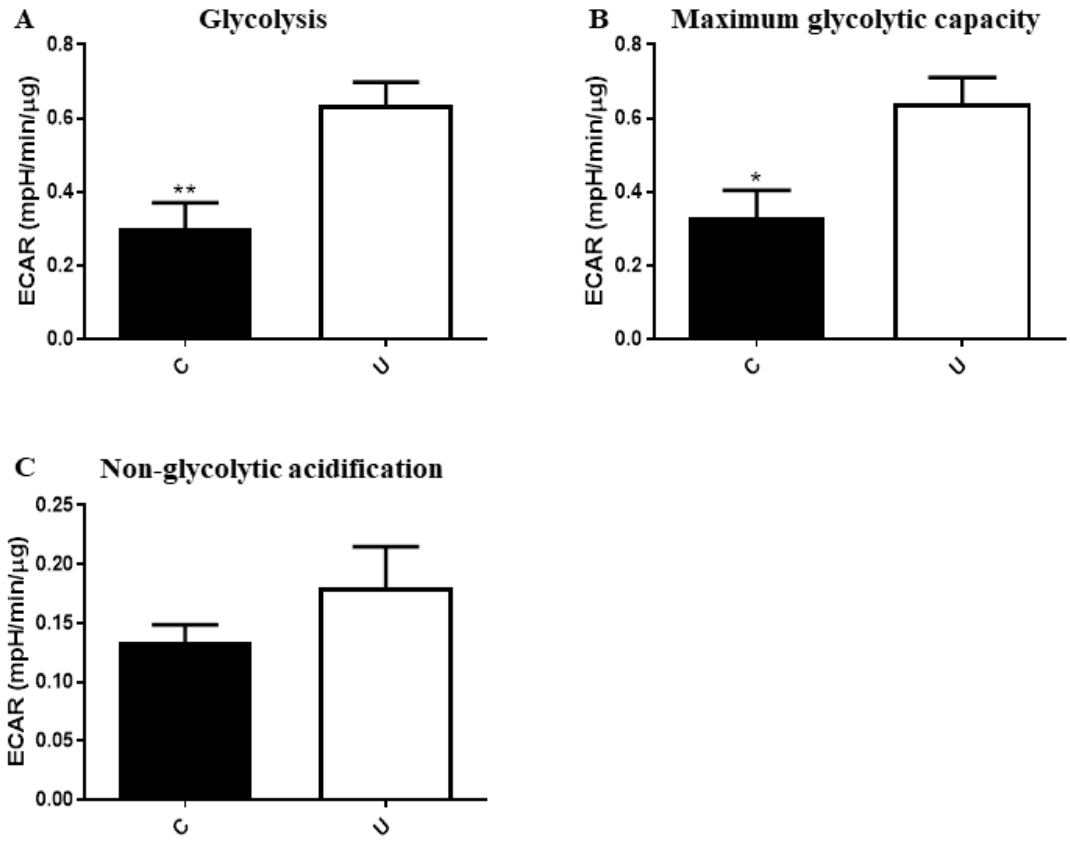


Figure 2.3: Increased glycolytic capacity in myotubes from *in utero* undernourished mice.

Extracellular acidification rate (ECAR) was measured to determine glycolysis (A), maximum glycolytic capacity (B), and non-glycolytic acidification (C) in myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars) in the presence of 10 mM glucose. Data are presented as mean ± SEM, n=7. Student's *t*-test, * $p<0.05$, ** $p<0.01$.

2.5.4 MITOCHONDRIAL CONTENT AND AMPK PHOSPHORYLATION PROTEIN LEVELS ARE UNALTERED

Given the metabolic differences observed, we assessed protein levels of mitochondrial complexes I-V as a marker of mitochondrial content and we compared U and C cultured in standard conditions in addition to restricted conditions (glucose- and glutamine- free medium). There were no differences in the protein levels of any of these mitochondrial complexes between the different groups (Figure 2.4A and 2.4C). Furthermore, we assessed protein levels of AMP-activated protein kinase (AMPK) and its phosphorylated form (pAMPK). Both individual protein levels (data not shown) and their ratio were not different between the groups (Figure 2.4B and 2.4 D).

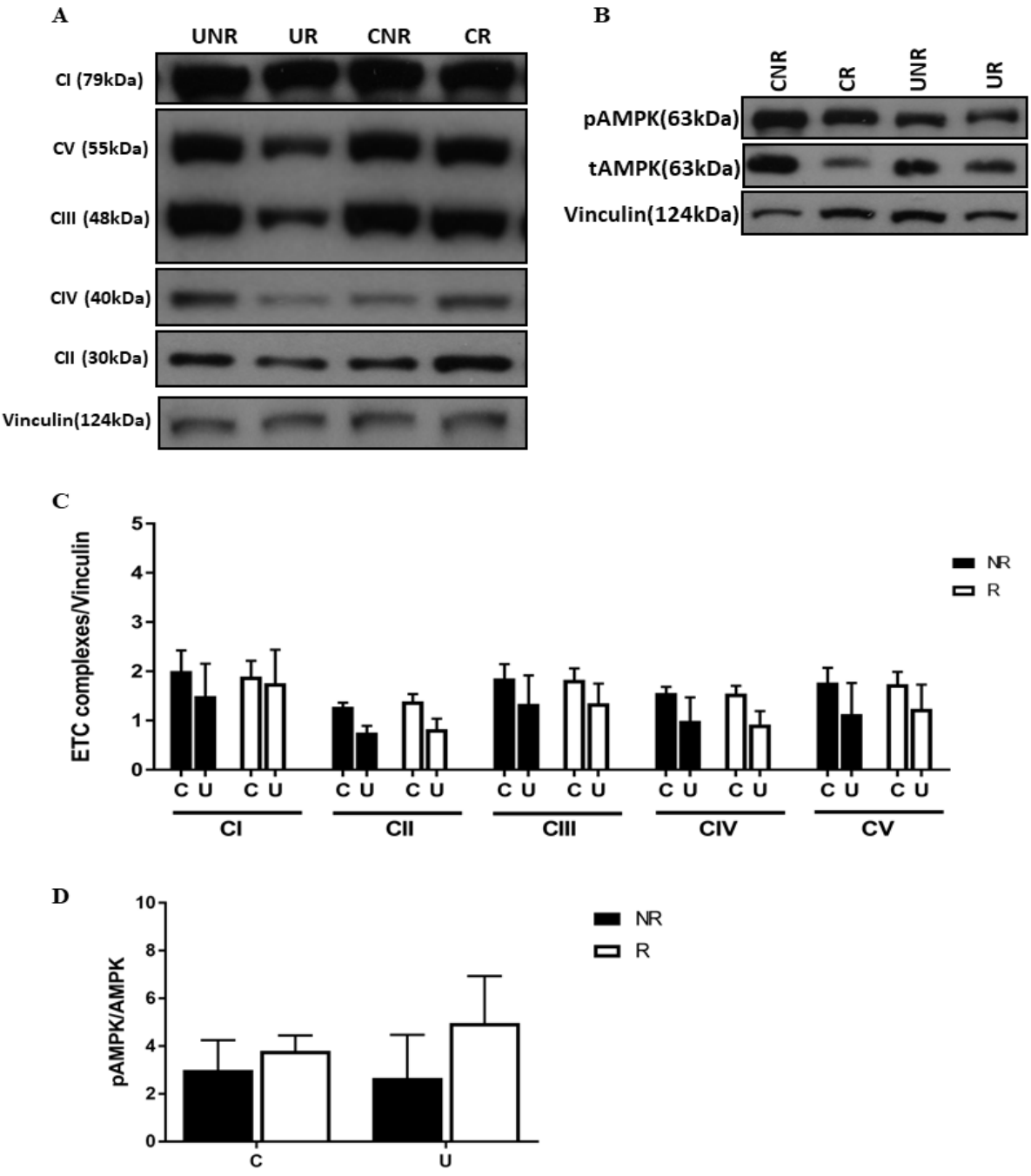

A
UNR
UR
CNR
CR
CI (79kDa)
CV (55kDa)
CIII (48kDa)
CIV (40kDa)
CII (30kDa)
Vinculin(124kDa)
B
CNR
CR
UNR
UR
pAMPK(63kDa)
tAMPK(63kDa)
Vinculin(124kDa)
C
ETC complexes/Vinculin
NR
R
C U C U
CI
CII
CIII
CIV
CV
D
pAMPK/AMPK
NR
R
C
U

Figure 2.4: Unchanged mitochondrial content and AMPK protein levels.

Western blot representatives showing protein expression of: (A) mitochondrial complexes (CI-CV) and (B) AMPK and pAMPK. (C) and (D) show protein quantification of (A) and (B) respectively. Data are presented as mean ± SEM, n=3-5. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$.

2.5.5 GENE SET ENRICHMENT ANALYSIS

A selection of representative gene sets found to be strongly enriched in the different comparisons was summarised in Tables 1-4. Gene expression analyses showed that genes implicated in oxidative phosphorylation (*e.g.* CI-CV subunits) were upregulated when C were grown in glucose-poor medium (Figure 2.5A and 2.5B). In the case of U, glucose deprivation was associated with an activation of the unfolded protein response (*e.g.* CCL2, BAX), as evidenced by a strong enrichment for the Gene Ontology category "Response to topologically incorrect proteins" (Figure 2.5C and 2.5D). Under conditions of glucose deprivation, cells from undernourished mice had increased expression of a group of genes (*e.g.* HSPA1A, SLC7A1) that are downregulated in multiple myeloma cells when treated with a DNA hypomethylating agent (Figure 2.6A and 2.6B). Under normal glucose culture conditions, U cells expressed less of a group of genes (*e.g.* LOX, WNT5A) normally downregulated during adipogenic differentiation of 3T3-L1 cells (Figure 2.6C and 2.6 D).

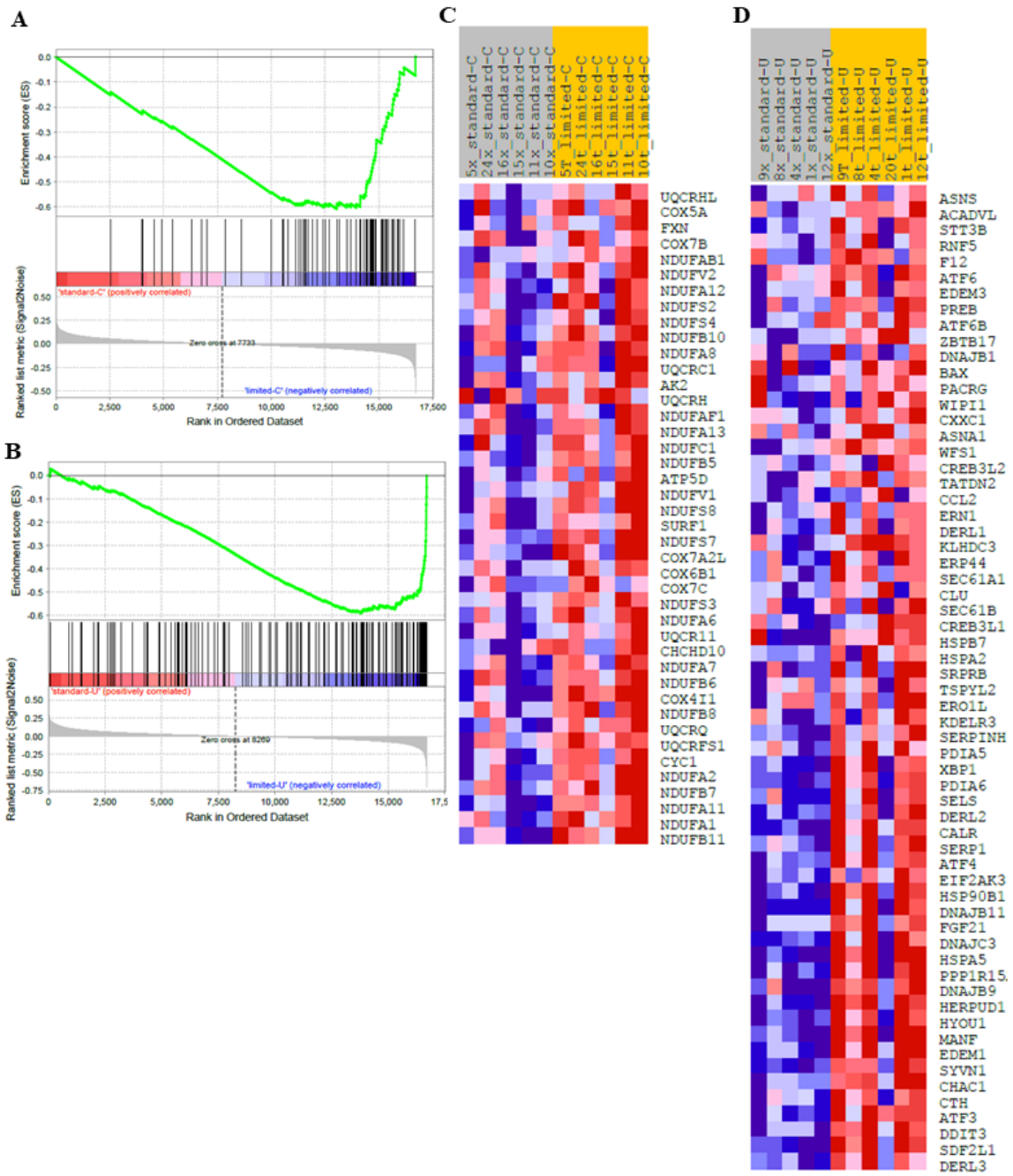

A
B
C
D
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'standard-C' (positively correlated)
'limited-C' (negatively correlated)
Zero cross at 7733
Rank in Ordered Dataset
'standard-U' (positively correlated)
'limited-U' (negatively correlated)
Zero cross at 8269
5x_standard-C
24x_standard-C
16x_standard-C
15x_standard-C
11x_standard-C
10x_standard-C
5t_limited-C
24t_limited-C
16t_limited-C
15t_limited-C
11t_limited-C
10t_limited-C
UQCRHL
COX5A
FXN
COX7B
NDUFAB1
NDUFV2
NDUFA12
NDUFS2
NDUFS4
NDUFB10
NDUFA8
UQCRC1
AK2
UQCRH
NDUFAF1
NDUFA13
NDUFC1
NDUFB5
ATP5D
NDUFV1
NDUFS8
SURF1
NDUFS7
COX7A2L
COX6B1
COX7C
NDUFS3
NDUFA6
UQCR11
CHCHD10
NDUFA7
NDUFB6
COX4I1
NDUFB8
UQCRQ
UQCRFS1
CYC1
NDUFA2
NDUFB7
NDUFA11
NDUFA1
NDUFB11
9x_standard-U
8x_standard-U
4x_standard-U
1x_standard-U
12x_standard-U
9t_limited-U
8t_limited-U
4t_limited-U
20t_limited-U
1t_limited-U
12t_limited-U
ASNS
ACADVL
STT3B
RNF5
F12
ATF6
EDEM3
PREB
ATF6B
ZBTB17
DNAJB1
BAX
PACRG
WIPI1
CXXC1
ASNA1
WFS1
CREB3L2
TATDN2
CCL2
ERN1
DERL1
KLHDC3
ERP44
SEC61A1
CLU
SEC61B
CREB3L1
HSPB7
HSPA2
SRPRB
TSPYL2
ERO1L
KDELR3
SERPINH
PDIA5
XBP1
PDIA6
SELS
DERL2
CALR
SERP1
ATF4
EIF2AK3
HSP90B1
DNAJB11
FGF21
DNAJC3
HSPA5
PPP1R15
DNAJB9
HERPUD1
HYOU1
MANF
EDEM1
SYVN1
CHAC1
CTH
ATF3
DDIT3
SDF2L1
DERL3

Figure 2.5: GSEA results for the comparison of:

(A-C) GO_OXIDATIVE_PHOSPHORYLATION gene set in cells from control offspring cultured in standard vs limited medium and
(B-D) GO_RESPONSE_TO_TOPOLOGICALLY_INCORRECT_PROTEIN gene set in cells from undernourished offspring cultured in standard vs limited medium.
The right panel shows the enrichment plot, with the green curve indicating the running-sum enrichment score along the list of genes ranked by decreasing expression in the corresponding condition. The left panel show a heatmap of the genes belonging to the leading edge, defined as the ranked genes appearing in the list before the point where the running-sum enrichment score deviates the most from zero. The heatmap colors are based on each gene's mean, minimum and maximum expression values across samples with blue meaning lower and red meaning higher than the mean.

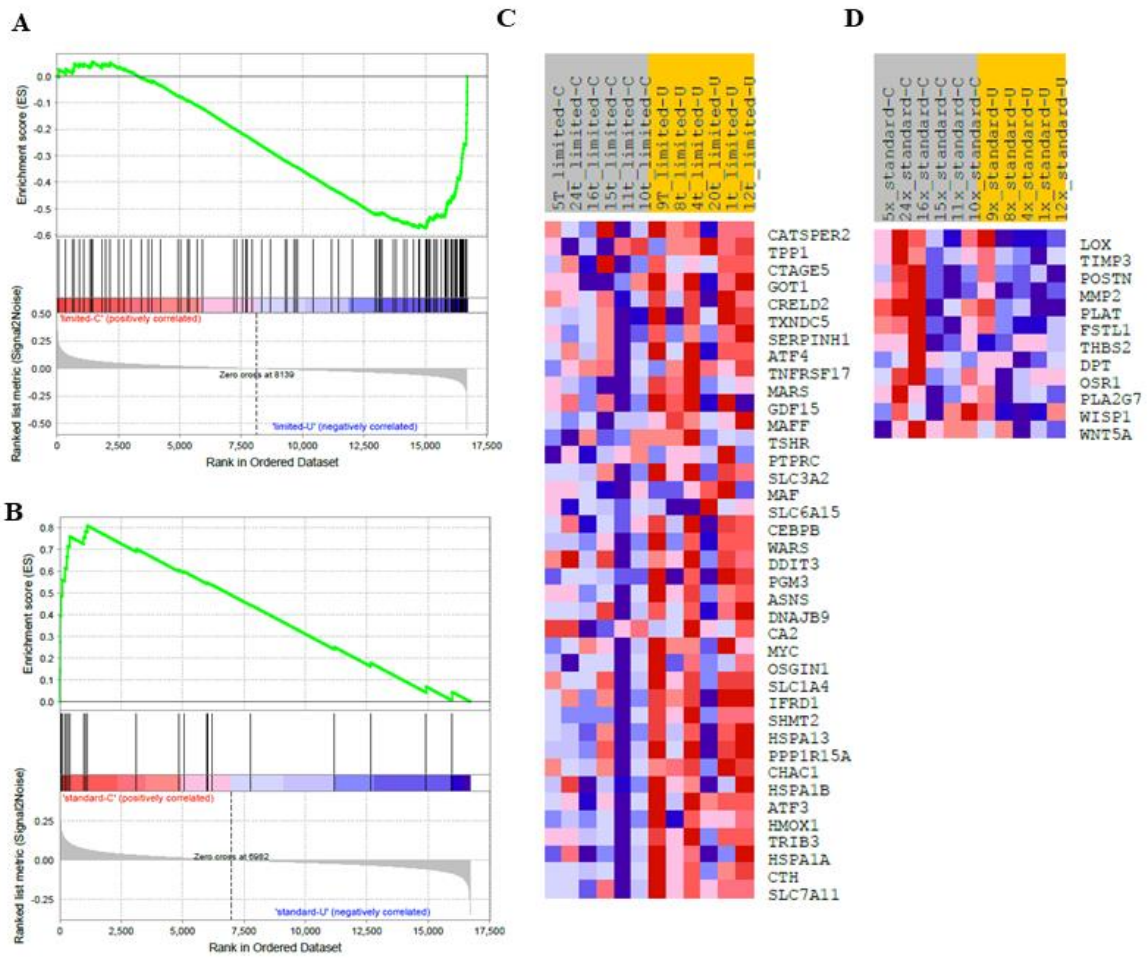


Figure 2.6: GSEA results for the comparison of:

(A-C) HELLER_SILENCED_BY_METHYLATION_DN gene set in cells from control and undernourished offspring cultured in limited medium and
(B-D) STEGER_ADIPOGENESIS_DN gene set in cells from control and undernourished offspring cultured in standard medium.

Table 2.1: A selection of representative gene sets found to be strongly enriched in Standard C vs Limited C. (NES: Normalized enrichment score; FDR: False discovery rate <0.05).

	Standard C vs Limited C		
	Gene set	**NES**	**FDR**
Upregulated in Standard C	**HORTON_SREBF_TARGETS**	2.56	0.000
	SCHMIDT_POR_TARGETS_IN_LIMB_BUD_UP	2.46	0.000
	REICHERT_MITOSIS_LIN9_TARGETS	2.44	0.000
	GSE7509_UNSTIM_VS_FCGRIIB_STIM_MONOCYTE_DN	2.32	0.002
	FARMER_BREAST_CANCER_CLUSTER_2	2.31	0.002
Upregulated in Limited C	**GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY**	2.34	0.000
	GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_I_BIOGENESIS	2.33	0.000
	REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	2.26	0.000
	GO_MITOCHONDRIAL_TRANSLATION	2.26	0.000
	GO_OXIDATIVE_PHOSPHORYLATION	2.23	0.000

Table 2.2: A selection of representative gene sets found to be strongly enriched in Limited C vs Limited U. (NES: Normalized enrichment score; FDR: False discovery rate <0.05).

	Limited C vs Limited U		
	Gene set	**NES**	**FDR**
Upregulated in Limited C	**ANASTASSIOU_MULTICANCER_INVASIVENESS_SIGNATURE**	2.70	0.000
	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	2.49	0.000
	CROMER_TUMORIGENESIS_UP	2.46	0.000
	STEGER_ADIPOGENESIS_DN	2.30	0.000
	GO_EXTRACELLULAR_MATRIX_DISASSEMBLY	2.11	0.004
Upregulated in Limited U	**KRIGE_AMINO_ACID_DEPRIVATION**	2.61	0.000
	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	2.53	0.000
	MARCINIAK_ER_STRESS_RESPONSE_VIA_CHOP	2.42	0.000
	GO_CELLULAR_RESPONSE_TO_TOPOLOGICALLY_INCORRECT_PROTEIN	2.34	0.000
	HELLER_SILENCED_BY_METHYLATION_DN	2.31	0.000

Table 2.3: A selection of representative gene sets found to be strongly enriched in Standard U vs Limited U. (NES: Normalized enrichment score; FDR: False discovery rate <0.05).

	Standard U vs Limited U		
	Gene set	**NES**	**FDR**
Upregulated in Standard U	**ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER**	2.54	0.000
	ODONNELL_TFRC_TARGETS_DN	2.54	0.000
	LEE_EARLY_T_LYMPHOCYTE_UP	2.52	0.000
	CHANG_CYCLING_GENES	2.49	0.000
	GSE15750_DAY6_VS_DAY10_EFF_CD8_TCELL_UP	2.49	0.000
Downregulated in Limited U	**GO_CELLULAR_RESPONSE_TO_TOPOLOGICALLY_INCORRECT_PROTEIN**	-2.49	0.000
	HELLER_SILENCED_BY_METHYLATION_DN	-2.48	0.000
	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	-2.42	0.000
	PACHER_TARGETS_OF_IGF1_AND_IGF2_UP	-2.36	0.000
	REACTOME_UNFOLDED_PROTEIN_RESPONSE	-2.35	0.000

Table 2.4: A selection of representative gene sets found to be strongly enriched in Standard C vs Standard U. (NES: Normalized enrichment score; FDR: False discovery rate <0.05).

	Standard C vs Standard U		
	Gene set	**NES**	**FDR**
Upregulated in Standard C	**ANASTASSIOU_MULTICANCER_INVASIVENESS_SIGNATURE**	3.00	0.000
	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	2.79	0.000
	STEGER_ADIPOGENESIS_DN	2.53	0.000
	GO_MULTICELLULAR_ORGANISMAL_MACROMOLECULE_METABOLIC_PROCESS	2.49	0.000
	URS_ADIPOCYTE_DIFFERENTIATION_DN	2.43	0.000
Downregulated in Standard U	**ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER**	-3.00	0.000
	GSE15750_DAY6_VS_DAY10_TRAF6KO_EFF_CD8_TCELL_UP	-2.75	0.000
	SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	-2.74	0.000
	CHANG_CYCLING_GENES	-2.70	0.000
	KONG_E2F3_TARGETS	-2.69	0.000

2.6 DISCUSSION

In this study, we provide the first evidence that transcriptional and functional characteristics of muscle cells established from low birth weight mice have 'programmed' metabolic defects. We and others have previously reported on the increased risk of metabolic disease, including obesity and type 2 diabetes, associated with low birth weight in mice [19]. Here we show pronounced differences in transcriptional profiles, indicating that *in utero* undernutrition can impact primary cells potentially affecting their metabolic behaviour. These findings are consistent with the observed dysfunctional mitochondrial oxidative characteristics, and thus an enhanced reliance on glycolytic processes. Our findings are thereby consistent with previous observations of impaired oxidative metabolism in human populations and in mouse models of low birth weight [19] suggesting that this impairment is likely associated with cell autonomous mechanisms.

Previously, we have shown that adult mouse offspring that were undernourished *in utero* have decreased skeletal muscle mitochondrial respiration and decreased respiration as assessed in permeabilized muscle fibers [19]. This current research confirms these previous observations and extends them by showing that the decreased respiration in skeletal muscle is a primary defect in muscle, *i.e.*, as opposed to a secondary effect of metabolic dysfunctions in other tissues or systemically. Studies have repeatedly shown that skeletal muscle oxidative activity is decreased in individuals with obesity and type 2 diabetes [15, 16, 28, 29], but the etiological role of mitochondrial function in disordered muscle metabolism and insulin resistance is debated [40]. Nevertheless, decreased oxidative capacity combined with an increased glycolytic capacity has been found in humans with obesity and type 2 diabetes

with changes in the proportions of skeletal muscle fiber types [15, 30-35]. Furthermore, an increased ratio of glycolytic to oxidative enzymes has been shown to contribute to insulin resistance in skeletal muscle. Activities of skeletal muscle oxidative enzymes have been found to be decreased and activities of glycolytic enzymes increased in obesity and type 2 diabetes [30]. It seems counterintuitive that there would be an increase in glycolytic capacity and a decrease in oxidative capacity in obesity and insulin resistance, situations in which there are typically higher circulating levels of fatty acids, and impaired muscle uptake of glucose. However, in situations of mitochondrial energetic dysfunction cells need to rely on glycolytic supplies of ATP to meet cellular energy demands.

We observed decreased mitochondrial respiration in primary myotubes from U compared to C under resting conditions when provided media containing glucose. When myotubes were forced to use palmitate as a substrate, respiration in myotubes from U was decreased compared to C under resting and maximal uncoupled conditions consistent with oxidative dysfunction and dysfunctional fatty acid oxidation. We then assessed the contribution of exogenous and endogenous fatty acid oxidation to further understand the observed differences. Results suggest that the decrease in fatty acid oxidation under resting conditions in myotubes from U is due to a decreased capacity to oxidize exogenous fatty acids. In contrast under maximal uncoupled conditions, there was impaired fatty acid oxidation in myotubes from U, and this was associated with lower fatty acid oxidation in the presence of etomoxir. This suggests that, although myotubes from U have the ability to use similar levels of exogenous fatty acids, under resting conditions they oxidize less. This study was limited to the effect of a specific fatty acid, palmitate. Palmitate was chosen since it is the most

abundant dietary saturated fatty acid and has been shown to impair insulin signaling in muscle cells [36]. However, other fatty acids may have different effects on metabolism. Further studies are needed to determine the specific molecular defects in fatty acid oxidation in U since decreased fatty acid oxidative capacity has been shown to be associated with obesity and insulin resistance [17, 18, 37] and since lipid oxidation is reduced in myotubes from type 2 diabetic patients [38]. Overall, findings are consistent with the conclusion that myotubes established from U inherited 'programmed' defects in fatty acid metabolism that contribute to the development of obesity and insulin resistance in adulthood.

We hypothesized that the metabolic/functional defects were due to decreased mitochondrial content since we previously found decreased mitochondrial content in muscle of adult U compared to C mice [19]. In contrast, in the primary cells studied herein, there were no differences in mitochondrial ETC content between U and C primary myotubes studied under substrate restricted cell culture conditions or not. Therefore, our findings indicate that the observed energetic differences in the myotubes are due to altered mitochondrial function, rather than content. We also assessed protein levels of AMPK and its activated form pAMPK, given this protein's central role in cellular energy homeostasis. However, there were no differences between groups.

To determine if the observed functional defects were due to altered gene expression in the muscle cells, we assessed gene expression using microarray. Gene set enrichment results demonstrate a strong impact of *in utero* undernutrition on gene expression in offspring primary cells. Genes involved in oxidative phosphorylation were upregulated when cells from C were cultured in low glucose medium, potentially as a compensatory response for a

decrease in glucose-derived energy production via glycolysis. On the other hand, cells from U responded to low availability of glucose by encoding genes involved in the response to unfolded or misfolded proteins and this was expected given the well-known association between glucose deprivation, N-linked glycosylation defects and accumulation of misfolded proteins [39]. Furthermore and under the same condition of glucose deprivation, genes previously shown to be downregulated after treatment with a hypomethylating agent were higher in expression in U myotubes. These effects could be an energy saving mode or stress mode that these cells apply when facing low availability of essential nutrients like glucose. In normal culture conditions, U cells when compared to C have less genes downregulated during adipogenesis in the well characterized cell line 3T3-1L. This suggests that the process of storing energy as fat in the myotubes from U is put on hold.

In summary, our findings demonstrate that muscle satellite cell-derived myotubes from low birth weight mice have inherited defects in gene transcription and in the metabolism of glucose and fatty acids. Findings are consistent overall with the hypothesis that impaired mitochondrial function in muscle of U precedes the onset of metabolic disease associated with obesity and type 2 diabetes. The altered muscle metabolism may be a compensatory mechanism programmed *in utero* to handle times of limited nutrient availability that becomes detrimental in adult life when nutrients are abundant.

2.7 REFERENCES

1. Gluckman, P.D., Hanson, M.A., Cooper, C., and Thornburg, K.L. (2008). Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med. *359*, 61–73.

2. Barker, D.J., Osmond, C., Golding, J., Kuh, D., and Wadsworth, M.E. (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ *298*, 564–567.

3. Barker, D.J.P. (2004). The developmental origins of adult disease. J Am Coll Nutr *23*, 588S–595S.

4. Luquet, S., Lopez-Soriano, J., Holst, D., Fredenrich, A., Melki, J., Rassoulzadegan, M., and Grimaldi, P.A. (2003). Peroxisome proliferator-activated receptor delta controls muscle development and oxidative capability. FASEB J. *17*, 2299–2301.

5. Flück, M., and Hoppeler, H. (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. Rev. Physiol. Biochem. Pharmacol. *146*, 159–216.

6. Rolfe, D.F., and Brown, G.C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. *77*, 731–758.

7. Zurlo, F., Larson, K., Bogardus, C., and Ravussin, E. (1990). Skeletal muscle metabolism is a major determinant of resting energy expenditure. J. Clin. Invest. *86*, 1423–1427.

8. DeFronzo, R.A., Gunnarsson, R., Björkman, O., Olsson, M., and Wahren, J. (1985). Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J. Clin. Invest. *76*, 149–155.

9. Wells, J.C.K., Chomtho, S., and Fewtrell, M.S. (2007). Programming of body composition by early growth and nutrition. Proc Nutr Soc *66*, 423–434.

10. Kensara, O.A., Wootton, S.A., Phillips, D.I., Patel, M., Jackson, A.A., Elia, M., and Hertfordshire Study Group (2005). Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen. Am. J. Clin. Nutr. *82*, 980–987.

11. Hediger, M.L., Overpeck, M.D., Kuczmarski, R.J., McGlynn, A., Maurer, K.R., and Davis, W.W. (1998). Muscularity and fatness of infants and young children born small- or large-for-gestational-age. Pediatrics *102*, E60.

12. Jimenez-Chillaron, J.C., Hernandez-Valencia, M., Reamer, C., Fisher, S., Joszi, A., Hirshman, M., Oge, A., Walrond, S., Przybyla, R., Boozer, C., et al. (2005). Beta-cell secretory dysfunction in the pathogenesis of low birth weight-associated diabetes: a murine model. Diabetes *54*, 702–711.

13. Jensen, C.B., Storgaard, H., Madsbad, S., Richter, E.A., and Vaag, A.A. (2007). Altered skeletal muscle fiber composition and size precede whole-body insulin resistance in young men with low birth weight. J. Clin. Endocrinol. Metab. *92*, 1530–1534.

14. Kelley, D.E., He, J., Menshikova, E.V., and Ritov, V.B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes *51*, 2944–2950.

15. Oberbach, A., Bossenz, Y., Lehmann, S., Niebauer, J., Adams, V., Paschke, R., Schön, M.R., Blüher, M., and Punkt, K. (2006). Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. Diabetes Care *29*, 895–900.

16. Mogensen, M., Sahlin, K., Fernström, M., Glintborg, D., Vind, B.F., Beck-Nielsen, H., and Højlund, K. (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes *56*, 1592–1599.

17. Kelley, D.E., and Simoneau, J.A. (1994). Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. J. Clin. Invest. *94*, 2349–2356.

18. Kim, J.Y., Hickner, R.C., Cortright, R.L., Dohm, G.L., and Houmard, J.A. (2000). Lipid oxidation is reduced in obese human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *279*, E1039-1044/

19. Beauchamp, B., Ghosh, S., Dysart, M.W., Kanaan, G.N., Chu, A., Blais, A., Rajamanickam, K., Tsai, E.C., Patti, M.-E., and Harper, M.-E. (2015). Low birth weight is associated with adiposity, impaired skeletal muscle energetics and weight loss resistance in mice. Int J Obes (Lond) *39*, 702–711.

20. Woo, M., Isganaitis, E., Cerletti, M., Fitzpatrick, C., Wagers, A.J., Jimenez-Chillaron, J., and Patti, M.E. (2011). Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair. Stem Cells Dev. *20*, 1763–1769.

21. Mailloux, R.J., Seifert, E.L., Bouillaud, F., Aguer, C., Collins, S., and Harper, M.-E. (2011). Glutathionylation acts as a control switch for uncoupling proteins UCP2 and UCP3. J. Biol. Chem. *286*, 21865–21875.

22. Aguer, C., Mercier, J., Man, C.Y.W., Metz, L., Bordenave, S., Lambert, K., Jean, E., Lantier, L., Bounoua, L., Brun, J.F., et al. (2010). Intramyocellular lipid accumulation is associated with permanent relocation ex vivo and in vitro of fatty acid translocase (FAT)/CD36 in obese patients. Diabetologia *53*, 1151–1163.

23. Carvalho, B.S., and Irizarry, R.A. (2010). A framework for oligonucleotide microarray preprocessing. Bioinforma. Oxf. Engl. *26*, 2363–2367.

24. Mootha, V.K., Lindgren, C.M., Eriksson, K.-F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., et al. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. *34*, 267–273.

25. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. *102*, 15545–15550.

26. Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. *1*, 417–425.

27. Turnbull, D.M., Bartlett, K., Younan, S.I., and Sherratt, H.S. (1984). The effects of 2[5(4-chlorophenyl)pentyl]oxirane-2-carbonyl-Co-A on mitochondrial oxidations. Biochem. Pharmacol. *33*, 475–481.

28. He, J., Watkins, S., and Kelley, D.E. (2001). Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. Diabetes *50*, 817–823.

29. Bruce, C.R., Anderson, M.J., Carey, A.L., Newman, D.G., Bonen, A., Kriketos, A.D., Cooney, G.J., and Hawley, J.A. (2003). Muscle oxidative capacity is a better predictor of insulin sensitivity than lipid status. J. Clin. Endocrinol. Metab. *88*, 5444–5451.

30. Simoneau, J.A., and Kelley, D.E. (1997). Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. J. Appl. Physiol. *83*, 166–171.

31. Simoneau, J.A., Colberg, S.R., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. FASEB J. *9*, 273–278.

32. Gaster, M., Staehr, P., Beck-Nielsen, H., Schrøder, H.D., and Handberg, A. (2001). GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 fiber disease? Diabetes *50*, 1324–1329.

33. Lillioja, S., Young, A.A., Culter, C.L., Ivy, J.L., Abbott, W.G., Zawadzki, J.K., Yki-Järvinen, H., Christin, L., Secomb, T.W., and Bogardus, C. (1987). Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. J. Clin. Invest. *80*, 415–424.

34. Mårin, P., Andersson, B., Krotkiewski, M., and Björntorp, P. (1994). Muscle fiber composition and capillary density in women and men with NIDDM. Diabetes Care *17*, 382–386.

35. Hickey, M.S., Carey, J.O., Azevedo, J.L., Houmard, J.A., Pories, W.J., Israel, R.G., and Dohm, G.L. (1995). Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. Am. J. Physiol. *268*, E453-457.

36. Dimopoulos, N., Watson, M., Sakamoto, K., and Hundal, H.S. (2006). Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. Biochem. J. *399*, 473–481.

37. Kelley, D.E., Goodpaster, B., Wing, R.R., and Simoneau, J.A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. Am. J. Physiol. *277*, E1130-1141.

38. Gaster, M., Rustan, A.C., Aas, V., and Beck-Nielsen, H. (2004). Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. Diabetes *53*, 542–548.

39. Xu, C., Bailly-Maitre, B., and Reed, J.C. (2005). Endoplasmic reticulum stress: cell life and death decisions. J. Clin. Invest. *115*, 2656–2664.

CHAPTER 3

Glutaredoxin-2 Controls Cardiac Mitochondrial Dynamics and Energetics in Mice, and Protects Against Human Cardiac Pathologies

Georges N. Kanaan[1], Bianca Ichim[1], Lara Gharibeh[1], Wael Maharsy[1], David Patten[1], Jian Ying Xuan[1], Arkadiy Reunov[2], Philip Marshall[3], John Veinot[2,4,5], Keir Menzies[1,3], Mona Nemer[1] and Mary-Ellen Harper[1*]

[1]Department of Biochemistry, Microbiology and Immunology, and Ottawa Institute of Systems Biology, Faculty of Medicine, 451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5;
[2]Ottawa Heart Institute, University of Ottawa, 40 Ruskin Street, Ottawa, ON Canada, K1Y 4W7;
[3]Interdisciplinary School of Health Sciences, University of Ottawa, Faculty of Health Sciences, 451 Smyth Road, Ottawa, ON Canada, K1H 8M5;
[4]The Ottawa Hospital, 501 Smyth Road, Ottawa Canada, ON K1H8L6; and
[5]Department of Pathology and Laboratory Medicine, and University of Ottawa, Faculty of Medicine, 451 Smyth Road, Ottawa, ON Canada, K1H 8M5.

[*] **To whom correspondence should be addressed:**
Dr. Mary-Ellen Harper, PhD
Professor
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine, University of Ottawa
451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5
Email: mharper@uottawa.ca
Tel: +1-613-562-5800 Ext 8235

3.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

3.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript "Glutaredoxin-2 Controls Cardiac Mitochondrial Dynamics and Energetics in Mice, and Protects Against Human Cardiac Pathologies" has been accepted for publication in the journal *Redox Biology*. PMID: 29101900

Redox Biology (14), April 2018, In Press. https://doi.org/10.1016/j.redox.2017.10.019

3.1.2 CONTRIBUTION STATEMENT

GNK and MEH conceived the idea for the project. GNK conducted most of the experiments and analyzed most of the results. MN conceived and coordinated the neonatal cardiomyocyte isolations and the echocardiographic determinations while LG and WM conducted the analyses. AR prepared samples for electron microscopy and collected the micrographs. BI helped in fibrosis analysis and Seahorse bioenergetics analyses of cardiomyocytes. DP helped with HPLC analyses. JYX helped with animal handling and genotyping. PM and KM acquired the human data from GTEx consortium and analyzed the data. JV conducted the histopathological analysis of the human left ventricles. GNK and MEH wrote the paper and all authors reviewed and approved it.

3.1.3 ACKNOWLEDGMENTS AND FUNDING

Authors are grateful for the help of the members of the University of Ottawa Pathology Laboratory in for their histological work. This research was supported by the Canadian

Institute of Health Research (FDN 143278) and by Heart and Stroke Foundation of Canada (NA7301). Georges N. Kanaan was supported by the University of Ottawa PhD full admission scholarship.

3.1.4 CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

3.2 ABSTRACT

Glutaredoxin 2 (GRX2), a mitochondrial glutathione-dependent oxidoreductase, is central to glutathione homeostasis and mitochondrial redox, which is crucial in highly metabolic tissues like the heart. Previous research showed that absence of Grx2, leads to impaired mitochondrial complex I function, hypertension and cardiac hypertrophy in mice but the impact on mitochondrial structure and function in intact cardiomyocytes and in humans has not been explored. We hypothesized that Grx2 controls cardiac mitochondrial dynamics and function in cellular and mouse models, and that low expression is associated with human cardiac dysfunction. Here we show that Grx2 absence impairs mitochondrial fusion, ultrastructure and energetics in primary cardiomyocytes and cardiac tissue. Moreover, provision of the glutathione precursor, N-acetylcysteine (NAC) to *Grx2-/-* mice did not restore glutathione redox or prevent impairments. Analysis of genetic and histopathological data from the human Genotype-Tissue Expression consortium we demonstrate that low GRX2 is associated with fibrosis, hypertrophy, and infarct in the left ventricle. Altogether, GRX2 is important in the control of cardiac mitochondrial structure and function, and protects against human cardiac pathologies.

Keywords: Human heart; mitochondria; oxidative stress; redox; cardiac metabolism; cardiac hypertrophy.

3.3 INTRODUCTION

Disordered cellular redox underlies the development of many chronic diseases and fundamental processes of aging. Cellular redox balance is maintained through the coordinated regulation of oxidation and reduction processes, and, in turn, plays essential roles in the control of fuel oxidation processes and oxidative stress [1]. During mitochondrial oxidative phosphorylation process, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can overwhelm the antioxidant systems and cause oxidative stress [2, 19]. While excessive reactive species levels are detrimental, low levels play key roles in processes such as cellular signaling and protection against infectious agents [3-5]. Within the battery of cellular antioxidant systems, glutathione, which is central to redox homeostasis, is thought to be the most important non-protein antioxidant within cells [6, 7, 19].

The glutaredoxin (GRX) enzymes are glutathione-dependent oxidoreductases and can protect proteins from oxidative damage [9, 10]. Glutaredoxin 2 (Grx2) is expressed in mitochondria of many cell types including cardiomyocytes. We and others previously demonstrated that *Grx2-/-* mice exhibit fibrotic cardiac hypertrophy, hypertension and early onset age-dependent cataract formation [11, 42]. However, until now Grx2 implications for human cardiac diseases have been unaddressed. With regard to underlying mechanisms, previous work in isolated cardiac mitochondria, showed that the absence of Grx2 was associated with impaired mitochondrial complex I activity [11]. However, no studies, to-date have assessed the impact of Grx2 deletion at the levels of the intact cell and tissue. Since it is now widely recognised that mitochondria exist in dynamic reticular structures in cells,

analyses of mitochondrial function should be conducted, as much as possible, in intact cells and tissues.

Indeed, recent research has demonstrated that mitochondrial fusion is controlled by glutathione redox; specifically, increased levels of oxidized glutathione were shown to increase mitochondrial fusion [8]. Our previous work showed high levels of oxidized glutathione in isolated mitochondria from the ventricular cardiac muscle [11] but the repercussions for mitochondrial fusion and ultrastructure remained unknown. Furthermore, the possible implications of glutathione redox for mitochondrial fusion in non-transformed cells and *in vivo* have not been elucidated. Thus the overall aims of this research were to address: 1) the impact of Grx2 deficiency on mitochondrial structure and function in intact cellular systems, 2) the effect of *in vivo* supplementation of the glutathione precursor, N-acetylcysteine (NAC), and 3) the role of GRX2 in human heart in association with cardiac pathologies.

Here we report that GRX2 absence results in disordered cardiac mitochondrial ultrastructure, a hyperfused mitochondrial reticulum and impaired oxidative and glycolytic capacities that are not reversed by NAC. The *in vivo* functional and structural abnormalities, cardiac hypertrophy, fibrosis and hypertension cannot be resolved by NAC treatment. Finally, using publically available datasets from the human Genotype-Tissue Expression (GTEx) consortium, we demonstrate for the first time in humans that low levels of *GRX2* transcripts are associated with fibrosis, hypertrophy, and infarct in the left ventricle, thus demonstrating that this mitochondrial oxidoreductase is essential for heart health.

3.4 METHODS

3.4.1 ANIMALS

All experimental procedures involving mice were conducted according to the guidelines and principles of the Canadian Council of Animal Care and after the approval of the Animal Care Committee of the University of Ottawa. In this study, male C57BL/6 mice wild type (WT) and Grx2 whole body knock-out (*Grx2-/-*) were used. Grx2 knock-out was confirmed by PCR before experimentation. All mice were housed in an environment in which temperature, humidity and light cycles (06:00-18:00h) were controlled.

3.4.2 PRIMARY CARDIOMYOCYTE ISOLATION

Primary cultures of cardiomyocytes were prepared from 1-3 day old WT and *Grx2-/-* pups. Each preparation required 15-17 hearts, which were isolated and digested for 10 minutes, 3 to 4 times, in Joklik's modified Eagle's medium (M0518-10X1L; Sigma-Aldrich) containing 0.1% collagenase (C-2139; Sigma-Aldrich). Enzymatic digestion was stopped with fetal bovine serum (FBS; A12617DJ; Invitrogen), and the undigested tissue was removed by filtration through nylon mesh (pore size, 100μm). Cardiomyocytes were purified by two pre-platings of 30 min each to remove residual non-myocytes by differential adhesion. Cardiomyocytes were then plated at 120-135 × 10^4 cells/well in a 24-well plate. Cells were cultured for 16 to 24 h in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% FBS. The following day, the medium was exchanged for serum-free hormonally defined medium (SFHD).

3.4.3 BIOENERGETIC DETERMINATIONS OF PRIMARY CARDIOMYOCYTES

Neonatal cardiomyocytes were washed and SFHD media was replaced with Seahorse medium (bicarbonate-free DMEM, 5mM D-glucose, 4mM L-glutamine, 1mM sodium pyruvate; pH 7.4) and incubated in a non-CO_2 incubator at 37ºC for 30 minutes. The assay cartridge was hydrated with XF calibrant solution one day prior to experiment and left at 37ºC overnight. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were determined using a Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience, Agilent Technologies). Calibration was conducted prior to collection of data. Leak and maximal respiration were measured after injection of 2µM oligomycin and 1µM FCCP, respectively (Sigma-Aldrich). Non-mitochondrial respiration was measured after injection of 1µM antimycin A (Sigma-Aldrich). Following the experiment, cardiomyocytes were lysed with 50µL of 0.5M NaOH to conduct protein quantification determination (Bradford assay). Rates were normalized to protein content in each well.

3.4.4 *IN VIVO* NAC SUPPLEMENTATION STUDIES

Mice were fed *ad libitum* a standard diet (44.2% carbohydrate, 6.2% fat, 18.6% crude protein; diet T.2018, Harlan Teklad, Indianapolis). Mice were divided into 4 groups (6mice/group): WT untreated, *Grx2-/-* untreated, WT NAC treated and *Grx2-/-* NAC treated. NAC (Sigma-Aldrich, US) was administered to the mice in their drinking water in a dose of 1g/kg/day from 5–11 weeks of age. Previous work established that cardiac hypertrophy develops between 9-10 weeks of age [11]. Body weights and food intake were monitored

weekly and NAC dosage was adjusted accordingly. Experiments were performed at 12 weeks of age, when body weight was measured in addition to the weights of the hearts, kidneys, liver, interscapular brown adipose tissue, epididymal white adipose tissue and hindlimb muscles.

3.4.5 ECHOCARDIOGRAPHY ANALYSES

For echocardiography measurements, a VEVO 2100 system (Visual Sonics, Amsterdam) with a 30-MHz linear array transducer was used. Mice were anesthetized (2.0% isoflurane, 80 ml/min 100% O_2); their anterior chests were shaved and pre-warmed transmission gel was applied. Parasternal long-axis view, short-axis view, two dimensional guided M-mode and Pulsed-Wave Doppler (PWD) images were recorded. Images were analysed using the VEVO 2100 analysis software.

3.4.6 BLOOD PRESSURE DETERMINATIONS

The non-invasive blood pressure analyzer for mice BP-2000 Blood Pressure Analysis System (Visitech Systems; Apex, NC) was used to determine systolic and diastolic blood pressure. At 10 weeks of age, restrained mice were placed on the pre-warmed platform (30°C) and tails were inserted into the tail cuffs. Measurements were taken between 7:00 and 9:00 am daily for 5 consecutive days. At each session, mice were acclimatized to the system for 10 min before the 20 min measurement period.

3.4.7 ANALYSES OF CARDIAC MUSCLE MITOCHONDRIAL ULTRASTRUCTURE

Left ventricle fragments were dissected, and small pieces of the left ventricle were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.5) at 4 °C. Fixed pieces were washed in cacodylate buffer, post-fixed in 2% OsO_4 in 0.1M cacodylate buffer for 1 hour, rinsed in 0.1M cacodylate buffer and distilled water, dehydrated in an ethanol series and embedded in Spurr's resin. Resin blocks were sectioned using an ultramicrotome (EM UC6; Leica Microsystems, Canada) using a diamond knife. Ultra-thin sections were mounted on copper grids coated with formvar film. Sections were stained with 2% alcoholic uranyl acetate and Reynold's lead citrate. Stained sections were examined with a transmission electron microscope (JEOL 1230; JEOL Ltd., Tokyo). Morphometric analyses were completed on 178 images (39 images *Grx2+/+* untreated, 37 images *Grx2-/-* untreated, 42 images *Grx2+/+* NAC treated and 60 images *Grx2-/-* NAC treated). In total 6833 mitochondria were analyzed and classified. Irregular mitochondria were defined as weirdly branched, tortuous and non-ovular.

3.4.8 CARDIAC FIBROSIS ANALYSIS

Mouse hearts were placed in 10% formalin and then in 70% ethanol prior to paraffin embedding. At the vertical midpoint, 4 micron transverse sections were obtained and stained with Sirius Red, to stain Type 1 and Type 3 collagen fibers. Fibrosis was assessed using Imagescope software (Leica Biosystems) in which stained fibers were quantified and normalized to the tissue area.

3.4.9 CARDIAC AND HEPATIC GSH:GSSG DETERMINATIONS

After isolation, heart left ventricle and liver tissues were weighed and put directly into homogenization bead tubes containing 125mM sucrose, 5mM TRIS, 1.5mM EDTA, 0.5%TFA and 0.5%MPA in mobile phase. A MagNA lyser (Roche, USA) was used to homogenize the tissue. Then samples were spun at 14000xg at 4°C for 20min. Supernatants were collected and either analyzed directly using an Agilent HPLC system equipped with a Pursuit C_{18} column (150 ×4.6mm, 5μm; Agilent Technologies) operating at a flow rate of 1 mL/min or stored at -80°C for later analysis. The mobile phase consisted of 0.09% trifluoroacetic acid diluted in ddH2O and mixed with HPLC-grade methanol in a 90:10 ratio. Standard solutions were used to estimate the retention times for GSH and GSSG. Using Agilent Chemstation software, absolute amounts of GSH and GSSG were acquired by integrating the area under the corresponding peaks, and values were calculated from standard curves.

3.4.10 HIGH RESOLUTION RESPIROMETRY OF PERMEABILIZED CARDIAC MYOFIBERS

In separate cohorts of mice, the left ventricle was removed and fibers were permeabilized with 50μg/ml of saponin. Characteristics of mitochondrial respiration were determined in duplicate and at 37°C (0.5mM ethylene glycol tetraacetic acid, 3mM $MgCl_26H_2O$, 20mM taurine, 10mM KH_2PO_4, 20mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 110mM D-sucrose, 0.1% bovine serum albumin and 60mM lactobionic acid; pH 7.1) using the Oxygraph-2k (Oroboros, Austria). To assess adenylate-free leak respiration and complex

I driven respiration, malate (2mM), pyruvate (5mM) and glutamate (10mM) were added to the incubation medium followed by addition of adenosine diphosphate and Mg^{2+} (5mM). To assess the maximum oxidative phosphorylation capacity (Complex I and II), succinate (10mM) and ADP (5mM) were added. Leak supported respiration was assessed by adding oligomycin (2µg/ml). By adding complex III inhibitor antimycin A (2.5µM), non-mitochondrial oxygen consumption was determined. N,N,N′,N′-Tetramethyl-p-phenylenediamine (TMPD) (0.5mM), ascorbate (2mM) and sodium azide (15mM) were subsequently added to assess complex IV activity. All values were corrected for residual non-mitochondrial oxygen consumption.

3.4.11 MITOCHONDRIAL FUSION CHARACTERISTICS IN PRIMARY CARDIOMYOCYTES

Isolated neonatal cardiomyocytes were washed with PBS and fixed with 4% PFA for 20min. Cells were rinsed, then permeabilized in PBS with 0.1% Tween-20 for 30min at room temperature. An incubation of 30 min in PBS with 1% BSA and 0.1% Triton X-100 solution was used to block non-specific binding. Using the same buffer, cells were incubated with anti-Tom20 (Santa Cruz 11415) and with Oregon green 488 antibodies (Thermo Fisher O-11038) for 1 hour each at room temperature. Nuclear staining was performed with Hoechst (Thermo Fisher H1399). The concentration of the antibodies was 1:100. Cells were imaged using Zeiss AxioImager M2 microscope (Carl Zeiss, USA). Mitochondrial length was assessed using Image J software (NIH, USA).

3.4.12 IDENTIFICATION OF TRANSCRIPT CORRELATIONS IN GTEX HUMAN TISSUE DATA SETS

Human left ventricle heart microarray data (Affymetrix Human Gene 1.1 ST Array) were analyzed for correlations between *GRX2* (*GLRX2*) transcript expression and mitochondrial-associated genes using the GeneNetwork program. Raw microarray data are also publicly available on Gene Expression Omnibus (GEO; http: //www.ncbi.nlm. nih.gov/geo) under the accession number GSE45878 (GTEx, 2015) and on GeneNetwork (www.genenetwork.org).

3.4.13 STATISTICAL ANALYSES

All data are represented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Prism, La Jolla, CA, USA). Data were analyzed by two-way repeated measures analysis of variance (ANOVA) with Bonferroni or Tukey post-hoc tests, as indicated. $P<0.05$ was considered significant.

3.4.14 DATA AVAILABILITY

GTEx expression data for *GRX2* (*GLRX2*) is available here: http://www.gtexportal.org/home/gene/GLRX2. GTEx histology images and pathological notes are available using the GTEx Histology Image Viewer: https://gtexportal.org/home/histologyPage. The data were obtained from the GTEx Portal on 05/17.

3.5 RESULTS

3.5.1 *GRX2-/-* MALE MICE DEVELOP CARDIAC HYPERTROPHY AND DIASTOLIC DYSFUNCTION THAT NAC FAILS TO REVERSE

To address our first aim, we conducted *in vivo* analyses of heart and *ex vivo* high resolution respirometry in permeabilized cardiac myofibers, in which mitochondria remain in reticular structures. We also queried whether NAC would prevent any dysfunction. Thus, half of all *Grx2-/-* and WT mice were treated with NAC for 6 weeks prior to the *ex vivo* cardiac myofiber analyses, which were conducted at 12 weeks of age. *In vivo* echocardiography and hypertension determinations were conducted at 9 and 10 weeks of age, respectively. No difference in water consumption between the groups was observed (data not shown). Results from echocardiography revealed that interventricular septum (IVS) length was increased in *Grx2-/-* mice during systole and diastole compared to WT mice; surprisingly NAC treatment had no effect in *Grx2-/-* or WT mice (Figure 3.1A). There was no difference in the left ventricular internal dimension (LVID) length in both systole and diastole (Figure 3.1B). The left ventricular posterior wall (LVPW) thickness was increased in systole but not in diastole (Figure 3.1C). Even with NAC treatment, left ventricle (LV) mass was still elevated in *Grx2-/-* mice (Figure 3.1D). Thus cardiac hypertrophy in *Grx2-/-* mice was not prevented by *in vivo* NAC treatment.

To further investigate cardiac function, we examined cardiac ejection fraction (EF), an indicator of the blood fraction ejected by the left ventricle during systole, and found no significant differences between groups (Figure 3.1E), consistent with the conclusion that left

ventricle function was not affected. Then, the velocity ratio of the early filling wave peak (E), the atrial contraction wave peak (A) and the E/A ratio were determined. E/A provides a proxy measure of mitral valve function. Given that E/A was lower in NAC treated *Grx2-/- vs* WT mice, with no differences in the untreated groups (Figure 3.1F), our results show that NAC treatment induces a mitral valve abnormality in *Grx2-/-* mice. Representative echocardiographic left ventricle images are shown in Supplementary Figure 6.1.

3.5.2 NAC TREATMENT DOES NOT MITIGATE HYPERTENSION IN *GRX2-/-* MICE

We next determined if *in vivo* NAC treatment would alleviate the hypertension that develops in *Grx2-/-* mice. It is well known that left ventricular hypertrophy can be caused by hemodynamic instability [12]. We found that the hypertension in untreated *Grx2-/-* mice was not diminished by NAC treatment (Figure 3.1G). In *Grx2 -/-* mice treated with NAC, diastolic blood pressure was significantly higher than that in NAC treated WT mice; there were no differences in untreated groups (Figure 3.1G). These findings are again consistent with detrimental effects of NAC treatment in the absence of GRX2.

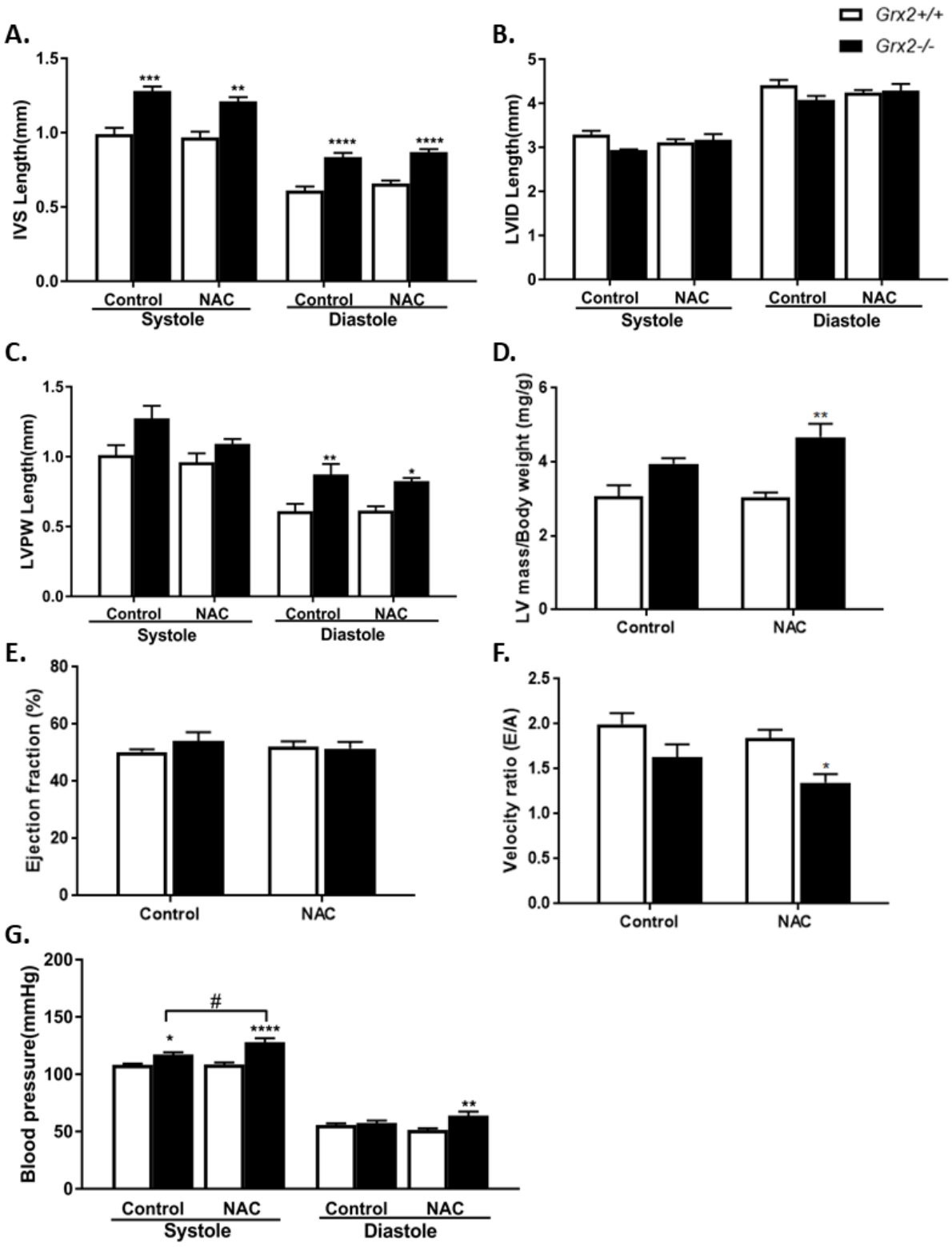

A.
IVS Length(mm)
1.5
1.0
0.5
0.0
***
**
****
****
Control
NAC
Control
NAC
Systole
Diastole
B.
Grx2+/+
Grx2-/-
LVID Length(mm)
5
4
3
2
1
0
Control
NAC
Control
NAC
Systole
Diastole
C.
LVPW Length(mm)
1.5
1.0
0.5
0.0
**
*
Control
NAC
Control
NAC
Systole
Diastole
D.
LV mass/Body weight (mg/g)
6
4
2
0
**
Control
NAC
E.
Ejection fraction (%)
80
60
40
20
0
Control
NAC
F.
Velocity ratio (E/A)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
NAC
G.
Blood pressure(mmHg)
200
150
100
50
0
#
*
****
**
Control
NAC
Control
NAC
Systole
Diastole

Figure 3.1: Echocardiographic and blood pressure analyses of untreated and NAC treated male mice.

(A-F) Echocardiographic measurements. All mice were 9 weeks of age and measurements were performed on M-mode images. N=4-5, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. **LV;** Left ventricle, **IVS;** interventricular septum, **LVID;** left ventricular internal dimension, **LVPW;** left ventricular posterior wall. (G) Blood pressure determinations during systole and diastole. N=6, data are represented as mean ± SEM. Two-way ANOVA with Bonferroni post-hoc test; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.5.3 DYSFUNCTIONAL CARDIAC MYOFIBER ENERGETICS IN *GRX2-/-* MICE

Given our previous finding that deficiency of Grx2 leads to dysfunctional energetics in isolated cardiac mitochondria [11], we sought to more comprehensively examine metabolic characteristics in cardiac myofibers in which mitochondria remain intact. We also tested the hypothesis that *in vivo* NAC supplementation restores cardiac myofiber energetics in *Grx2-/-* mice. High resolution respirometry of left ventricular myofibers showed significantly lower complex I -driven phosphorylating respiration in NAC treated *Grx2-/-* mice compared to treated WT, but no difference between the untreated groups (Figure 3.2A). For complex I and II -driven respiration (maximal phosphorylating respiration), a significant decrease was noted between the untreated groups (genotype effect), but this was no longer apparent in the treated groups (Figure 3.2A). This was not due to a rescue of respiration in *Grx2-/-* mice by NAC but due to lower respiration in the WT mice, indicative of an inhibitory effect of NAC on respiratory capacity (Figure 3.2A). There was no difference in leak respiration or complex IV activity (Figure 3.2A). To test whether the impaired respiration was due to protein oxidation and glutathionylation, we then examined the effect of dithiothreitol (DTT), a powerful reducing agent. We hypothesized that it would abolish the differences in respiration between the groups if the above described effects were due to oxidation or glutathionylation. Findings showed that DTT increased maximal respiration and had no effects on leak or complex IV activities (Figure 3.2B), thereby confirming our hypothesis. Altogether, results in intact cardiac myofibers and primary cardiomyocytes show that *Grx2-/-* causes dysfunctional cellular energetics that cannot be prevented by NAC.

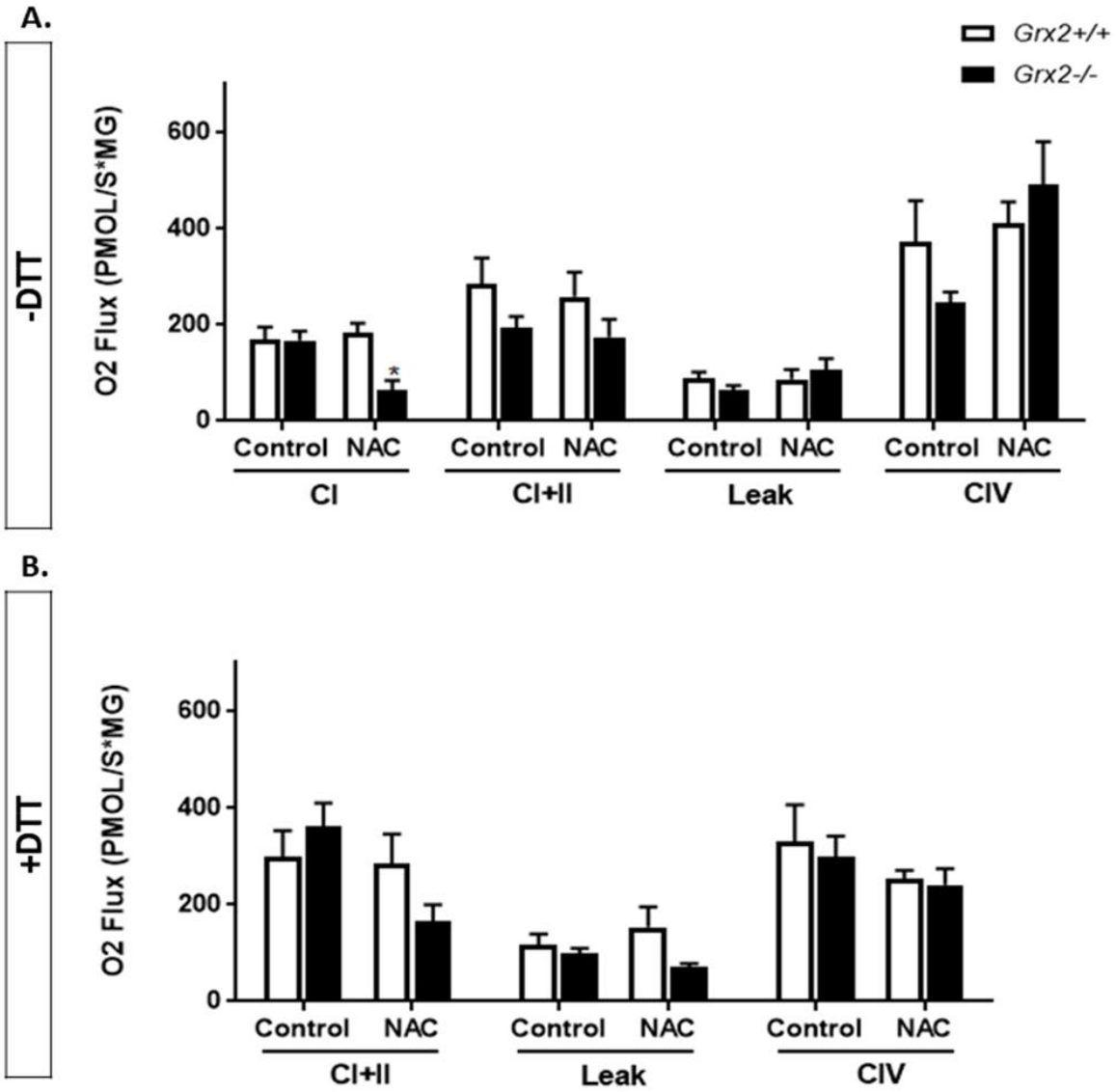


Figure 3.2: Impaired respiration in *Grx2-/-* intact cardiac myofibers isolated from the left ventricle tissue.

Myofibers were either treated with DTT (B) or not (A). Complex I respiration, complex I+II respiration, leak respiration, complex IV activity were determined. N=6, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$.

3.5.4 *IN VIVO* NAC TREATMENT DOES NOT PREVENT CARDIAC HYPERTROPHY AND FIBROSIS

Heart weight (normalized to body weight) of untreated *Grx2-/-* mice at 12 weeks of age was 23% greater than untreated WT control mice. NAC treatment failed to prevent the cardiac hypertrophy (Figure 3.3A). There were no differences in body weights and, apart from the cardiac hypertrophy, there were no differences in tissue weights between the groups (data not shown). There was a strong trend for left ventricular fibrosis in the hearts of *Grx2-/-* as observed in our previous study (p=0.06) [11]. Surprisingly, NAC treatment resulted in increased fibrosis in *Grx2-/-* mice compared to WT (Figure 3.3B-F).

3.5.5 NAC TREATMENT INCREASES GSSG AND LOWERS GLUTATHIONE REDOX IN THE LIVER WITH NO EFFECTS IN THE HEART OF *GRX2-/-* MICE

We next determined if *Grx2-/-* and the *in vivo* NAC treatment affected cardiac muscle glutathione redox potential. In homogenates of left ventricular tissue, there were no significant genotype or treatment differences in reduced (GSH) or oxidized (GSSG) glutathione levels, or their ratio (GSH:GSSG), in the heart (Figure 3.4, A-C). Thus impaired (oxidized) glutathione redox in the hearts of *Grx2-/-* mice exists only at the mitochondrial level [11]. Given that our *in vivo* NAC treatments did not increase heart tissue glutathione levels, even in WT mice, we then queried whether levels increased in the livers of mice. HPLC determinations of liver homogenate demonstrated that NAC caused increased GSSG

levels, decreased GSH:GSSG, and no change in GSH levels in NAC treated *Grx2-/-* compared to all other conditions (Figure 3.4, D-F).

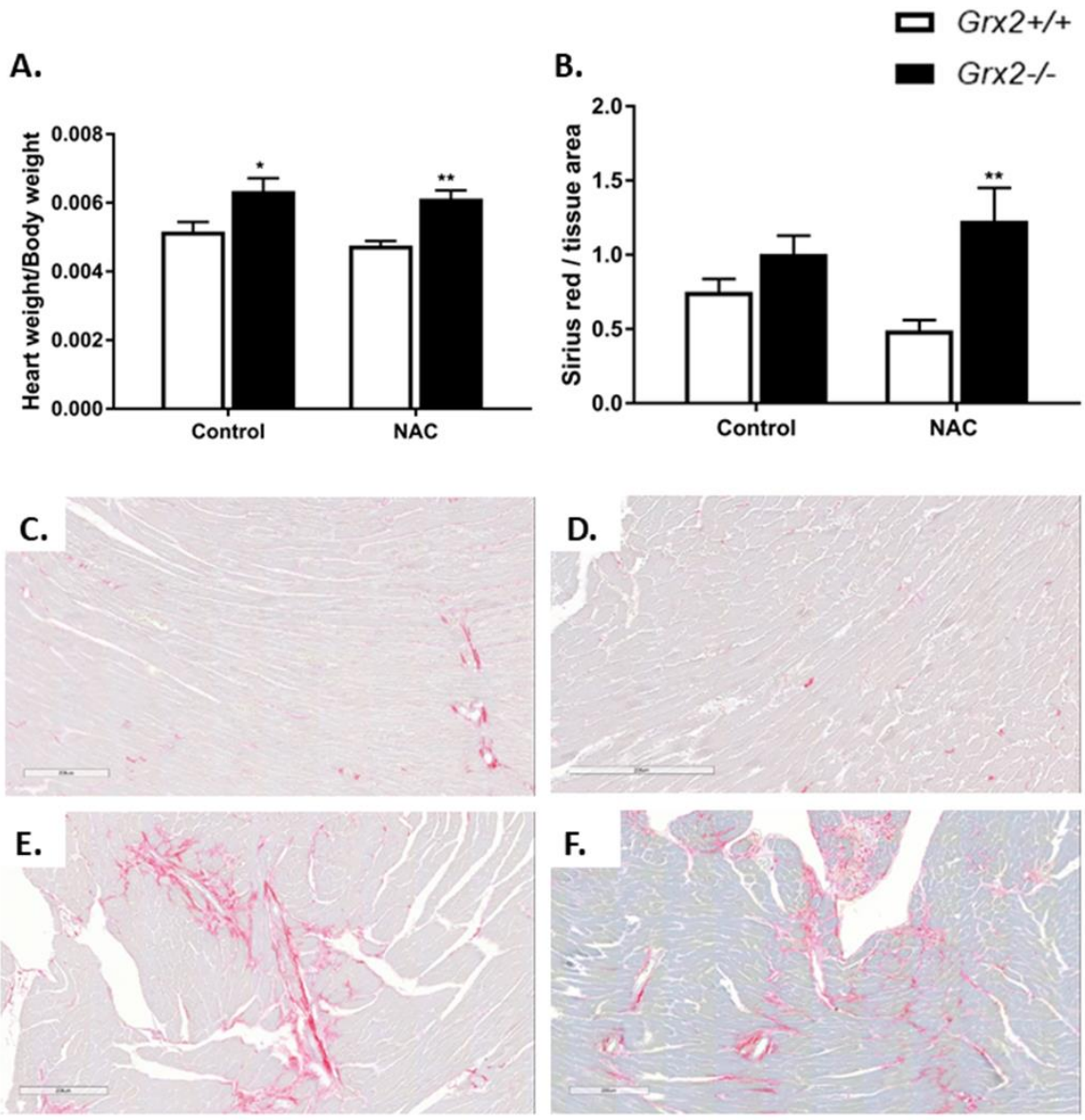


Figure 3.3: Cardiac hypertrophy and fibrosis analysis.

(A) Weight of hearts normalized to body weight. (B) Fibrosis quantification was done using Imagescope software. Images of left ventricle in (C) untreated WT mice, (D) NAC treated WT mice, (E) untreated *Grx2-/-* mice and (F) NAC treated *Grx2-/-* mice. N=3-6, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$, **$p<0.01$. Scale bar for all images: 200μm.

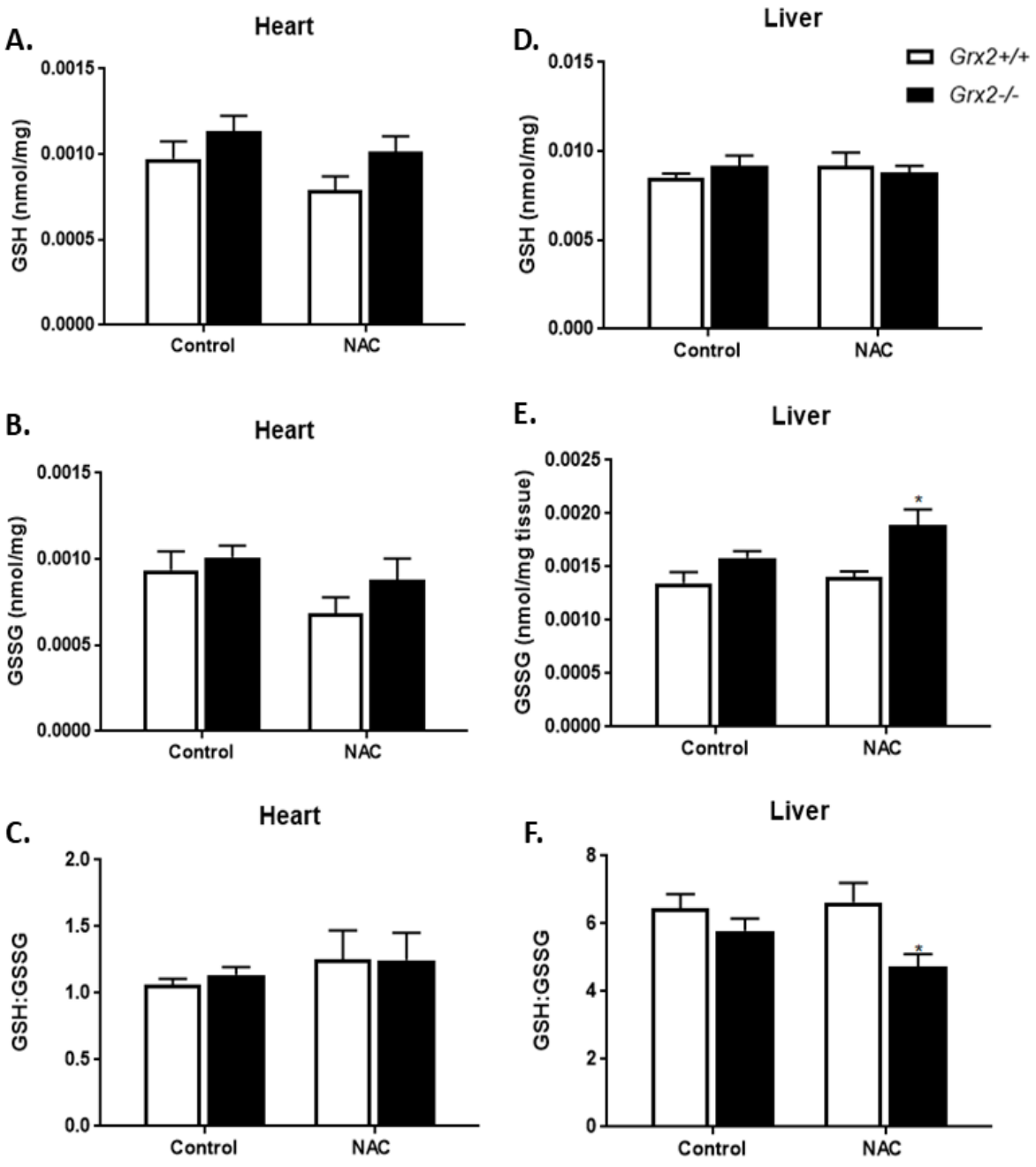

A.
Heart
GSH (nmol/mg)
0.0015
0.0010
0.0005
0.0000
Control
NAC
D.
Liver
Grx2+/+
Grx2-/-
GSH (nmol/mg)
0.015
0.010
0.005
0.000
Control
NAC
B.
Heart
GSSG (nmol/mg)
0.0015
0.0010
0.0005
0.0000
Control
NAC
E.
Liver
GSSG (nmol/mg tissue)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
*
Control
NAC
C.
Heart
GSH:GSSG
2.0
1.5
1.0
0.5
0.0
Control
NAC
F.
Liver
GSH:GSSG
8
6
4
2
0
*
Control
NAC

Figure 3.4: NAC treatment alters glutathione redox in the liver but not the heart of *Grx2-/-* mice.

Measurements of: (A) GSH, (B) GSSG and (C) GSH:GSSG ratio in the heart; (D) GSH, (E) GSSG and (F) GSH:GSSG ratio in the liver. N=6, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test.

3.5.6 NAC PARTIALLY RESTORES NORMAL MITOCHONDRIAL ULTRASTRUCTURE IN CARDIAC TISSUE. A HYPERFUSED MITOCHONDRIAL NETWORK IN *GRX2-/-* CARDIOMYOCYTES IS UNCHANGED BY NAC TREATMENT

Based on the findings of Shutt *et al.* [8] who demonstrated in HeLa cells that a decrease in glutathione redox causes mitochondrial fusion, and our previous findings of decreased glutathione redox in isolated mitochondria of *Grx2-/-* hearts [11], we hypothesized abnormal mitochondrial morphology in hearts of *Grx2-/-* mice. Transmission electron microscopic analysis of cross sections of the left ventricle revealed irregular mitochondrial shapes, despite normal cristae density, in untreated *Grx2-/-* mice (Figure 3.5B and C) compared to untreated WT mice (Figure 3.5A). While 6 weeks of NAC treatment had no effect on WT mitochondria (Figure 3.5D), the abnormal morphology of *Grx2-/-* mitochondria were no longer apparent after NAC treatment (Figure 3.5E and J).

Next we wanted to investigate the impact of Grx2 and NAC treatment on mitochondrial dynamics. We isolated and studied neonatal cardiomyocytes from *Grx2-/-* and WT mice, and then used immunocytochemistry and quantitative morphometry to assess mitochondrial length. Mitochondria in the *Grx2-/-* cells were hyperfused, with fewer punctate mitochondria than controls (Figure 3.5H). There were significantly longer mitochondria in both untreated and in NAC treated *Grx2-/-* cells, thus demonstrating that NAC has no effect on mitochondrial length under these conditions (Figure 3.5F-I and K).

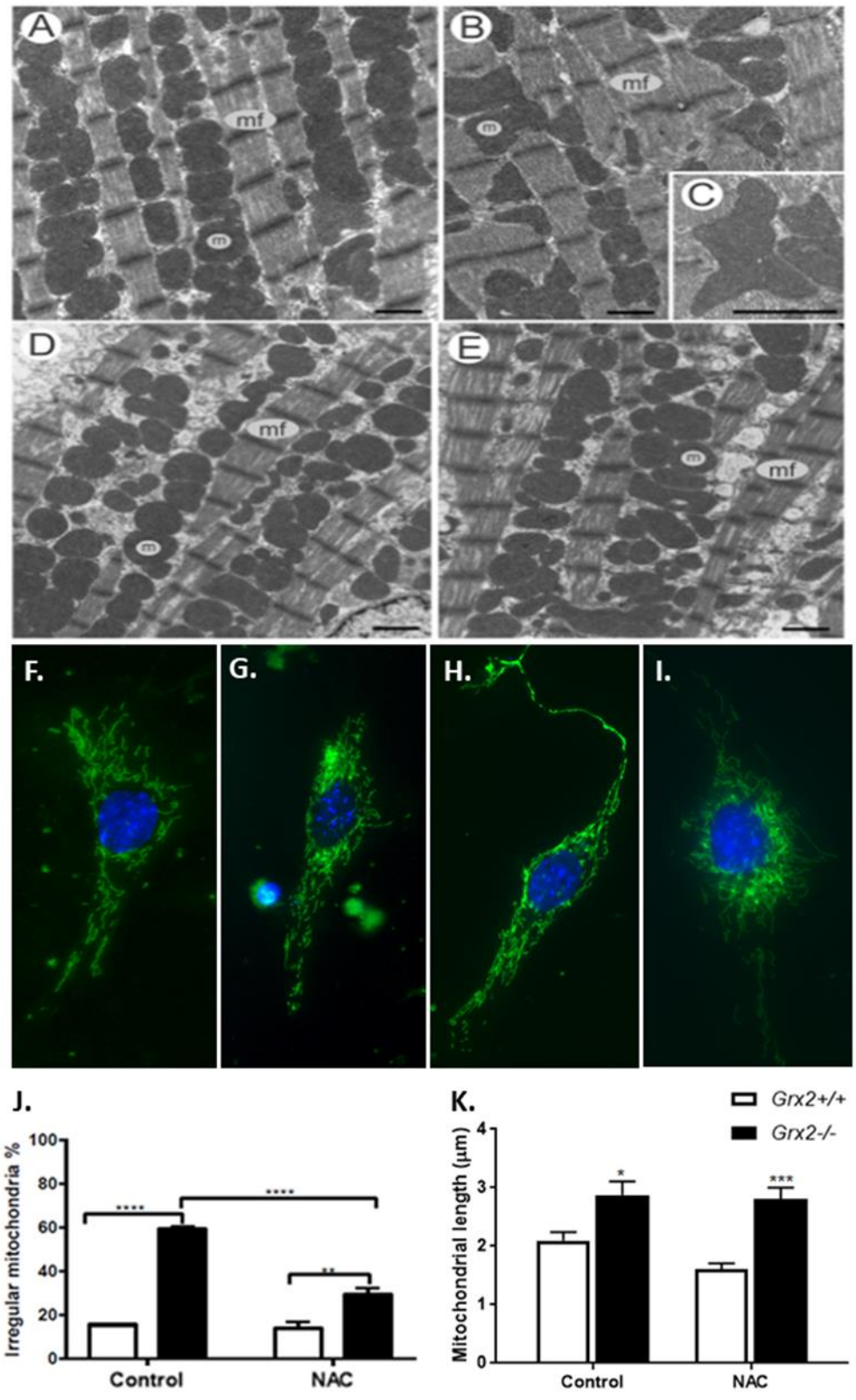

A
mf
m
B
mf
m
C
D
mf
m
E
m
mf
F.
G.
H.
I.
J.
Irregular mitochondria %
100
80
60
40
20
0
****
****
**
Control
NAC
K.
Grx2+/+
Grx2-/-
Mitochondrial length (µm)
4
3
2
1
0
*
***
Control
NAC

Figure 3.5: Abnormal mitochondrial ultrastructural in the myocytes of mouse heart accompanied by mitochondrial tubulation in neonatal cardiomyocytes.

(A) Untreated *Grx2+/+* and (B) *Grx2-/-* hearts. (C) Larger magnification showing irregular mitochondria in *Grx2-/-* heart. (D) NAC treated *Grx2+/+* and (E) *Grx2-/-* hearts. m; mitochondria, mf; myofibrils. Scale bar for EM images: 1µm. (F) Untreated *Grx2+/+*, (G) NAC treated *Grx2+/+*, (H) untreated *Grx2-/-*, (I) NAC treated *Grx2-/-* , (J) mitochondrial morphometric analyses and (k) quantification of mitochondrial length. Hoechst dye stains the nucleus (blue) and Tom20 antibody is used to observe the mitochondria (green). N=15-20, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$, ***$p<0.001$. Scale bar for IF images: 200µm

3.5.7 IMPAIRED CELLULAR ENERGETICS AND METABOLIC FLEXIBILITY IN *GRX2-/-* PRIMARY CARDIOMYOCYTES

We then investigated oxidative and glycolytic metabolic characteristics in intact primary cardiomyocytes. To also test the possible effects of the glutathione precursor, NAC, some cells were treated for 1 hour prior to analyses of cellular energetics. In *Grx2-/-* cardiomyocytes, resting and ATP-turnover dependent oxygen consumption rates (OCRs) were abnormally low (Figure 3.6A and D). However there were no significant differences in leak respiration or in maximal or spare respiratory capacities (Figure 3.6B, C and E). Treating with NAC did not improve respiration in *Grx2-/-* cells compared to treated WT cells in any of the tested conditions. We then probed the metabolic flexibility of the cardiomyocytes through the combined analyses of OCRs and extracellular acidification rates (ECARs). Results clearly demonstrate profoundly limited metabolic flexibility of *Grx2-/-* cells, both in the absence and presence of NAC (Figure 3.6F).

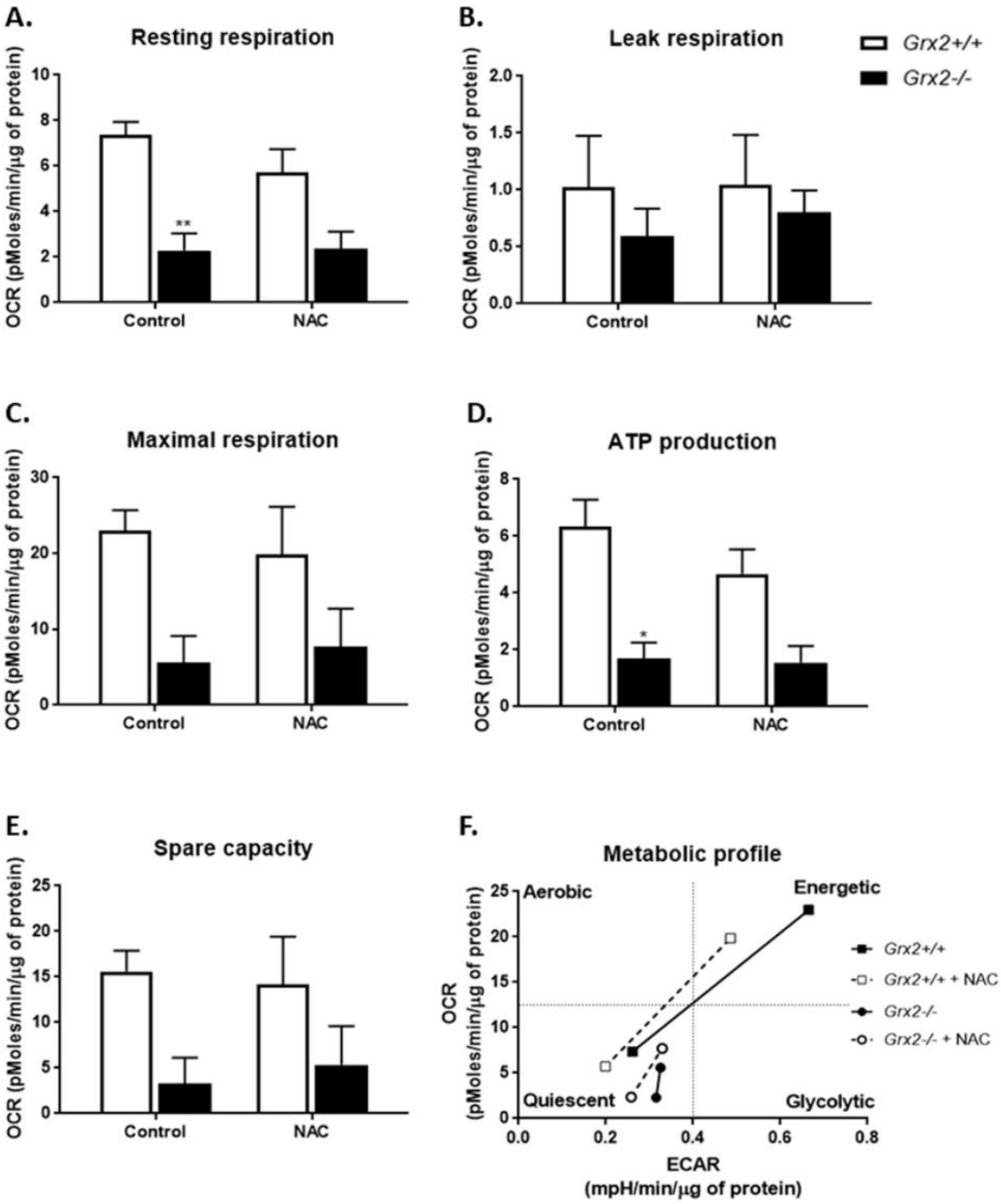

A.
Resting respiration
OCR (pMoles/min/µg of protein)
10
8
6
4
2
0
**
Control
NAC
B.
Leak respiration
Grx2+/+
Grx2-/-
OCR (pMoles/min/µg of protein)
2.0
1.5
1.0
0.5
0.0
Control
NAC
C.
Maximal respiration
OCR (pMoles/min/µg of protein)
30
20
10
0
Control
NAC
D.
ATP production
OCR (pMoles/min/µg of protein)
8
6
4
2
0
*
Control
NAC
E.
Spare capacity
OCR (pMoles/min/µg of protein)
25
20
15
10
5
0
Control
NAC
F.
Metabolic profile
OCR (pMoles/min/µg of protein)
25
20
15
10
5
0
Aerobic
Energetic
Quiescent
Glycolytic
0.0
0.2
0.4
0.6
0.8
ECAR
(mpH/min/µg of protein)
Grx2+/+
Grx2+/+ + NAC
Grx2-/-
Grx2-/- + NAC

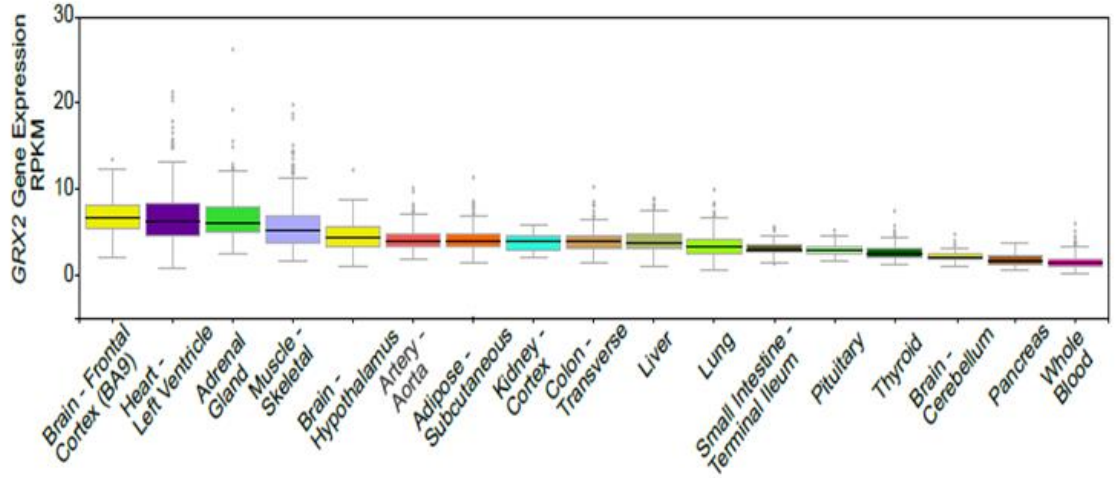
Figure 3.6: Impaired bioenergetics in neonatal cardiomyocytes from *Grx2-/-* mice.

Mitochondrial respiration was measured using a Seahorse XF 24 analyzer. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized to protein content. (A) Resting respiration, (B) leak respiration, (C) maximal respiration, (D) ATP production, (E) spare capacity and (F) metabolic profile. N=3, data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$, **$p<0.01$.

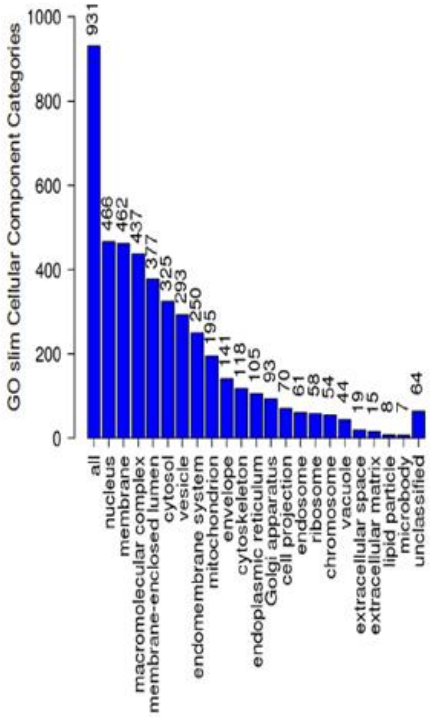
3.5.8 *GRX2* TRANSCRIPT EXPRESSION CORRELATES WITH KEY MITOCHONDRIAL GENES IN HUMAN HEART AND INVERSELY CORRELATES WITH ADVERSE HEART PATHOLOGIES

To examine the potential role of GRX2 in the human heart we first examined the level of *GRX2* transcript expression across the tissue samples from the GTEx consortium (GTEx, 2015). *GRX2* transcript levels were expressed at highest levels in the brain, heart (Figure 3.7A and Supp. Figure 6.2A) and testis (not shown), and demonstrated a wide range of expression levels across the human left ventricle heart samples (Supp. Figure 6.2B). Using GTEx left ventricle heart transcriptome data downloaded from the GeneNetwork program (http://www.genenetwork.org), we then performed a gene ontology slim term cellular component analysis of the top 1000 genes that correlated with *GRX2* and found 195 genes classified as components of the mitochondrion (Figure 3.7B). Notably, *GRX2* transcript expression was positively correlated to a large sampling of mitochondrial-related genes (Figure 3.7C and Supp. Figure 6.3). We then grouped GTEx left ventricle tissue samples into those that expressed the highest and lowest levels of *GRX2* transcripts (each n=50) (Figure 3.8A), and compared histopathological phenotypes. Using available images of left ventricle H&E histology sections and pathologist's notes from the GTEx Portal, there was substantially greater evidence of moderate to extensive fibrosis, hypertrophy and infarct in the group of 50 samples with low *GRX2* expression *vs* those with high *GRX2* expression (Figure 3.8B and C). These results are consistent and complement our key findings from the *Grx2-/-* mouse model by demonstrating an inverse relationship between *GRX2* expression and cardiac disease in humans.

A.


GRX2 Gene Expression
RPKM
30
20
10
0
Brain - Frontal Cortex (BA9)
Heart - Left Ventricle
Adrenal Gland
Muscle - Skeletal
Brain - Hypothalamus
Artery - Aorta
Adipose - Subcutaneous
Kidney - Cortex
Colon - Transverse
Liver
Lung
Small Intestine - Terminal Ileum
Pituitary
Thyroid
Brain - Cerebellum
Pancreas
Whole Blood

B.


GO slim Cellular Component Categories
1000
800
600
400
200
0
931
466
462
437
377
325
293
250
195
141
118
105
93
70
61
58
54
44
19
15
8
7
64
all
nucleus
membrane
macromolecular complex
membrane-enclosed lumen
cytosol
vesicle
endomembrane system
mitochondrion
envelope
cytoskeleton
endoplasmic reticulum
Golgi apparatus
cell projection
endosome
ribosome
chromosome
vacuole
extracellular space
extracellular matrix
lipid particle
microbody
unclassified

C.

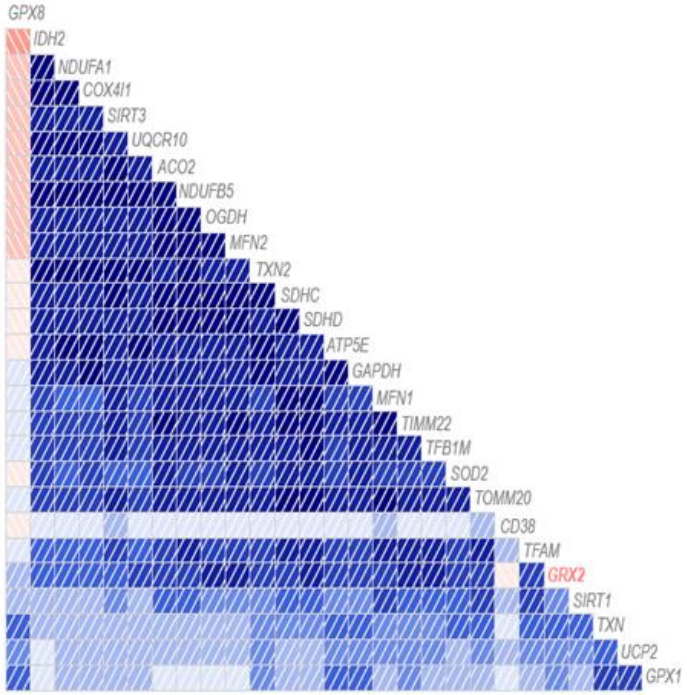

GPX8
IDH2
NDUFA1
COX4I1
SIRT3
UQCR10
ACO2
NDUFB5
OGDH
MFN2
TXN2
SDHC
SDHD
ATP5E
GAPDH
MFN1
TIMM22
TFB1M
SOD2
TOMM20
CD38
TFAM
GRX2
SIRT1
TXN
UCP2
GPX1

Figure 3.7: Positive correlation between the human GRX2 transcript expression and key mitochondrial genes.

(A) The expression values of *GRX2* (also known as *GLRX2*) in 17 tissue types was plotted using GTEx Analysis Release v6 (dbGaP accession number: phs000424.v6.p1). Expression values were plotted as log(RPKM) (Reads Per Kilobase of transcript per Million mapped reads). Box plots are shown as median and 25th and 75th percentiles; points are displayed as outliers if they are above or below 1.5 times the interquartile range. (B) Gene ontology slim term cellular component category analysis using WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) was performed on the top 1000 genes that were significantly ($P < 0.05$) correlated to GRX2 in data sets derived from the GTEx left ventricle heart tissue transcriptome (n=246; downloaded from the GeneNetwork program at http://www.genenetwork.org). (C) Transcript expression for *GRX2* (shown in red font) were positively correlated with mitochondrial-associated genes using custom-designed data sets derived from the GTEx left ventricle heart tissue transcriptome. As seen on the correlogram, blue correlations are positive (red correlations are negative—intensity of the colors correlates with level of significance).

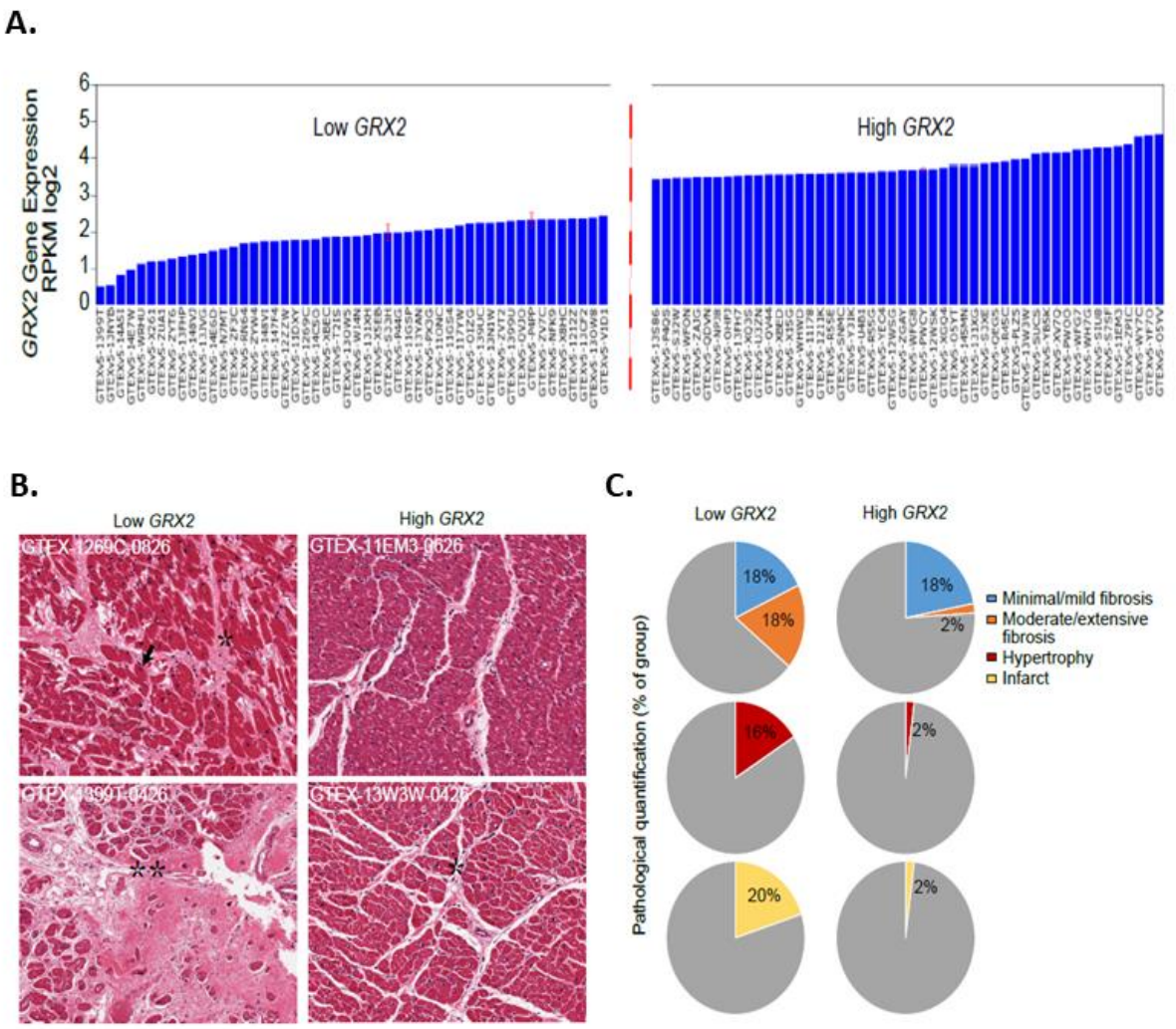


Figure 3.8: Low *GRX2* expression in humans is associated with extensive fibrosis, hypertrophy and infarct.

(A) *GRX2* transcript expression data from GTEx left ventricle tissues samples were downloaded from the GeneNetwork program as RPKM log2 values and grouped into those that expressed the highest and lowest levels of *GRX2* transcripts (top n=50 per group). (B) Representative images for the GTEx left ventricle H&E histology sections for high and low *GRX2* groups (Arrow: hypertrophy; *: fibrosis; **: infarct). Images were downloaded from the GTEx Portal (C) Corresponding pathological notes for all 50 GTEx left ventricle heart samples in the high or low *GRX2* groups were downloaded from the GTEx Portal and quantified to summarize evidence for extensive fibrosis, hypertrophy and infarct.

3.6 DISCUSSION

Mitochondrial glutathione redox balance in the heart is important to support the exceptionally high rates of oxidative reactions while minimizing oxidative stress. In the present study, we investigated mechanisms impacted by Grx2 deficiency in intact primary cardiomyocytes and permeabilized *ex vivo* cardiac myofibers in mice, and complimented this with studies into the GTEx human data resource database and associated tissue bank. We also examined the effects of mouse *in vivo* and *in vitro* supplementation of the glutathione precursor, NAC. We hypothesized that NAC would increase glutathione redox and thereby mitigate, at least in part, the functional impairments. This was based on previous findings showing that oxidative stress is important in establishing cardiac remodeling and fibrosis, leading to a failing myocardium and thus heart failure [23,24]. Uncontrolled oxidative stress increases cardiac collagen type I and IV, fibronectin and impairs cardiac contractility [25]. Previous studies also showed that NAC can protect against oxidative stress-mediated cardiac dysfunction. Moreover, in a heart failure rat model, the glutathione content of the left ventricle was shown to be decreased and treatment with NAC was able to lower oxidative stress, the expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and its receptor, and to restore cardiac function and damage [26]. In this context it is important to note that while we previously observed clearly lower glutathione redox in isolated mitochondria of in *Grx2-/-* mice, there is no evidence of increased ROS production or oxidative stress in the heart of these mice [11]. Thus we anticipated that NAC would mitigate dysfunction through the restoration of glutathione redox in *Grx2-/-* mice.

Echocardiography showed that NAC did not affect cardiac functions in WT mice, and more importantly did not abolish cardiac hypertrophy in *Grx2-/-* mice. Moreover, NAC treatment of *Grx2-/-* mice was associated with a possible mitral valve abnormality and diastolic dysfunction. NAC treated *Grx2-/-* mice had a decreased velocity ratio of the early filling wave (E) to the atrial contraction peak (A). In addition, non-invasive blood pressure measurements show that NAC did not mitigate hypertension in *Grx2-/-* mice. It is possible that in the absence of Grx2, NAC treatment causes glutathionylation of key cardiac proteins leading to slower kinetics and increased calcium sensitivity, which in turn can result in sarcomere dysfunction, cardiac hypertrophy and hypertension [27].

It appears that the absence of Grx2 in mice causes profound defects in mitochondrial function that are intransigent to glutathione precursor supplementation. This may be why our results differ from those in which diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy were reversed by NAC [27, 28].

Furthermore, given the importance of mitochondrial structure to mitochondrial functions, we investigated characteristics of mitochondrial OXPHOS in left ventricle myofibers in which mitochondria remain intact. We observed impaired maximal respiration, and no effect of NAC treatment in *Grx2-/-*. Surprisingly NAC lowered oxygen consumption in WT tissue, without rescuing *Grx2-/-* mice respiration. In a previous study we showed that the reducing agent DTT restored respiration in isolated mitochondria [11]. In the current study, we tested the effects of DTT in intact cardiac myofibers, and indeed we were able to rescue maximal respiration in *Grx2-/-* cardiac myofibers. DTT is a powerful reducing agent

that reduces the cellular environment and induces protein deglutathionylation. These findings are consistent with the possibility that in the absence of Grx2 an oxidized environment results in protein glutathionylation (a reversible redox sensitive post-translational modification), and that DTT reduces and reactivates the proteins. Glutathionylation in mitochondria can be enzymatically mediated by Grx2 [16-20]. Disrupted mitochondrial glutathionylation of complex I and ATP synthase can lead to cardiac dysfunction, consistent with the importance of mitochondrial glutathione redox to heart function [21,22].

In a murine model of heart failure, NAC attenuated cardiac remodeling and fibrosis and accelerated wound healing, and had beneficial effects in acute bronchiolitis and congenital heart defects [29, 30]. To the contrary, our fibrosis analyses demonstrated higher inflammation in heart tissues from *Grx2-/-* mice treated with NAC compared to other groups. These results further indicate that NAC exacerbates cellular redox in *Grx2-/-*. Interestingly, a recent study in humans showed that NAC was ineffective in treating idiopathic pulmonary fibrosis [31].

In our model, cardiac GSH, GSSG and GSH:GSSG levels were unchanged by *in vivo* NAC treatment. We thus assessed levels in the liver and found increased levels of GSSG and a decrease in GSH:GSSG ratio in treated *Grx2-/-* mice. Many studies in experimental animals and in humans have shown that NAC can increase, decrease or not change glutathione levels. Along with these effects, there is a strong evidence to support the notion that NAC is only beneficial in increasing glutathione levels when GSH levels are depleted in the target tissue [35-40].

In our analyses of cardiac tissue levels of GSH and GSSG, we found no effect of *Grx2-/-*. This is in contrast to our previous findings in isolated mitochondria from the hearts of *Grx2-/-* and WT mice [11]. Together, our findings are consistent with the conclusion that there is impaired mitochondrial uptake and/or metabolism of glutathione in the absence of Grx2, and further research is needed to investigate this possibility. Traditionally it has been thought that glutathione is transported into the mitochondrial matrix via the oxoglutarate carrier and/or the dicarboxylate carrier [46], but recent research has challenged this [47].

Mitochondrial dynamics is central in both normal physiology and disease states [43]. High levels of oxidized glutathione have previously been shown to control mitochondrial fusion [8], and thus we hypothesized that mitochondrial structure would be abnormal in tissue and primary cell systems in the absence of Grx2. Our electron microscopy analyses demonstrated that mitochondrial ultrastructure in *Grx2-/-* hearts is abnormal, despite normal cristae density. The cross sections of mitochondria revealed unusual angular shapes, which were partially restored to normal ovular-like mitochondrial structures by i*n vivo* NAC supplementation. We also assessed mitochondrial length in mouse neonatal primary cardiomyocytes of *Grx2-/-* and WT mice, and demonstrated increased mitochondrial fusion in *Grx2-/-* cells. NAC treatment of the primary cells however did not affect mitochondrial tubulation. While Shutt *et al* [8] previously showed that GSH redox regulates mitochondrial fusion, our findings are the first to show that the absence of Grx2 causes mitochondrial elongation in primary cells.

Our analyses of neonatal cardiomyocytes revealed that the absence of Grx2 impacted mitochondrial oxidative phosphorylation as demonstrated by the impaired resting respiration and ATP production in *Grx2-/-* cells. Moreover, contrary to our hypothesis, our results show that in the absence of Grx2, NAC did not ameliorate the respiratory defects; instead it worsened them, consistent with what we observed in cardiac myofibers.

Finally, our findings are the first to show in adult humans that low levels of *GRX2* expression are associated with cardiac disease risk. Through examining GTEx human left ventricle samples, we demonstrate that GRX2 transcript expression is correlated with the expression of various mitochondrial-associated genes. These data agree with the observed reductions in maximal respiration of intact myofibers from the left ventricle of *Grx2-/-* mice. Notably, consistent with the hypertrophy and fibrosis in *Grx2-/-* mouse hearts, we report moderate/extensive fibrosis, hypertrophy and infarct in human heart samples having low *vs* high expression of GRX2 transcripts. These results support the idea that GRX2 has a cardioprotective role; indeed this would be consistent with the finding of an attenuation of cardiac injury in Grx2 transgenic mice treated with the cardiotoxin, doxorubicin [41]. Thus, these findings clearly emphasize the potential role of GRX2 in protecting against left ventricle pathologies in adult humans.

Altogether, our study is the first to show that Grx2 plays a key role in the control of cellular oxidative and glycolytic functions in cardiomyocytes; that Grx2 is essential for normal mitochondrial dynamics and morphology in cardiomyocytes and heart tissue in mice and humans, and that the GSH precursor, NAC, does not improve mitochondrial energetics

or dynamics in mouse *in vitro* or *in vivo* systems. The impact of Grx2 deficiency on the transport of glutathione into mitochondria requires further investigation. Future research into the impact and potential therapeutic implications of *GRX2* in human heart disease is warranted.

3.7 REFERENCES

1. Schafer, F. Q., & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology Medicine,* 30, 1191–1212

2. Kovacic, P., Pozos, R. S., Somanathan, R., Shangari, N., and O'Brien, P. J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: Focus on electron transfer, free radicals, and structure–activity relationships. *Current Medicinal Chemistry,* 12, 2601–2623.

3. Ridnour, L. A., Isenberg, J. S., Espey, M. G., Thomas, D. D., Roberts, D. D., and Wink, D. A. (2005). Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *Proceedings of the Natural Academy of Sciences of the United States of America,* 102, 13147–13152.

4. Bartosz G. (2009). Reactive oxygen species: Destroyers or messengers? *Biochemical Pharmacology,* 77, 1303-1315.

5. Lambeth, J.D. (2004). NOX enzymes and the biology of reactive oxygen. *Nature Reviews Immunology* 4, 181–189.

6. Butler, J. (2000). Thermodynamic considerations of free radical reactions. In C. J. Rhodes (Ed.), *Toxicology of the human environment* (pp. 437–453). London: Taylor and Francis

7. Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews*, 82, 47–95.

8. Shutt, T., Geoffrion, M., Milne, R., and McBride, H.M. (2012). The intracellular redox state is a core determinant of mitochondrial fusion. *EMBO Reports, 13*, 909–915.

9. Koehler, C.M., Beverly, K., and Leverich, E.P. (2006). Redox pathways in the mitochondrion. *Antioxidants and Redox Signaling* 8: 813–822.

10. Jacob, C., Knight, I., and Winyard, P. G. (2006). Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. *The Journal of Biological Chemistry* 387:1385–1397

11. Mailloux, R. J., Xuan, J. Y., McBride, S., Maharsy, W., Thorn, S., Holterman, C. E., Kennedy, C.R.J., Rippstein, P., deKemp, R., DaSilva, J., Nemer, M., Lou, M., and Harper, M. E. (2014). Glutaredoxin-2 is required to control oxidative phosphorylation in cardiac muscle by mediating deglutathionylation reactions. *The Journal of Biological Chemistry,* 289(21), 14812-14828.

12. Lorell, B.H., and Carabello, B.A. (2000). Left Ventricular Hypertrophy. *Circulation, 102*, 470–479.

13. Rosca, M.G., and Hoppel, C.L. (2010). Mitochondria in heart failure. *Cardiovascular Research 88*, 40–50.

14. Lundberg M, Johansson C, Chandra J, Enoksson M, Jacobsson G, Ljung J, Johansson M, and Holmgren A. (2001). Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *The Journal of Biological Chemistry* 276: 26269–26275.

15. Mailloux R. J., Xuan J. Y., Beauchamp B., Jui L., Lou M., Harper M. E. (2013)Glutaredoxin-2 is required to control proton leak through uncoupling protein-3. *The Journal of Biological Chemistry*,288, 8365–8379.

16. Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A., and Shelton, M. D. (2008). Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxidants & Redox Signaling*, 10(11), 1941-1988.

17. Grek, C. L., Zhang, J., Manevich, Y., Townsend, D. M., and Tew, K. D. (2013). Causes and consequences of cysteine S-glutathionylation. *The Journal of Biological Chemistry*, 288(37), 26497-26504.

18. Beer, S. M., Taylor, E. R., Brown, S. E., Dahm, C. C., Costa, N. J., Runswick, M. J., and Murphy, M. P. (2004). Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: Implications for mitochondrial redox regulation and antioxidant DEFENSE. *The Journal of Biological Chemistry*, 279(46), 47939-47951.

19. Mailloux, R. J., McBride, S.L., and Harper, M. E. (2013). Unearthing the secrets of mitochondria ROS and glutathione in bioenergetics. *Trends in Biochemical Sciences*, 38(12), 592-602.

20. Hurd T. R., Requejo R., Filipovska A., Brown S., Prime T. A., Robinson A. J., Fearnley I. M.,Murphy M. P. (2008) Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: potential role of Cys residues in decreasing oxidative damage *The Journal of Biological Chemistry*, 283, 24801–24815.

21. Wang S. B., Foster D. B., Rucker J., O'Rourke B., Kass D. A., Van Eyk J. E. (2011) Redox regulation of mitochondrial ATP synthase: implications for cardiac resynchronization therapy. *Circulation Research*, 109, 750–757.

22. Passarelli C., Tozzi G., Pastore A., Bertini E.,Piemonte F.(2010) GSSG-mediated complex I defect in isolated cardiac mitochondria. *International Journal of Molecular Medicine*, 26, 95–99.

23. Tsutsui, H., Ide, T., & Kinugawa, S. (2006). Mitochondrial oxidative stress, DNA damage, and heart failure. *Antioxidants & Redox Signaling*, *8*(9–10), 1737–1744. http://doi.org/10.1089/ars.2006.8.1737.

24. Tham, Y. K., Bernardo, B. C., Ooi, J. Y. Y., Weeks, K. L., & McMullen, J. R. (2015). Pathophysiology of cardiac hypertrophy and heart failure: signaling pathways and novel therapeutic targets. *Archives of Toxicology*, *89*(9), 1401–1438. http://doi.org/10.1007/s00204-015-1477-x.

25. Aragno, M., Mastrocola, R., Alloatti, G., Vercellinatto, I., Bardini, P., Geuna, S., … Boccuzzi, G. (2008). Oxidative stress triggers cardiac fibrosis in the heart of diabetic rats. *Endocrinology*, *149*(1), 380–388. http://doi.org/10.1210/en.2007-0877.

26. Adamy, C., Mulder, P., Khouzami, L., Andrieu-abadie, N., Defer, N., Candiani, G., … Pecker, F. (2007). Neutral sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats. *Journal of Molecular and Cellular Cardiology*, *43*(3), 344–353. http://doi.org/10.1016/j.yjmcc.2007.06.010.

27. Wilder, T., Ryba, D. M., Wieczorek, D. F., Wolska, B. M., & Solaro, R. J. (2015). N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy. *American Journal of Physiology. Heart and Circulatory Physiology*, *309*(10), H1720-1730. http://doi.org/10.1152/ajpheart.00339.2015.

28. Chaumais, M.-C., Ranchoux, B., Montani, D., Dorfmüller, P., Tu, L., Lecerf, F., … Perros, F. (2014). N-acetylcysteine improves established monocrotaline-induced pulmonary hypertension in rats. Respiratory Research, 15, 65. http://doi.org/10.1186/1465-9921-15-65.

29. Giam, B., Chu, P.-Y., Kuruppu, S., Smith, A. I., Horlock, D., Kiriazis, H., … Rajapakse, N. W. (2016). N-acetylcysteine attenuates the development of cardiac fibrosis and remodeling in a mouse model of heart failure. *Physiological Reports*, *4*(7). http://doi.org/10.14814/phy2.12757 .

30. AlMatar, M., Batool, T., & Makky, E. A. (2016). Therapeutic Potential of N-Acetylcysteine for Wound Healing, Acute Bronchiolitis, and Congenital Heart Defects. Current Drug Metabolism, 17(2), 156–167.

31. Myllärniemi, M., & Kaarteenaho, R. (2015). Pharmacological treatment of idiopathic pulmonary fibrosis - preclinical and clinical studies of pirfenidone, nintedanib, and N-

acetylcysteine. European Clinical Respiratory Journal, 2. http://doi.org/10.3402/ecrj.v2.26385

32. Picard, M., Shirihai, O.S., Gentil, B.J., and Burelle, Y. (2013). Mitochondrial morphology transitions and functions: implications for retrograde signaling? *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 304*, R393–R406.

33. Willems, P.H.G.M., Rossignol, R., Dieteren, C.E.J., Murphy, M.P., and Koopman, W.J.H. (2015). Redox Homeostasis and Mitochondrial Dynamics. *Cell Metabolism 22*, 207–218.

34. Samuni, Y., Goldstein, S., Dean, O. M., & Berk, M. (2013). The chemistry and biological activities of N-acetylcysteine. Biochimica Et Biophysica Acta, 1830(8), 4117–4129. https://doi.org/10.1016/j.bbagen.2013.04.016

35. Forman, M. B., Puett, D. W., Cates, C. U., McCroskey, D. E., Beckman, J. K., Greene, H. L., & Virmani, R. (1988). Glutathione redox pathway and reperfusion injury. Effect of N-acetylcysteine on infarct size and ventricular function. Circulation, 78(1), 202–213.

36. Kleinveld, H. A., Demacker, P. N., & Stalenhoef, A. F. (1992). Failure of N-acetylcysteine to reduce low-density lipoprotein oxidizability in healthy subjects. European Journal of Clinical Pharmacology, 43(6), 639–642.

37. Meyer, A., Buhl, R., Kampf, S., & Magnussen, H. (1995). Intravenous N-acetylcysteine and lung glutathione of patients with pulmonary fibrosis and normals. American Journal of Respiratory and Critical Care Medicine, 152(3), 1055–1060. https://doi.org/10.1164/ajrccm.152.3.7663783

38. Treweeke, A. T., Winterburn, T. J., Mackenzie, I., Barrett, F., Barr, C., Rushworth, G. F., … Megson, I. L. (2012). N-Acetylcysteine inhibits platelet-monocyte conjugation in patients with type 2 diabetes with depleted intraplatelet glutathione: a randomised controlled trial. Diabetologia, 55(11), 2920–2928. https://doi.org/10.1007/s00125-012-2685-z

39. Rushworth, G. F., & Megson, I. L. (2014). Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. Pharmacology & Therapeutics, 141(2), 150–159. https://doi.org/10.1016/j.pharmthera.2013.09.006

40. Lasram, M. M., Dhouib, I. B., Annabi, A., El Fazaa, S., & Gharbi, N. (2015). A review on the possible molecular mechanism of action of N-acetylcysteine against insulin resistance and type-2 diabetes development. Clinical Biochemistry, 48(16–17), 1200–1208. https://doi.org/10.1016/j.clinbiochem.2015.04.017

41. Diotte, N. M., Xiong, Y., Gao, J., Chua, B. H. L., & Ho, Y.-S. (2009). Attenuation of doxorubicin-induced cardiac injury by mitochondrial glutaredoxin 2. Biochimica Et Biophysica Acta, 1793(2), 427–438. https://doi.org/10.1016/j.bbamcr.2008.10.014

42. Wu, H., Yu, Y., David, L., Ho, Y.-S., & Lou, M. F. (2014). Glutaredoxin 2 (Grx2) gene deletion induces early onset of age-dependent cataracts in mice. The Journal of Biological Chemistry, 289(52), 36125–36139. https://doi.org/10.1074/jbc.M114.620047

43. Mishra, P., & Chan, D. C. (2016). Metabolic regulation of mitochondrial dynamics. The Journal of Cell Biology, 212(4), 379–387. https://doi.org/10.1083/jcb.20151103

44. Peake, J., & Suzuki, K. (2004). Neutrophil activation, antioxidant supplements and exercise-induced oxidative stress. Exercise Immunology Review, 10, 129–141.

45. Zembron-Lacny, A., Slowinska-Lisowska, M., Szygula, Z., Witkowski, K., & Szyszka, K. (2009). The comparison of antioxidant and hematological properties of N-acetylcysteine and alpha-lipoic acid in physically active males. Physiological Research, 58(6), 855–861.

46. Lash, L. H. (2015). Mitochondrial Glutathione in Diabetic Nephropathy. Journal of Clinical Medicine, 4(7), 1428–1447. https://doi.org/10.3390/jcm4071428

47. Booty, L. M., King, M. S., Thangaratnarajah, C., Majd, H., James, A. M., Kunji, E. R. S., & Murphy, M. P. (2015). The mitochondrial dicarboxylate and 2-oxoglutarate carriers do not transport glutathione. FEBS Letters, 589(5), 621–628. https://doi.org/10.1016/j.febslet.2015.01.027

CHAPTER 4

Atrial fibrillation is associated with impaired atrial mitochondrial energetics and supercomplex formation in type 2 diabetes

Georges N. Kanaan[1], David Patten[1], Calum Redpath[2*] and Mary-Ellen Harper[1*]

[1] Department of Biochemistry, Microbiology and Immunology, Ottawa Institute of Systems Biology, Faculty of Medicine, University of Ottawa, Ottawa, Canada.
[2] Ottawa Heart Institute, University of Ottawa, Ottawa, Canada.

[*] **To whom correspondence should be addressed:**

Dr. Mary-Ellen Harper, PhD
Professor
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine, University of Ottawa
451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5
Email: mharper@uottawa.ca
Tel: +1-613-562-5800 Ext 8235

Dr. Calum Redpath, MD, PhD
Assistant Professor and Staff Cardiologist
Department of Electrophysiology and Department of Medicine
Ottawa Heart Institute, University of Ottawa
40 Ruskin Street, Ottawa, Ontario, Canada, K1Y 4W7.
Email: credpath@ottawaheart.ca
Tel: +613-696-7000 Ext13654

4.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

4.1.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript “Atrial fibrillation is associated with impaired atrial mitochondrial energetics and supercomplex formation in type 2 diabetes” has been submitted for publication to the *Canadian Journal of Diabetes (CJD_2017_417)* on December 15, 2017.

4.1.2 CONTRIBUTION STATEMENT

GNK, CR and MEH conceived the idea for the project. GNK conducted most of the experiments and analyzed most of the results. DP conducted and analyzed BN-PAGE determinations. GNK, CR and MEH wrote the paper and all authors reviewed and approved it.

4.1.3 ACKNOWLEDGMENTS AND FUNDING

Authors are deeply grateful to the patients for their generous participation in this study. We would also like to thank University of Ottawa Heart Institute for their invaluable contribution to patient recruitment and sample collection. This work was funded by support from CIHR (FDN 143278; MEH). Georges N. Kanaan received University of Ottawa PhD full admission scholarship.

4.1.4 CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

4.2 ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is a chronic progressive disease associated with increased risk for cardiovascular diseases (CVDs) and with impaired mitochondrial metabolism in cardiac and skeletal muscles. Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with significant morbidity and mortality. T2DM is also one of the prevalent concomitant diseases in patients with AF. During AF, myocardial energy demand is high due to electrical activity. To-date however, very little is known about the effects of AF on atrial muscle mitochondrial energetics.

Hypothesis: We hypothesized that pre-existing fibrillation or T2DM impacts atrial mitochondrial energetics and electron transport chain (ETC) supercomplexes.

Methods: Atrial appendages were collected from consented patients with and without pre-existent AF undergoing coronary artery bypass graft (CABG) surgery. Mitochondrial functional analyses were conducted in permeabilized myofibers using high resolution respirometry.

Results and conclusion: Results show impaired complex I and II function in addition to impaired ETC supercomplex assembly in diabetic patients with AF compared to diabetic patients without AF. There were no differences in mitochondrial content in atrial muscle between groups. There was a strong trend for increased oxidative damage (protein carbonyls) in diabetic patients with AF, compared to diabetic patients without AF. Overall, findings demonstrate impaired mitochondrial function in AF and T2DM.

Keywords: Diabetes; heart; atrial fibrillation; mitochondria; oxidative stress; cardiac metabolism.

4.3 INTRODUCTION

Insulin resistance and abnormal or impaired glucose metabolism are early signs of type 2 diabetes mellitus (T2DM). T2DM is a chronic progressive disease that represents 90% of all diabetes cases, and is on the rise internationally. According to the World Health Organization, there were 422 million adults living with diabetes in 2014 with 1.6 million deaths directly caused by diabetes in 2015 (World Health Organization, 2016; NCD Risk Factor Collaboration (NCD-RisC), 2016). In Canada, there were 3.4 million people with a diagnosis of diabetes, and the number is expected to increase to 5 million in 2025 (Diabetes Canada, 2017).

T2DM commonly coexists (≤ 20%) in patients with atrial fibrillation (AF) and is considered by some to independently promote AF development (Iguchi *et al.,* 2008; Sun and Hu, 2010). However, a causal relationship between T2DM and AF is not yet confirmed. It is established that T2DM-associated metabolic defects cause endothelial dysfunction, acceleration of atherogenesis, and activation of the renin-angiotensin-aldosterone system which alone, or in combination with other factors, may promote AF development (Tadic and Cuspidi, 2015). AF is the most common sustained cardiac arrhythmia in humans and is increasing in prevalence (Benjamin *et al.,* 2017). Currently, approximately 350,000 Canadians suffer AF, which confers significantly increased morbidity and mortality (Andrade *et al.,* 2014; Benjamin *et al.,* 2017). AF is associated with an irregular tachycardia in humans as a result of a ten-fold increase in atrial activation frequency. This rapid electrical activity promotes profound alterations in atrial electrical, structural and mechanical function (Heijman e*t al.,* 2014, 2016). These changes, collectively referred to as AF induced

remodelling account for the clinical progression from paroxysmal to persistent AF (Wijffels *et al.,* 1995; Heijman *et al.,* 2014). The molecular origins of AF are complex and despite recent progress, little, if anything, is known of the fundamental mechanisms linking T2DM and AF (Heijman *et al.,* 2014, 2016). It is intuitive that AF must increase the energy demands of atrial muscle; however our understanding of the metabolic mechanisms and overall impact of fibrillation is still preliminary (Opacic *et al.,* 2016). During AF, rapid electrical activity increases ATP demands that need to be met through mitochondrial oxidative phosphorylation processes and concomitant increases in oxygen and nutrient supply (Opacic *et al.,* 2016). It is unknown if these increased ATP demands place an excessive demand on atrial mitochondria, leading to collateral negative effects due to prolonged stress responses. Recent research has demonstrated that high ATP demands in muscle are in part met by the formation of mitochondrial supercomplexes, which are higher order protein structures that improve OXPHOS capacity (Antoun *et al.,* 2015; Greggio *et al.,* 2017). Moreover, we previously demonstrated that T2DM is associated with impaired mitochondrial supercomplex formation in skeletal muscle (Antoun *et al.,* 2015). Thus the overall aim of this study was to assess atrial muscle mitochondrial energetics and supercomplexes in patients with AF in the presence and absence of T2DM. Here we show that atrial mitochondrial respiration and supercomplex assembly are impaired in patients with AF and T2DM.

4.4 METHODS

4.4.1 ATRIAL APPENDAGE COLLECTION

Atrial appendages were collected from consented patients undergoing coronary artery bypass graft surgery (CABG). This study was approved by the Ottawa Hospital Research Ethics Board (OHREB) and patients provided informed written consent. Tissue was collected, placed immediately in a respiratory buffer (0.5mM ethylene glycol tetraacetic acid, 3mM $MgCl_2 6H_2O$, 20mM taurine, 10mM KH_2PO_4, 20mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 110mM D-sucrose, 0.1% bovine serum albumin and 60mM lactobionic acid; pH 7.1) and analyzed in less than 3 hours after CABG surgery.

4.4.2 HIGH RESOLUTION RESPIROMETRY OF PERMEABILIZED ATRIAL MYOFIBERS

A small fraction (roughly 15 mg) of atrial tissue was used to prepare atrial fibers for high resolution respirometry (HRR) analyses of mitochondrial energetics. The remainder of the atrial appendage was frozen for storage at -80^{o}C. Fibers were mechanically teased apart using fine forceps under a dissection microscope, and were then permeabilized with 50µg/ml of saponin. HRR was conducted at 37°C and in duplicate (0.5mM ethylene glycol tetraacetic acid, 3mM $MgCl_2 6H_2O$, 20mM taurine, 10mM KH_2PO_4, 20mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 110mM D-sucrose, 0.1% bovine serum albumin and 60mM lactobionic acid; pH 7.1) using an Oxygraph-2k (Oroboros, Austria). Malate (2mM), pyruvate (5mM) and glutamate (10mM) were added to the incubation medium followed by addition of adenosine diphosphate and Mg2+ (5mM) to assess adenylate-free proton leak respiration and complex I driven respiration, respectively.

Succinate (10mM) and ADP (5mM) were then added to determine maximum oxidative phosphorylation capacity fueled by Complex I and II energy substrates. Proton leak respiration was assessed again in the presence of oligomycin (2μg/ml), which inhibits ATP synthase. The complex III inhibitor antimycin A (2.5μM) was added to inhibit mitochondrial respiration. Finally N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) (0.5mM), ascorbate (2mM) and sodium azide (15mM) were subsequently added to assess complex IV activity. All values were corrected for residual non-mitochondrial oxygen consumption.

4.4.3 BLUE NATIVE GEL ELECTROPHORESIS (BN-PAGE)

BN-PAGE was conducted as described previously in (Patten *et al.*, 2014; Antoun *et al.*, 2015). Briefly, atrial tissues were homogenized in sucrose buffer (250mM sucrose, 20mM imidazole/HCl, pH 7). The membrane fraction containing mitochondria was pelleted at 10,000xg for 10 minutes and resuspended in 50mM imidiazole/HCl pH 7.0, 50mM NaCl, 5mM 6-aminohexanoic acid, 1mM EDTA with 1% digitonin (experimentally determined; digitonin /tissue ratio (w/w) was 1:10) for 30 minutes. Then samples were centrifuged for 30 minutes at 14,000xg. 5% glycerol and a 1:10 dye:digitonin ratio of Coomassie Blue G-250 were added to the proteins. Proteins were loaded onto 3- 13% large gradient gels. Gels were run in high Coomassie Blue cathode buffer for 2 hours at 150V and switched to low Coomassie cathode buffer overnight at 200V. After transfer to nitrocellulose membrane, the following proteins were probed: Complex I [NDUFA9] (459100, Invitrogen), Complex II [Fp] (459200, Invitrogen), Complex III [UQCRC2] (Ab14745, MitoSciences), Complex IV [subunit I] (459600, Invitrogen), Complex V [ATP5A] (Ab14748, MitoSciences). Bands

were visualized using the Pierce ECL Western Blotting Substrate (32106, Thermo) and quantified using the software, Image J.

4.4.4 WESTERN BLOT ANALYSIS

Atrial tissues were weighed and homogenized in 10mM Tris HCl, 150mM NaCl, 1mM EDTA with 0.5% Triton-X100, pH 7.4 using homogenization bead tubes (Precellys, CK14) and the MagNa Lyser instrument (Roche). Protein content was measured using a Bradford assay and samples were stored at -80°C. Samples were prepared, subjected to reducing SDS-PAGE (4-12%) and transferred to a nitrocellulose membrane. Membranes were blocked for 1hour at room temperature using either 5% milk or 5% BSA in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST). All primary antibodies were incubated overnight at 4°C followed by a 2 hour-incubation at room temperature with the appropriate horseradish peroxidise-conjugated secondary antibody. The following primary antibodies were used at the indicated dilutions: MitoProfile Total OXPHOS Human WB Antibody Cocktail (ab110411, Abcam; 1:3000) and COL1A antibody (sc-59772, Santa Cruz; 1:1000). Blots developed with the Pierce ECL Western Blotting Substrate (32106, Thermo) according to the manufacturer's protocols. Bands were quantified using Image J software.

4.4.5 OXYBLOT ANALYSIS

To determine levels of oxidative stress we measured protein carbonyls (OxyBlot; S7510, EmdMillipore). Briefly, after homogenization, 15ug of protein in each sample was derivatized, separated on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. After the transfer, membranes were blocked with 5% BSA in PBST for 1 hour at

room temperature then incubated with the provided primary rabbit anti-DNPH protein antibody (1:200, overnight at 4°C). Secondary goat anti-rabbit diluted in the blocking solution was used in a dilution of 1:5000 for 2 hours at room temperature. Blots were developed with the Pierce ECL Western Blotting Substrate (32106, Thermo). Bands were quantified using the software, Image J.

4.4.6 STATISTICAL ANALYSES

All data are represented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Prism, La Jolla, CA, USA). Data were analyzed either by Student t-tests or by two-way repeated measures analysis of variance (ANOVA) with Tukey post-hoc tests, as indicated. $P<0.05$ was considered significant.

4.5 RESULTS

4.5.1 PATIENT CHARACTERISTICS

Our study investigated patients with or without T2DM, and with or without pre-operative AF. The clinical characteristics of these patients are shown in Table 1. There were no significant differences in age, weight, height, and body mass index (BMI). Glycated haemoglobin (A1C) percentage was higher in patients with diabetes than those without diabetes, but was not different within the groups without and with diabetes.

Table 4.1: Patient characteristics

Age, weight, height, BMI and A1C of with or without T2DM, and with or without pre-operative AF. Data are presented as mean, one-way ANOVA, bold text represents statistical significance.

	Patients Characteristics				
	No diabetes No AF	**No diabetes AF**	**Diabetes No AF**	**Diabetes AF**	
N	12	2	16	4	**P Values**
Age (years)	69	76	65	68	0.6471
Weight (Kg)	79.33	83.50	84.44	111.25	0.0849
Height (cm)	168.00	169.00	172.56	171.50	0.6541
BMI (kg/m^2)	28.17	29.16	28.42	36.81	0.1191
A1C (%)	**5.55**	**5.40**	**7.97**	**6.85**	**0.001**

4.5.2 AF IMPAIRS MITOCHONDRIAL RESPIRATION IN ATRIAL MYOFIBERS

High resolution respirometry of the atrial myofibers showed no differences in complex I (non-phosphorylating)-, maximal- or leak-dependent respiration, or in complex IV activity when we compared results between patients without AF with patients with AF (Figure 1A-D). On the other hand when we compared results from patients with T2DM with and without AF (D^+AF^+ vs D^+AF^-), we observed significantly lower complex I –driven phosphorylating respiration (Figure 1E). This impaired oxygen consumption was still observed when succinate was subsequently added to determine complex I and II –driven phosphorylating respiration (Figure 1F). The latter respiration rate is also referred to as maximal phosphorylating respiration since these respiratory chain substrates in the presence of ADP support the highest rates of ADP-coupled respiration. There were no differences in leak (non-phosphorylating) respiration or in complex IV activity (Figure 1G and H).

4.5.3 UNCHANGED LEVELS OF MITOCHONDRIAL OXPHOS PROTEINS

In order to determine if mitochondrial dysfunction is due to decreases in levels of OXPHOS proteins, we next used western blotting of atrial muscle homogenate to assess levels of proteins representing CI, CII, CIII, CIV and CV. There were no changes in protein levels, whether bands were normalized to GAPDH or vinculin (Figure 2A and B).

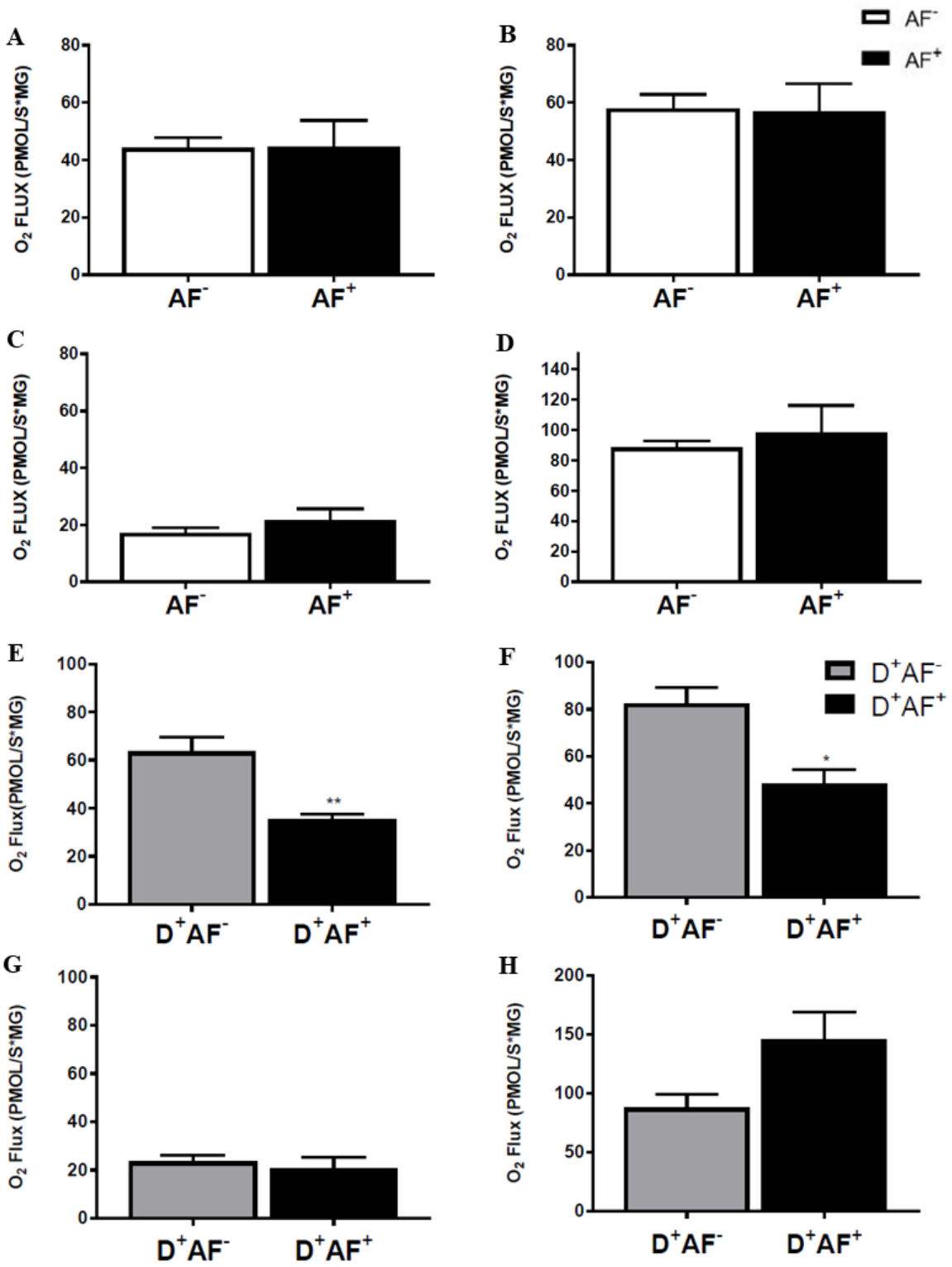

A
B
C
D
E
F
G
H
O2 FLUX (PMOL/S*MG)
O2 Flux(PMOL/S*MG)
O2 Flux (PMOL/S*MG)
AF-
AF+
D+AF-
D+AF+
**
*

Figure 4.1: Atrial mitochondrial respiration analyses in human patients.

Oxygen consumption in atrial appendages was measured using high resolution respirometry. In patients with and without AF complex I respiration, complex I+II respiration, leak respiration and complex IV activity were determined (A-D). Same determinations were conducted in patients with T2DM, with and without AF (E-H). Data are represented as mean ± SEM. Unpaired Student's t test; *$p<0.05$, **$p<0.01$.

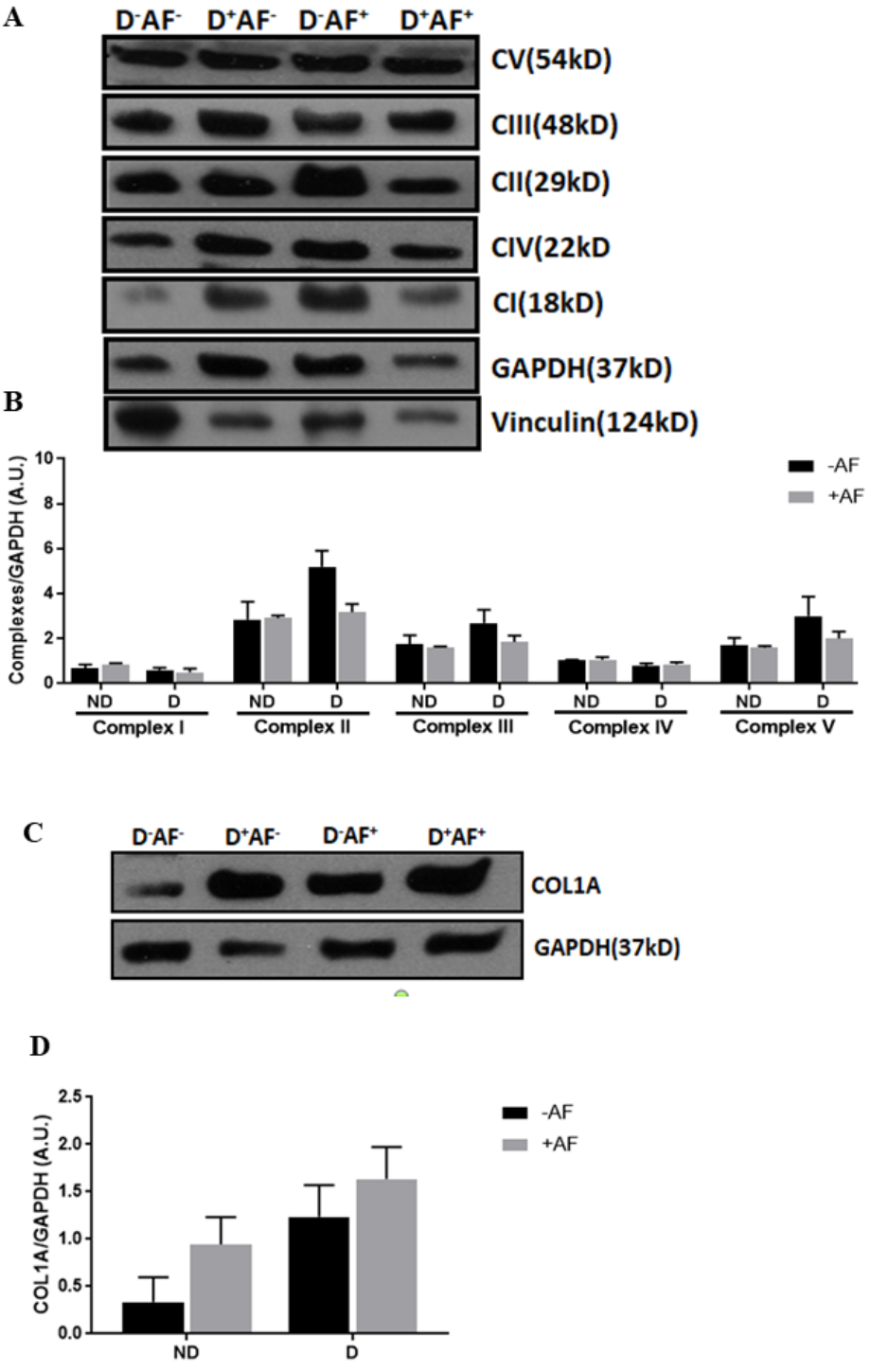
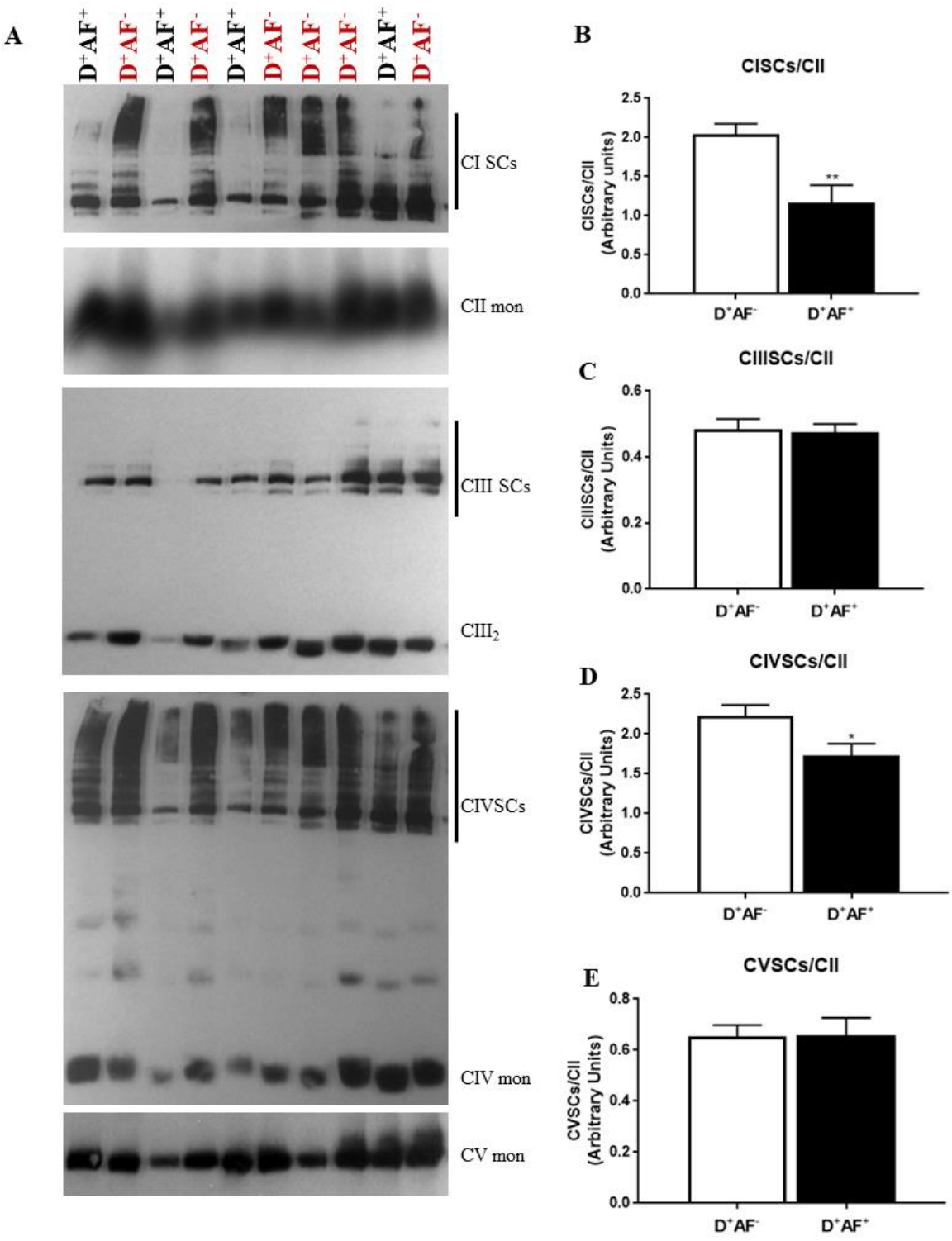

A
D-AF-
D+AF-
D-AF+
D+AF+
CV(54kD)
CIII(48kD)
CII(29kD)
CIV(22kD
CI(18kD)
GAPDH(37kD)
Vinculin(124kD)
B
Complexes/GAPDH (A.U.)
-AF
+AF
ND
D
Complex I
Complex II
Complex III
Complex IV
Complex V
C
COL1A
GAPDH(37kD)
D
COL1A/GAPDH (A.U.)

Figure 4.2: Mitochondrial content is not changed.

Protein expression normalised to GAPDH of: mitochondrial ETC complexes (I-V) (A and B) and collagen (C and D). Quantification was done by Image J and data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$.

4.5.4 AF IMPAIRS MITOCHONDRIAL SUPERCOMPLEX ASSEMBLY

Recently, we reported that skeletal muscle from obese individuals with T2DM had dysfunctional mitochondrial bioenergetics compared to obese controls, without concomitant decreases in mitochondrial content (Antoun *et al.*, 2015). Surprisingly, we discovered a significant decrease in ETC supercomplexes, which have been shown to affect respiration (Lapuente-Brun *et al.*, 2013). Here, we similarly rationalized that decreased CI, CII and CIV activities in atrial appendages from D^+AF^+ versus D^+AF^- could be affected by altered ETC supercomplex assembly. Because complex II does not participate in the formation of supercomplexes (Dudkina *et al.*, 2010; Chaban *et al.*, 2014), we normalised our densitometry data to complex II monomer, as others have done (Khacho *et al.*, 2014; Antoun *et al.*, 2015). Results show significantly lower complex I and complex IV related supercomplex formation, with no changes in complex III supercomplexes or in complex IV or V monomers in D^+AF^+ vs D^+AF^- (Figure 3A-E). Furthermore, in D^+AF^+ vs D^+AF^- there was a decrease in the high oligomeric supercomplexes. When these "high oligomeric" supercomplexes were normalized to CII and "traditional" supercomplexes were normalized to CII, it was clear that the reductions in supercomplex formation were due to drastic reductions in these high oligomeric supercomplexes in D^+AF^+ vs D^+AF^- (Figure 3F-G).


A
D+AF+
D+AF-
D+AF+
D+AF-
D+AF+
D+AF-
D+AF-
D+AF-
D+AF+
D+AF-
CI SCs
CII mon
CIII SCs
CIII2
CIVSCs
CIV mon
CV mon
B
CISCs/CII
CISCs/CII (Arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
**
D+AF-
D+AF+
C
CIIISCs/CII
CIIISCs/CII (Arbitrary Units)
0.6
0.4
0.2
0.0
D+AF-
D+AF+
D
CIVSCs/CII
CIVSCs/CII (Arbitrary Units)
2.5
2.0
1.5
1.0
0.5
0.0
*
D+AF-
D+AF+
E
CVSCs/CII
CVSCs/CII (Arbitrary Units)
0.8
0.6
0.4
0.2
0.0
D+AF-
D+AF+

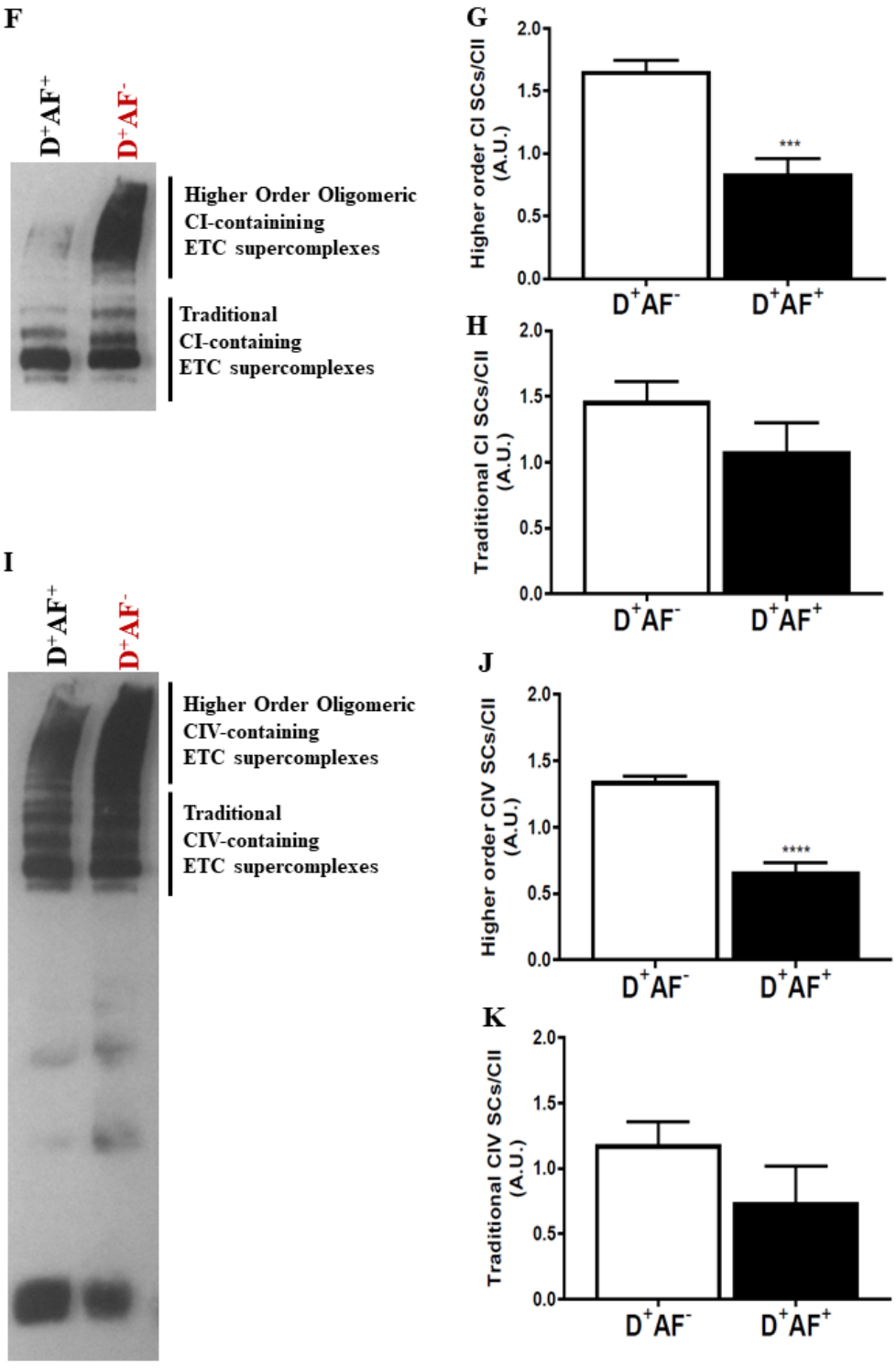

F
D+AF+
D+AF-
Higher Order Oligomeric CI-containining ETC supercomplexes
Traditional CI-containing ETC supercomplexes
G
Higher order CI SCs/CII (A.U.)
2.0
1.5
1.0
0.5
0.0
***
D+AF-
D+AF+
H
Traditional CI SCs/CII (A.U.)
2.0
1.5
1.0
0.5
0.0
D+AF-
D+AF+
I
D+AF+
D+AF-
Higher Order Oligomeric CIV-containing ETC supercomplexes
Traditional CIV-containing ETC supercomplexes
J
Higher order CIV SCs/CII (A.U.)
2.0
1.5
1.0
0.5
0.0
****
D+AF-
D+AF+
K
Traditional CIV SCs/CII (A.U.)
2.0
1.5
1.0
0.5
0.0
D+AF-
D+AF+

Figure 4.3: Supercomplex assembly is altered in patients with T2DM and AF.

(A) Representative BN-PAGE blot of the indicated respiratory complexes (CI–V), supercomplexes (SCs), and monomers (Mon) using anti-NDUFA9 (complex I), anti-flavoprotein (complex II), anti-UQCRC2 (complex III), anti-complex IV and anti-ATP5a (complex V) antibodies. (B–E) Quantification of expression of the indicated respiratory supercomplex normalised to complex II monomer levels. Higher order oligomeric CI and CIV-containining ETC supercomplexes were separated from traditional ETC supercomplexes and quantified (F-K). Quantification was done by Image J and data are represented as mean ± SEM. Unpaired student's t test; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

4.5.5 AF TENDS TO INCREASE OXIDATIVE DAMAGE IN THE ABSENCE OF FIBROSIS

Oxyblot determinations of protein carbonyl adducts in atrial tissue demonstrated a strong trend for increased protein oxidation in D^+AF^+ vs D^+AF^- patients (Figure 4B). In order to check for inflammation and fibrosis, we then measured collagen protein levels. There were no significant differences between groups (Figure 2C and D).

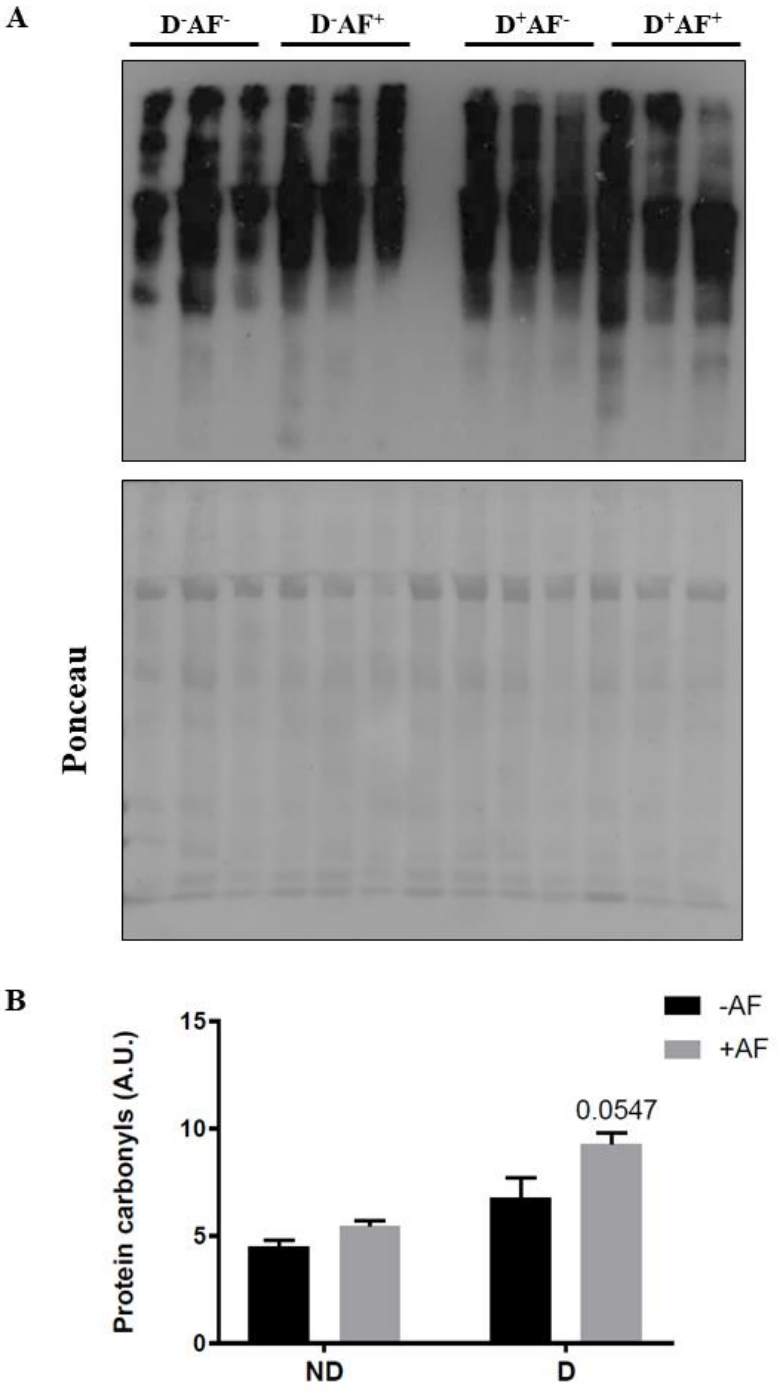

A
D-AF-
D-AF+
D+AF-
D+AF+
Ponceau
B
-AF
+AF
Protein carbonyls (A.U.)
15
10
5
0
0.0547
ND
D

Figure 4.4: Protein carbonyls in atrial tissue of patients with T2DM and AF.

(A) Representative blot of an Oxyblot and Ponceau stain. (B) Quantification was done by Image J and data are represented as mean ± SEM. Two-way ANOVA with Tukey post-hoc test; *$p<0.05$.

4.6 DISCUSSION

Clinical risk factors for AF are well recognised (*e.g.*, aging, obesity, T2DM) and recent research has elucidated aspects of AF-induced remodelling (Watanabe *et al.*, 2008; Andrade *et al.*, 2014; Nyström *et al.*, 2015). However, our understanding of metabolic factors in the atrium remain limited (Opacic *et al.*, 2016). Indeed atrial metabolism is not well studied although some important advances have been made in recent years. In 2003, Lai *et al.* showed that in older patients with AF there is an accumulation of mtDNA mutations in atrial tissue (Lai *et al.*, 2003). Furthermore, there is an association between the fibrillating atrium and mitochondrial dysfunction and morphology (Mihm *et al.*, 2001; Bukowska *et al.*, 2008; Reilly *et al.*, 2011; Redpath *et al.*, 2013; Zou *et al.*, 2016). Thus in order to understand the role of mitochondrial oxidative phosphorylation in AF, we examined characteristics of mitochondrial structure and function in human cardiac myofibers. Our study is the first to address such factors in human patients with T2DM, and, is the first to our knowledge to analyze mitochondrial bioenergetics or supercomplexes in the human atrium.

Mitochondrial oxidative phosphorylation (OXPHOS) is responsible for the production of more than 90% of myocardial cell ATP production (Ventura-Clapier *et al.*, 2004). Mitochondrial respiration is driven by the flow of electrons or "reducing equivalents" through the ETC complexes, which drives the pumping of protons into the mitochondrial intermembrane space, thereby generating protonmotive force. Protons move back into the matrix through ATP synthase resulting in ATP production and the overall process is referred to as oxidative phosphorylation. Myocardial ATP demand is constant, due to its vital role in maintaining cardiac output (Neubauer, 2007a), and is largely met in the healthy heart through

fatty acid oxidation (60-90%) and through glycolysis to a lesser extent (10-40%) (Stanley *et al.,* 2005). In patients with T2DM, the heart relies on lipid oxidation more than glucose oxidation, even during hyperglycemia (Heather and Clarke, 2011; Aon *et al.,* 2015). The implications of sustained high frequency activation on cardiac tissue, as occurs in AF, are unknown.

Thus the application of novel approaches for the study of mitochondrial structure and function is important for an improved understanding atrial function. We used high resolution respirometry of atrial muscle fibers, which were studied immediately (< 3h) after CABG surgery. Results from these experiments showed impaired complex I and maximal (I+II) phosphorylating respiration in diabetic patients with AF compared to diabetic patients without AF. This was associated with an increase in maximal complex IV activity. In a recent study, homogenates of atrial tissues from non-diabetic patients with and without AF were studied and enzyme activities of the complexes were determined spectrophotometrically. Lower enzymatic activities were recorded for complex I and complex II, and an increase in complex V (ATP synthase) activity was observed, consistent with the possibility of impaired mitochondrial respiration in patients with AF (Emelyanova *et al.,* 2016). However, these determinations were in homogenates of frozen tissues by using enzymatic assays. Our study has addressed these questions by measuring the oxidative phosphorylation (OXPHOS) system in fresh atrial tissue. Using this approach, mitochondria remain in their reticular structures *in situ* in the muscle fibers, and the activities of the complexes are assessed in the intact OXPHOS system with the coordinated activities of electron flow and proton efflux.

The idea that OXPHOS complexes can bind together to form supercomplexes was first proposed by Chance and Williams over 60 years ago (Chance and Williams, 1955), but our understanding of these structures remains very limited. It was only recently discovered that supercomplexes assembly factors can affect mitochondrial respiration and ROS production (Lapuente-Brun *et al.,* 2013; Maranzana *et al.,* 2013). Here we investigated supercomplex formation and show impaired supercomplex assembly in AF patients. Supercomplexes can be divided into 4 main groups according to their assembly of ETC complexes: I+ III_2 (abundant in plants), $III_2 + IV_{1–2}$ (abundant in fungi) and $I + III_2 + IV_{1–4}$ (abundant in mammals) in addition to complex V dimer formation (Chaban *et al.,* 2014).

In addition to traditional $I + III_2 + IV_{1–4}$ –supercomplexes, we found much larger higher order supercomplexes than had been previously been documented for other human tissues (Antoun *et al.,* 2015; Greggio *et al.,* 2017). These high order supercomplexes are consistent with the respiratory string theory (Wittig *et al.,* 2006), which contends that supercomplex formation is not the highest level of OXPHOS organization. The string form is likely due to complex IV interaction with a neighbouring complex IV aligning 2 traditional $I+ III_2 + IV_4$ supercomplexes side by side (Wittig *et al.,* 2006), although little is known about the species and tissue specificity of respiratory strings. It has been hypothesized that strings of complexes enhance electron flow as a result of reduced distances between the complexes and reduced ROS production, especially at complexes I and III (Vartak *et al.,* 2013; Dudkina *et al.,* 2015). Further experimentation is required to clarify why higher order respiratory strings were detectable when immunoblotting for Complex I and Complex IV, but not with Complex III antibodies. Speculatively, the Complex III epitope could be masked

in the higher order string confirmation, and is thus not detectable. Nevertheless, our findings are consistent with the notion that the reduction in respiratory strings in the atrial myocardium of AF patients could impair electron flow and cause an increase in ROS production, which could, in turn, result in oxidative damage. As there were no changes in the levels of atrial collagen, it appears that the mitochondrial dysfunction and atrial protein oxidation was not associated with concomitant fibrosis.

In summary, our findings show that AF is associated with impaired mitochondrial OXPHOS, decreased supercomplex assembly into respiratory strings, and increased protein oxidative damage in atrial myocardial tissue of patients with T2DM. Despite our small sample size, our findings represent important 'proof-of-concept' discoveries. This is the first study, to our knowledge, that has examined mitochondrial energetics in intact atrial myofibers from patients with atrial fibrillation. Future research should address the factors underlying the impaired mitochondrial energetics and supercomplex formation.

4.7 REFERENCES

Andrade, J., Khairy, P., Dobrev, D., Nattel, S., 2014. The clinical profile and pathophysiology of atrial fibrillation: relationships among clinical features, epidemiology, and mechanisms. Circ. Res. 114, 1453–1468. doi:10.1161/CIRCRESAHA.114.303211

Antoun, G., McMurray, F., Thrush, A. B., Patten, D. A., Peixoto, A. C., Slack, R. S., … Harper, M.-E. (2015). Impaired mitochondrial oxidative phosphorylation and supercomplex assembly in rectus abdominis muscle of diabetic obese individuals. *Diabetologia*, *58*(12), 2861–2866. https://doi.org/10.1007/s00125-015-3772-8

Benjamin, E. J., Wolf, P. A., D'Agostino, R. B., Silbershatz, H., Kannel, W. B., & Levy, D. (1998). Impact of atrial fibrillation on the risk of death: the Framingham Heart Study. *Circulation*, *98*(10), 946–952.

Benjamin, E.J., Blaha, M.J., Chiuve, S.E., Cushman, M., Das, S.R., Deo, R., de Ferranti, S.D., Floyd, J., Fornage, M., Gillespie, C., Isasi, C.R., Jiménez, M.C., Jordan, L.C., Judd, S.E., Lackland, D., Lichtman, J.H., Lisabeth, L., Liu, S., Longenecker, C.T., Mackey, R.H., Matsushita, K., Mozaffarian, D., Mussolino, M.E., Nasir, K., Neumar, R.W., Palaniappan, L., Pandey, D.K., Thiagarajan, R.R., Reeves, M.J., Ritchey, M., Rodriguez, C.J., Roth, G.A., Rosamond, W.D., Sasson, C., Towfighi, A., Tsao, C.W., Turner, M.B., Virani, S.S., Voeks, J.H., Willey, J.Z., Wilkins, J.T., Wu, J.H., Alger, H.M., Wong, S.S., Muntner, P., American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2017. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation 135, e146–e603. doi:10.1161/CIR.0000000000000485

Bukowska, A., Schild, L., Keilhoff, G., Hirte, D., Neumann, M., Gardemann, A., … Lendeckel, U. (2008). Mitochondrial dysfunction and redox signaling in atrial tachyarrhythmia. *Experimental Biology and Medicine (Maywood, N.J.)*, *233*(5), 558–574. https://doi.org/10.3181/0706-RM-155

Chaban, Y., Boekema, E. J., & Dudkina, N. V. (2014). Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. *Biochimica Et Biophysica Acta*, *1837*(4), 418–426. https://doi.org/10.1016/j.bbabio.2013.10.004

Diabetes Canada. (2017, September 7). Diabetes Statistics in Canada. Retrieved June 19, 2017, from http://www.diabetes.ca/how-you-can-help/advocate/why-federal-leadership-is-essential/diabetes-statistics-in-canada

Dudkina, N. V., Folea, I. M., & Boekema, E. J. (2015). Towards structural and functional characterization of photosynthetic and mitochondrial supercomplexes. *Micron (Oxford, England: 1993)*, *72*, 39–51. https://doi.org/10.1016/j.micron.2015.03.002

Dudkina, N. V., Kouril, R., Peters, K., Braun, H.-P., & Boekema, E. J. (2010). Structure and function of mitochondrial supercomplexes. *Biochimica Et Biophysica Acta*, *1797*(6–7), 664–670. https://doi.org/10.1016/j.bbabio.2009.12.013

Greggio, C., Jha, P., Kulkarni, S. S., Lagarrigue, S., Broskey, N. T., Boutant, M., … Amati, F. (2017). Enhanced Respiratory Chain Supercomplex Formation in Response to Exercise in Human Skeletal Muscle. Cell Metabolism, 25(2), 301–311. https://doi.org/10.1016/j.cmet.2016.11.004

Heart and Stroke. (2017, September 7). Atrial fibrillation. Retrieved September 8, 2017, from https://www.heartandstroke.ca:443/heart/conditions/atrial-fibrillation

Heijman, J., Voigt, N., Nattel, S., Dobrev, D., 2014. Cellular and molecular electrophysiology of atrial fibrillation initiation, maintenance, and progression. Circ. Res. 114, 1483–1499. doi:10.1161/CIRCRESAHA.114.302226

Heijman, J., Algalarrondo, V., Voigt, N., Melka, J., Wehrens, X.H.T., Dobrev, D., Nattel, S., 2016. The value of basic research insights into atrial fibrillation mechanisms as a guide to therapeutic innovation: a critical analysis. Cardiovasc Res 109, 467–479. doi:10.1093/cvr/cvv275

Iguchi, Y., Kimura, K., Aoki, J., Kobayashi, K., Terasawa, Y., Sakai, K., & Shibazaki, K. (2008). Prevalence of atrial fibrillation in community-dwelling Japanese aged 40 years or older in Japan: analysis of 41,436 non-employee residents in Kurashiki-city. *Circulation Journal: Official Journal of the Japanese Circulation Society*, *72*(6), 909–913.

Khacho, M., Tarabay, M., Patten, D., Khacho, P., MacLaurin, J. G., Guadagno, J., … Slack, R. S. (2014). Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival. *Nature Communications,* 5, 3550. https://doi.org/10.1038/ncomms4550

Lai, L.-P., Tsai, C.-C., Su, M.-J., Lin, J.-L., Chen, Y.-S., Tseng, Y.-Z., & Huang, S. K. S. (2003). Atrial fibrillation is associated with accumulation of aging-related common type mitochondrial DNA deletion mutation in human atrial tissue. *Chest*, *123*(2), 539–544.

Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellicer, A., Colás, C., Balsa, E., … Enríquez, J. A. (2013). Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science (New York, N.Y.), 340(6140), 1567–1570. https://doi.org/10.1126/science.1230381

Maranzana, E., Barbero, G., Falasca, A. I., Lenaz, G., & Genova, M. L. (2013). Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. Antioxidants & Redox Signaling, 19(13), 1469–1480. https://doi.org/10.1089/ars.2012.4845

Murphy, N. F., Simpson, C. R., Jhund, P. S., Stewart, S., Kirkpatrick, M., Chalmers, J., … McMurray, J. J. V. (2007). A national survey of the prevalence, incidence, primary care burden and treatment of atrial fibrillation in Scotland. *Heart (British Cardiac Society)*, *93*(5), 606–612. https://doi.org/10.1136/hrt.2006.107573

NCD Risk Factor Collaboration (NCD-RisC). (2016). Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet

(London, England), 387(10027), 1513–1530. https://doi.org/10.1016/S0140-6736(16)00618-8

Opacic, D., van Bragt, K. A., Nasrallah, H. M., Schotten, U., & Verheule, S. (2016). Atrial metabolism and tissue perfusion as determinants of electrical and structural remodelling in atrial fibrillation. Cardiovascular Research, 109(4), 527–541. https://doi.org/10.1093/cvr/cvw007

Redpath, C. J., Bou Khalil, M., Drozdzal, G., Radisic, M., & McBride, H. M. (2013). Mitochondrial hyperfusion during oxidative stress is coupled to a dysregulation in calcium handling within a C2C12 cell model. *PloS One*, *8*(7), e69165. https://doi.org/10.1371/journal.pone.0069165

Reilly, S. N., Jayaram, R., Nahar, K., Antoniades, C., Verheule, S., Channon, K. M., … Casadei, B. (2011). Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. *Circulation*, *124*(10), 1107–1117. https://doi.org/10.1161/CIRCULATIONAHA.111.029223

Sun, Y., & Hu, D. (2010). The link between diabetes and atrial fibrillation: cause or correlation? *Journal of Cardiovascular Disease Research*, *1*(1), 10–11. https://doi.org/10.4103/0975-3583.59978

Tadic, M., Cuspidi, C., 2015. Type 2 diabetes mellitus and atrial fibrillation: From mechanisms to clinical practice. Arch Cardiovasc Dis 108, 269–276. doi:10.1016/j.acvd.2015.01.009

Vartak, R., Porras, C. A.-M., & Bai, Y. (2013). Respiratory supercomplexes: structure, function and assembly. *Protein & Cell*, *4*(8), 582–590. https://doi.org/10.1007/s13238-013-3032-y

Ventura-Clapier, R., Garnier, A., & Veksler, V. (2004). Energy metabolism in heart failure. The Journal of Physiology, 555(Pt 1), 1–13. https://doi.org/10.1113/jphysiol.2003.055095

Wijffels, M.C., Kirchhof, C.J., Dorland, R., Allessie, M.A., 1995. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation 92, 1954–1968.

Wittig, I., Carrozzo, R., Santorelli, F. M., & Schägger, H. (2006). Supercomplexes and subcomplexes of mitochondrial oxidative phosphorylation. *Biochimica Et Biophysica Acta*, *1757*(9–10), 1066–1072. https://doi.org/10.1016/j.bbabio.2006.05.006

World Health Organization. (2016). *WHO Global Report on Diabetes* (p. 25). Retrieved from http://www.who.int/diabetes/global-report/en/

Zou, D., Geng, N., Chen, Y., Ren, L., Liu, X., Wan, J., … Wang, S. (2016). Ranolazine improves oxidative stress and mitochondrial function in the atrium of acetylcholine-CaCl2

induced atrial fibrillation rats. *Life Sciences*, *156*, 7–14. https://doi.org/10.1016/j.lfs.2016.05.026

CHAPTER 5 – GENERAL DISCUSSION

In the last few decades the incidence of obesity, diabetes, cardiovascular diseases and atrial fibrillation has increased globally. These diseases are now affecting more people every year, increasing the financial burden on healthcare systems globally. Our understanding of the etiology of these diseases has improved significantly over the last few decades, and this has led to a better preventative and treatment strategies. However, many fundamentally important questions remain regarding the etiology of these prevalent metabolic diseases. Advances in research allowed us to determine a common feature in these diseases: mitochondrial dysfunction. The overarching goal of this doctoral research was to investigate mitochondrial dysfunction in muscle cells and tissues of mice and humans.

The goal of chapter 2 of this thesis was to assess if in utero growth retardation (IUGR) impacts primary muscle cells derived from undernourished mouse dams. IUGR is caused by an inadequate supply of oxygen and nutrients to the growing fetus and is also associated with the development of obesity, insulin resistance and cardiovascular disease in adulthood in humans and in experimental animals (Barker *et al.*, 1989, 1993a, 1993b; Hales *et al.*, 1991; Ravelli *et al.*, 1999; Gluckman *et al.*, 2008). It is hypothesized that hostile conditions during early life development periods increase susceptibility to disease in adulthood (Barker, 2004, 2007; Gluckman and Hanson, 2004).

Previous work done by our group showed that female adult offspring from mouse dams undergoing 50% food restriction during the third week of gestation, have decreased mitochondrial content in skeletal muscle, decreased mitochondrial respiration in skeletal and

cardiac muscles in addition to increased adiposity and decreased glucose tolerance (Beauchamp *et al.,* 2014, 2015; Beauchamp and Harper, 2015). These metabolic adaptations are thought to occur to maintain energy homeostasis in the offspring in an 'anticipated' nutrient-limited environment (Barker, 2007). In chapter 2 we wanted to extend our observations in skeletal muscle and investigate if these metabolic characteristics are also present in primary muscle cells isolated from in utero undernourished offspring. Our findings show that myotubes from undernourished mice have dysfunctional fatty acid oxidation, enhanced glycolysis, no alterations in mitochondrial content and pronounced differences in transcriptional profiles. For example, an upregulation of genes implicated in oxidative phosphorylation was observed in control cells cultured in glucose-poor medium. On the other hand, cells from offspring of undernourished dams have a decreased expression of a group of genes normally downregulated during adipogenic differentiation of 3T3-L1 cells. These results are align with the decreased respiration in fibers and isolated mitochondria of skeletal muscle from offspring of food restricted dams and are consistent with the decreased oxidative activity and increased glycolytic capacity observed in humans with obesity and T2DM (Beauchamp *et al.,* 2014; Mogensen *et al.,* 2007; Colberg *et al.,* 1995; Kelley *et al.,* 1999; Simoneau *et al.,* 1995; Simoneau and Kelley, 1997). Altogether, our microarray analyses provide the first evidence that in utero undernutrition impacts profoundly gene expression in skeletal muscle primary cells "programming" these cells to be adapted for times of limited nutrient availability, but that becomes detrimental if nutrient supplies are abundant.

Over the years, many studies have investigated the effects of IUGR in different animal models and in humans but gaps in knowledge still exist. From what we know, IUGR impacts skeletal muscle heavily predisposing the offspring to obesity, insulin resistance, T2DM and cardiovascular disease in adult life. The exact mechanisms mediating these effects remain largely unknown. Studies in sheep found that in utero reductions in nutrients reduce muscle mass and insulin sensitive fibers, which may help to explain the link between IUGR and adulthood insulin resistance and T2DM (Yates *et al.,* 2016). It is also predicted that reduced fetal muscle mass due to IUGR may augment catch-up growth, and in turn, adiposity and obesity (Brown and Hay, 2016). This is consistent with our previous findings of lower muscle mass in offspring of food restricted dams (Beauchamp *et al.,* 2014). Furthermore IUGR was shown to impact insulin signaling, myoblast incorporation, fiber hypertrophy, and glucose oxidation by affecting adrenergic receptor expression profile in sheep, thereby lowering their fatty acid oxidation capabilities, an effect that was observed even at 9 months of age (Yates *et al.,* 2012). In addition, in an intrauterine-restricted sheep model, myoblasts from fetuses exhibited intrinsic deficiencies in proliferation that did not affect differentiation (Yates *et al.,* 2014). The molecular mechanisms behind these effects are mostly unidentified but it has been shown that some of these effects are transgenerational suggesting that in utero undernutrition drives heritable changes in gene expression (Liguori *et al.,* 2010). For example, obesity and impaired glucose metabolism linked to low birth weight were shown to be transmitted across generations in mice (Jimenez-Chillaron *et al.,* 2009, 2016). This intergenerational transmission is due to epigenetic mechanisms modifying DNA and histones and resulting in changes in gene expression without altering the DNA

sequence. One modification that can cause long-term changes in gene expression is altered DNA methylation. DNA methylation is linked to stable variations in gene expression and is established mainly in utero. Increased DNA methylation causes the suppression of gene transcription (Jeltsch and Jurkowska, 2014). Thus we can understand the importance of the fetal environment in inducing changes in gene expression especially through methylation. It has been shown that IUGR rats have increased methylation of the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α), along with decreases in PGC-1α transcription activity, mitochondrial content and proteins in the insulin signaling pathway (Xie *et al.,* 2015b).

Overall our findings in this chapter have revealed important new insights into the effects of fetal programming on skeletal muscle myotubes including profound impairments in oxidative metabolism, enhanced glycolytic metabolism and altered gene expression.

Research described in chapter 3 aimed to investigate mitochondrial glutathione redox homeostasis in both mouse and human hearts. Energy demands of the heart are unsurpassed by those of any other organ in the body; thus the control of oxidative phosphorylation is of central importance. The heart beats about 100,000 times daily to pump approximately 10 tons of blood delivering oxygen and nutrients to the entire body and consuming almost 6 kg of ATP per day (Neubauer, 2007). The crucial role of efficient OXPHOS while minimizing oxidative stress is the main task of the glutathione system. In chapter 3, we investigated the importance of Grx2, a glutathione-dependent oxidoreductase, in intact primary cardiomyocytes and in cardiac tissue in mice and followed this by studying Genotype-Tissue

Expression (GTEx), the human database and associated tissue bank. We also studied the effect of the glutathione precursor, N-acetylcysteine (NAC) on mice in vivo and on cells in vitro. It was found that the absence of Grx2 affects mitochondrial dynamics, morphology and energetics in primary cardiac cells and tissue. Supplementation of NAC to Grx2 deficient mice did not restore glutathione redox or prevent impairments. Using GTEx we demonstrated for the first time that low GRX2 expression is linked to increased fibrosis, hypertrophy and infarct in the left ventricle of the human heart.

The idea of studying Grx2 came from the fact that during OXPHOS reactive oxygen species (ROS) and reactive nitrogen species (RNS) can form and cause oxidative stress, if these species are not balanced by antioxidant systems (Kovacic *et al.,* 2005). Since glutathione is central to redox homeostasis and is thought to be the most important non-protein antioxidant in cells, we studied the mitochondrial isoform of the glutathione-dependent glutaredoxin enzymes, Grx2 (Dröge, 2002). These enzymes are disulfide oxidoreductases that reduce GSH-protein mixed disulfides and protect proteins from oxidative damage (Koehler *et al.,* 2006; Jacob *et al.,* 2006). There are important gaps in our knowledge about the function of Grx2 and its role in metabolism. Since its characterisation in humans in 2001 by Lundberg et al., Grx2 has been shown to catalyze de/glutathionylation reactions; to interact with other antioxidant enzymes like thioredoxin 1, thioredoxin 2 and peroxiredoxin 3; to function as a possible redox sensor; to control proton leak through uncoupling proteins -2 and -3 (UCP2 and UCP3) and to reduce oxidative stress (Lundberg *et al.,* 2001; Johansson et al., 2004; Hanschmann *et al.,* 2010; Wu *et al.,* 2011; Schütte *et al.,* 2013; Mailloux *et al.,* 2013; Zhang *et al.,* 2014). Furthermore previous work from our group

and others showed that when Grx2 is deficient in mice, they suffer from fibrotic cardiac hypertrophy and hypertension (in males) and from early onset age-dependent cataract formation (Mailloux *et al.,* 2014; Wu *et al.,* 2014). In rats, cardiac Grx2 expression was shown to be increased in the mitochondrial matrix by up to 2.6 fold in elderly vs young hearts and was activated by accumulation of ROS acting as a redox sensor and protecting the heart from oxidative damage (Gao *et al.,* 2013).

In our study, we used NAC, which is also a commercially available nutritional supplement used by athletes as an antioxidant and GSH precursor (Peake and Suzuki, 2004) to investigate hypothesized benefits of restoring glutathione redox in Grx2-/- mice. These mice were given NAC for 6 weeks in the period in which they develop hypertrophy and hypertension. Our results demonstrate that the supplementation was not effective in preventing the development of these pathologies. In addition, NAC did not ameliorate the disordered mitochondrial respiration or prevent fibrosis; it also did not change GSH levels in the heart and liver. On the contrary, NAC lowered mitochondrial respiration in cardiac tissue of WT mice and worsened fibrosis in Grx2-/- mice. These results were unexpected since some studies showed that NAC is able to lower oxidative stress, to restore cardiac function and damage, to reverse hypertension and to increase GSH levels in tissues (Adamy *et al.,* 2007; Treweeke *et al.,* 2012; Rushworth and Megson, 2014; Wilder *et al.,* 2015; Giam *et al.,* 2016). None of the studies mentioned above was conducted in Grx2 deficient mice. It could be that in the absence of this enzyme, NAC is causing glutathionylation of key cardiac proteins leading to sarcomere dysfunction, cardiac hypertrophy and hypertension, increased

fibrosis and mitochondrial dysfunction, which has been observed in other situations (Passarelli *et al.,* 2010; Wang *et al.,* 2011; Wilder *et al.,* 2015).

Moreover previous research has shown that mitochondrial dynamics are affected by glutathione redox (Shutt *et al.,* 2012; Mishra and Chan, 2016). Our findings demonstrate that the absence of Grx2 caused neonatal cardiomyocyte mitochondria to elongate, and NAC did not impact this mitochondrial tabulation, showing for the first time the impact of Grx2 absence on mitochondrial dynamics in primary cells. These findings show the importance of Grx2 in the heart and shed light on the use of NAC as a nutritional supplement. Our results are consistent with the conclusion that taking NAC in a Grx2 deficient environment could lead to harmful effects. A limitation to our study is the fact that it has been conducted in mice and not in humans. Finally we were the first to study GRX2 expression in the left ventricle of adult humans. Using GTEx, we demonstrated an inverse correlation between GRX2 expression and cardiac hypertrophy, fibrosis and infarct. This is the first evidence of a cardioprotective role for GRX2 in adult humans. More research is needed to elucidate the full mechanism of Grx2 and its targets in addition to its impact and potential therapeutic implications in human heart disease.

Overall, findings in chapter 3 show that Grx2 is a key player in controlling oxidative and glycolytic functions in cardiomyocytes and in cardiac tissue; that Grx2 is crucial for cardiac mitochondrial dynamics and morphology in both mice and humans, and that NAC supplementation did not ameliorate any defect in mice in vivo or in vitro. More research on Grx2 will allow a better understanding of this enzyme and its involvement in essential

cellular processes and make it a potential target for future therapeutics against cardiovascular diseases.

In chapter 4 we investigated atrial muscle mitochondrial energetics in patients with AF with or without T2DM. As mentioned earlier, T2DM is on the rise and the prevalence of AF is also increasing. It is estimated that T2DM will affect 5 million Canadians in 2025 (Diabetes Canada, 2017). On the other hand, AF currently affects 350,000 Canadians and these numbers are estimated to rise (Andrade *et al.*, 2014; Benjamin *et al.*, 2017). Beyond the substantial morbidity and mortality associated with these diseases, the financial and social burdens on the society and governing entities are tremendous. Although T2DM commonly coexists in patients with AF (≤ 20%), a relationship between the two is not yet confirmed (Sun and Hu, 2010). Additionally although some important advances have been made in recent years, our understanding of metabolic factors in the atrium remains limited (Opacic *et al.*, 2016). In our study we have for the first time assessed mitochondrial function and structure in fresh atrial appendages collected from patients undergoing CABG surgery. We showed impaired complex I and maximal (I+II) phosphorylating respiration in patients with T2DM and AF, compared to patients with T2DM but do not have AF. In addition we observed increased maximal complex IV activity. Our results extend previous observations in patients without T2DM but with AF in which enzymatic activities of complex I and complex II decrease, whereas complex V activity increased (Emelyanova *et al.*, 2016). In the aforementioned study, mitochondrial function determinations were conducted spectrophotometrically using frozen tissues, which is not ideal for functional analyses. Our approach in permeabilized cardiac myofibers is the most physiological method available, and

allows the study of mitochondrial energetics in situ in cells, without damaging the mitochondrial environment.

Moreover it is known that during AF there is a significant increase in atrial activation frequency, thereby altering atrial electrical, structural and mechanical functions (Heijman *et al.,* 2014, 2016). This rapid electrical activity increases ATP demands that need to be met through mitochondrial OXPHOS and concomitant increases in oxygen and nutrient supply (Opacic *et al.,* 2016). We also measured the levels of mitochondrial OXPHOS proteins and found that they were unchanged; thus the increased ATP demands are most likely being met by increased activity of OXPHOS. Furthermore, we have been the first to address the possibility that this increase in ATP demand could lead to increases in ETC supercomplex formation, e.g., as a compensatory mechanism. Supercomplexes are first mentioned over 60 years ago but it is only very recently that progress has been made in understanding their structure and function. Supercomplexes impact mitochondrial respiration and ROS production (Lapuente-Brun *et al.,* 2013; Maranzana *et al.,* 2013). Previous work from our group showed that supercomplex assembly is impaired in skeletal muscle of individuals with T2DM (Antoun *et al.,* 2015). Thus we aimed to investigate supercomplex formation in the atrium and our findings demonstrated impaired supercomplex assembly in AF patients. Specifically we observed impaired higher order supercomplexes similar to previous findings in human skeletal muscle tissue from diabetic patients and healthy exercising individuals (Antoun *et al.,* 2015; Greggio *et al.,* 2017). To our knowledge, our study is the first to investigate supercomplex assembly in human hearts and in AF patients. High order supercomplexes are consistent with the respiratory string theory (Wittig *et al.,* 2006), and are

hypothesized to enhance electron flow through the complexes due to shortened distances and reduced ROS production (Vartak *et al.,* 2013; Dudkina *et al.,* 2015). It is unknown if the decrease in supercomplexes in AF patients with T2DM can lead to increased ROS production resulting in oxidative damage and exacerbation of AF. Thus we checked protein carbonyl adducts in atrial tissue and found a strong trend for increased protein oxidation in patients with AF and T2DM. There were no differences in levels of tissue fibrosis. Increases in protein oxidation can have severe consequences on protein function; for example, it has been shown that increased oxidation of the ryanodine receptor (RyR2) can lead to calcium leak, which is fundamental in AF pathophysiology (Zhang *et al.,* 2011; Shan *et al.*, 2012; Xie *et al.,* 2013, 2015a).

Altogether our research described in chapter 4 shows impaired atrial energetics and supercomplex assembly in patients with T2DM and AF, and a strong trend for an increase in protein oxidation. While patient numbers were low, our results comprise the first proof-of-concept for mitochondrial dysfunction in fresh human atrial tissue in T2DM and AF. Future research is needed to fully understand the relationship between T2DM and AF, and more fundamentally, the role of mitochondria in arrhythmia. This will in the future allow researchers to design drugs and molecules to target specific mitochondrial proteins with specific functions, thus providing possible tools for the early diagnosis and treatment of AF.

In conclusion, research conducted during my doctoral studies has allowed a better understanding of mitochondrial dysfunction and its implications in metabolic diseases. From in utero undernutrition in mice and its effects on skeletal muscle primary cell oxidative and

glycolytic functions and gene programming through impaired mitochondrial respiration, dynamics and morphology in the absence of Grx2 in the heart of mice and humans resulting cardiac hypertrophy, fibrosis and hypertension to impaired oxidative function and supercomplex assembly in patients with T2DM and AF, there is one common element: mitochondrial dysfunction. More research is needed to better comprehend the full implications of mitochondria in metabolic diseases in order to achieve future personalized treatments that target these important organelles.

5.1 REFERENCES

Adamy, C., Mulder, P., Khouzami, L., Andrieu-abadie, N., Defer, N., Candiani, G., Pavoine, C., Caramelle, P., Souktani, R., Le Corvoisier, P., et al. (2007). Neutral sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats. J. Mol. Cell. Cardiol. *43*, 344–353.

Andrade, J., Khairy, P., Dobrev, D., and Nattel, S. (2014). The clinical profile and pathophysiology of atrial fibrillation: relationships among clinical features, epidemiology, and mechanisms. Circ. Res. *114*, 1453–1468.

Antoun, G., McMurray, F., Thrush, A.B., Patten, D.A., Peixoto, A.C., Slack, R.S., McPherson, R., Dent, R., and Harper, M.-E. (2015). Impaired mitochondrial oxidative phosphorylation and supercomplex assembly in rectus abdominis muscle of diabetic obese individuals. Diabetologia *58*, 2861–2866.

Barker, D.J.P. (2004). The developmental origins of adult disease. J. Am. Coll. Nutr. *23*, 588S–595S.

Barker, D.J.P. (2007). The origins of the developmental origins theory. J. Intern. Med. *261*, 412–417.

Barker, D.J., Osmond, C., Golding, J., Kuh, D., and Wadsworth, M.E. (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ *298*, 564–567.

Barker, D.J., Hales, C.N., Fall, C.H., Osmond, C., Phipps, K., and Clark, P.M. (1993a). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia *36*, 62–67.

Barker, D.J., Gluckman, P.D., Godfrey, K.M., Harding, J.E., Owens, J.A., and Robinson, J.S. (1993b). Fetal nutrition and cardiovascular disease in adult life. Lancet Lond. Engl. *341*, 938–941.

Beauchamp, B., and Harper, M.-E. (2015). In utero Undernutrition Programs Skeletal and Cardiac Muscle Metabolism. Front. Physiol. *6*, 401.

Beauchamp, B., Ghosh, S., Dysart, M.W., Kanaan, G.N., Chu, A., Blais, A., Rajamanickam, K., Tsai, E.C., Patti, M.E., and Harper, M.E. (2014). Low birth weight is associated with adiposity, impaired skeletal muscle energetics and weight loss resistance in mice. Int. J. Obes. 2005.

Beauchamp, B., Thrush, A.B., Quizi, J., Antoun, G., McIntosh, N., Al-Dirbashi, O.Y., Patti, M.-E., and Harper, M.-E. (2015). Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring. Biosci. Rep. *35*.

Benjamin, E.J., Blaha, M.J., Chiuve, S.E., Cushman, M., Das, S.R., Deo, R., de Ferranti, S.D., Floyd, J., Fornage, M., Gillespie, C., et al. (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation *135*, e146–e603.

Brown, L.D., and Hay, W.W. (2016). Impact of placental insufficiency on fetal skeletal muscle growth. Mol. Cell. Endocrinol. *435*, 69–77.

Colberg, S.R., Simoneau, J.A., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle utilization of free fatty acids in women with visceral obesity. J. Clin. Invest. *95*, 1846–1853.

Diabetes Canada. (2017, September 7). Diabetes Statistics in Canada. Retrieved June 19, 2017, from http://www.diabetes.ca/how-you-can-help/advocate/why-federal-leadership-is-essential/diabetes-statistics-in-canada

Dröge, W. (2002). Free radicals in the physiological control of cell function. Physiol. Rev. *82*, 47–95.

Dudkina, N.V., Folea, I.M., and Boekema, E.J. (2015). Towards structural and functional characterization of photosynthetic and mitochondrial supercomplexes. Micron Oxf. Engl. 1993 *72*, 39–51.

Emelyanova, L., Ashary, Z., Cosic, M., Negmadjanov, U., Ross, G., Rizvi, F., Olet, S., Kress, D., Sra, J., Tajik, A.J., et al. (2016). Selective downregulation of mitochondrial electron transport chain activity and increased oxidative stress in human atrial fibrillation. Am. J. Physiol. Heart Circ. Physiol. *311*, H54-63.

Gao, X.-H., Qanungo, S., Pai, H.V., Starke, D.W., Steller, K.M., Fujioka, H., Lesnefsky, E.J., Kerner, J., Rosca, M.G., Hoppel, C.L., et al. (2013). Aging-dependent changes in rat heart mitochondrial glutaredoxins—Implications for redox regulation. Redox Biol. *1*, 586–598.

Giam, B., Chu, P.-Y., Kuruppu, S., Smith, A.I., Horlock, D., Kiriazis, H., Du, X.-J., Kaye, D.M., and Rajapakse, N.W. (2016). N-acetylcysteine attenuates the development of cardiac fibrosis and remodeling in a mouse model of heart failure. Physiol. Rep. *4*.

Gluckman, P.D., and Hanson, M.A. (2004). Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr. Res. *56*, 311–317.

Gluckman, P.D., Hanson, M.A., Cooper, C., and Thornburg, K.L. (2008). Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med. *359*, 61–73.

Greggio, C., Jha, P., Kulkarni, S.S., Lagarrigue, S., Broskey, N.T., Boutant, M., Wang, X., Conde Alonso, S., Ofori, E., Auwerx, J., et al. (2017). Enhanced Respiratory Chain Supercomplex Formation in Response to Exercise in Human Skeletal Muscle. Cell Metab. *25*, 301–311.

Hales, C.N., Barker, D.J., Clark, P.M., Cox, L.J., Fall, C., Osmond, C., and Winter, P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. BMJ *303*, 1019–1022.

Hanschmann, E.-M., Lönn, M.E., Schütte, L.D., Funke, M., Godoy, J.R., Eitner, S., Hudemann, C., and Lillig, C.H. (2010). Both thioredoxin 2 and glutaredoxin 2 contribute to the reduction of the mitochondrial 2-Cys peroxiredoxin Prx3. J. Biol. Chem. *285*, 40699–40705.

Heijman, J., Voigt, N., Nattel, S., and Dobrev, D. (2014). Cellular and molecular electrophysiology of atrial fibrillation initiation, maintenance, and progression. Circ. Res. *114*, 1483–1499.

Heijman, J., Algalarrondo, V., Voigt, N., Melka, J., Wehrens, X.H.T., Dobrev, D., and Nattel, S. (2016). The value of basic research insights into atrial fibrillation mechanisms as a guide to therapeutic innovation: a critical analysis. Cardiovasc. Res. *109*, 467–479.

Jacob, C., Knight, I., and Winyard, P.G. (2006). Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. Biol. Chem. *387*, 1385–1397.

Jeltsch, A., and Jurkowska, R.Z. (2014). New concepts in DNA methylation. Trends Biochem. Sci. *39*, 310–318.

Jimenez-Chillaron, J.C., Isganaitis, E., Charalambous, M., Gesta, S., Pentinat-Pelegrin, T., Faucette, R.R., Otis, J.P., Chow, A., Diaz, R., Ferguson-Smith, A., et al. (2009). Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. Diabetes *58*, 460–468.

Jimenez-Chillaron, J.C., Ramon-Krauel, M., Ribo, S., and Diaz, R. (2016). Transgenerational epigenetic inheritance of diabetes risk as a consequence of early nutritional imbalances. Proc. Nutr. Soc. *75*, 78–89.

Johansson, C., Lillig, C.H., and Holmgren, A. (2004). Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. J. Biol. Chem. *279*, 7537–7543.

Kelley, D.E., Goodpaster, B., Wing, R.R., and Simoneau, J.A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. Am. J. Physiol. *277*, E1130-1141.

Koehler, C.M., Beverly, K.N., and Leverich, E.P. (2006). Redox pathways of the mitochondrion. Antioxid. Redox Signal. *8*, 813–822.

Kovacic, P., Pozos, R.S., Somanathan, R., Shangari, N., and O'Brien, P.J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. Curr. Med. Chem. *12*, 2601–2623.

Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellicer, A., Colás, C., Balsa, E., Perales-Clemente, E., Quirós, P.M., Calvo, E., Rodríguez-Hernández, M.A., et al. (2013). Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science *340*, 1567–1570.

Liguori, A., Puglianiello, A., Germani, D., Deodati, A., Peschiaroli, E., and Cianfarani, S. (2010). Epigenetic Changes Predisposing to Type 2 Diabetes in Intrauterine Growth Retardation. Front. Endocrinol. *1*.

Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001). Cloning and Expression of a Novel Human Glutaredoxin (Grx2) with Mitochondrial and Nuclear Isoforms. J. Biol. Chem. *276*, 26269–26275.

Mailloux, R.J., Xuan, J.Y., Beauchamp, B., Jui, L., Lou, M., and Harper, M.E. (2013). Glutaredoxin-2 is required to control proton leak through uncoupling protein-3. J. Biol. Chem. *288*, 8365–8379.

Mailloux, R.J., Xuan, J.Y., McBride, S., Maharsy, W., Thorn, S., Holterman, C.E., Kennedy, C.R., Rippstein, P., deKemp, R., da Silva, J., et al. (2014). Glutaredoxin-2 is required to control oxidative phosphorylation in cardiac muscle by mediating deglutathionylation reactions. J. Biol. Chem. *289*, 14812–14828.

Maranzana, E., Barbero, G., Falasca, A.I., Lenaz, G., and Genova, M.L. (2013). Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. Antioxid. Redox Signal. *19*, 1469–1480.

Mishra, P., and Chan, D.C. (2016). Metabolic regulation of mitochondrial dynamics. J. Cell Biol. *212*, 379–387.

Mogensen, M., Sahlin, K., Fernström, M., Glintborg, D., Vind, B.F., Beck-Nielsen, H., and Højlund, K. (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes *56*, 1592–1599.

Neubauer, S. (2007). The failing heart--an engine out of fuel. N. Engl. J. Med. *356*, 1140–1151.

Opacic, D., van Bragt, K.A., Nasrallah, H.M., Schotten, U., and Verheule, S. (2016). Atrial metabolism and tissue perfusion as determinants of electrical and structural remodelling in atrial fibrillation. Cardiovasc. Res. *109*, 527–541.

Passarelli, C., Tozzi, G., Pastore, A., Bertini, E., and Piemonte, F. (2010). GSSG-mediated Complex I defect in isolated cardiac mitochondria. Int. J. Mol. Med. *26*, 95–99.

Peake, J., and Suzuki, K. (2004). Neutrophil activation, antioxidant supplements and exercise-induced oxidative stress. Exerc. Immunol. Rev. *10*, 129–141.

Ravelli, A.C., van Der Meulen, J.H., Osmond, C., Barker, D.J., and Bleker, O.P. (1999). Obesity at the age of 50 y in men and women exposed to famine prenatally. Am. J. Clin. Nutr. *70*, 811–816.

Rushworth, G.F., and Megson, I.L. (2014). Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. Pharmacol. Ther. *141*, 150–159.

Schütte, L.D., Baumeister, S., Weis, B., Hudemann, C., Hanschmann, E.-M., and Lillig, C.H. (2013). Identification of potential protein dithiol-disulfide substrates of mammalian Grx2. Biochim. Biophys. Acta *1830*, 4999–5005.

Shan, J., Xie, W., Betzenhauser, M., Reiken, S., Chen, B.-X., Wronska, A., and Marks, A.R. (2012). Calcium leak through ryanodine receptors leads to atrial fibrillation in 3 mouse models of catecholaminergic polymorphic ventricular tachycardia. Circ. Res. *111*, 708–717.

Shutt, T., Geoffrion, M., Milne, R., and McBride, H.M. (2012). The intracellular redox state is a core determinant of mitochondrial fusion. EMBO Rep. *13*, 909–915.

Simoneau, J.A., and Kelley, D.E. (1997). Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. J. Appl. Physiol. Bethesda Md 1985 *83*, 166–171.

Simoneau, J.A., Colberg, S.R., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *9*, 273–278.

Sun, Y., and Hu, D. (2010). The link between diabetes and atrial fibrillation: cause or correlation? J. Cardiovasc. Dis. Res. *1*, 10–11.

Treweeke, A.T., Winterburn, T.J., Mackenzie, I., Barrett, F., Barr, C., Rushworth, G.F., Dransfield, I., MacRury, S.M., and Megson, I.L. (2012). N-Acetylcysteine inhibits platelet-monocyte conjugation in patients with type 2 diabetes with depleted intraplatelet glutathione: a randomised controlled trial. Diabetologia *55*, 2920–2928.

Vartak, R., Porras, C.A.-M., and Bai, Y. (2013). Respiratory supercomplexes: structure, function and assembly. Protein Cell *4*, 582–590.

Wang, S.-B., Foster, D.B., Rucker, J., O'Rourke, B., Kass, D.A., and Van Eyk, J.E. (2011). Redox regulation of mitochondrial ATP synthase: implications for cardiac resynchronization therapy. Circ. Res. *109*, 750–757.

Wilder, T., Ryba, D.M., Wieczorek, D.F., Wolska, B.M., and Solaro, R.J. (2015). N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy. Am. J. Physiol. Heart Circ. Physiol. *309*, H1720-1730.

Wittig, I., Carrozzo, R., Santorelli, F.M., and Schägger, H. (2006). Supercomplexes and subcomplexes of mitochondrial oxidative phosphorylation. Biochim. Biophys. Acta *1757*, 1066–1072.

Wu, H., Lin, L., Giblin, F., Ho, Y.-S., and Lou, M.F. (2011). Glutaredoxin 2 knockout increases sensitivity to oxidative stress in mouse lens epithelial cells. Free Radic. Biol. Med. *51*, 2108–2117.

Wu, H., Yu, Y., David, L., Ho, Y.-S., and Lou, M.F. (2014). Glutaredoxin 2 (Grx2) gene deletion induces early onset of age-dependent cataracts in mice. J. Biol. Chem. *289*, 36125–36139.

Xie, W., Santulli, G., Guo, X., Gao, M., Chen, B.-X., and Marks, A.R. (2013). Imaging atrial arrhythmic intracellular calcium in intact heart. J. Mol. Cell. Cardiol. *64*, 120–123.

Xie, W., Santulli, G., Reiken, S.R., Yuan, Q., Osborne, B.W., Chen, B.-X., and Marks, A.R. (2015a). Mitochondrial oxidative stress promotes atrial fibrillation. Sci. Rep. *5*, 11427.

Xie, X., Lin, T., Zhang, M., Liao, L., Yuan, G., Gao, H., Ning, Q., and Luo, X. (2015b). IUGR with infantile overnutrition programs an insulin-resistant phenotype through DNA methylation of peroxisome proliferator-activated receptor-γ coactivator-1α in rats. Pediatr. Res. *77*, 625–632.

Yates, D.T., Macko, A.R., Nearing, M., Chen, X., Rhoads, R.P., and Limesand, S.W. (2012). Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. J. Pregnancy *2012*, 631038.

Yates, D.T., Clarke, D.S., Macko, A.R., Anderson, M.J., Shelton, L.A., Nearing, M., Allen, R.E., Rhoads, R.P., and Limesand, S.W. (2014). Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. J. Physiol. *592*, 3113–3125.

Yates, D.T., Cadaret, C.N., Beede, K.A., Riley, H.E., Macko, A.R., Anderson, M.J., Camacho, L.E., and Limesand, S.W. (2016). Intrauterine growth-restricted sheep fetuses exhibit smaller hindlimb muscle fibers and lower proportions of insulin-sensitive Type I fibers near term. Am. J. Physiol. - Regul. Integr. Comp. Physiol. *310*, R1020–R1029.

Zhang, H., Du, Y., Zhang, X., Lu, J., and Holmgren, A. (2014). Glutaredoxin 2 reduces both thioredoxin 2 and thioredoxin 1 and protects cells from apoptosis induced by auranofin and 4-hydroxynonenal. Antioxid. Redox Signal. *21*, 669–681.

Zhang, Y., Fraser, J.A., Jeevaratnam, K., Hao, X., Hothi, S.S., Grace, A.A., Lei, M., and Huang, C.L.-H. (2011). Acute atrial arrhythmogenicity and altered Ca(2+) homeostasis in murine RyR2-P2328S hearts. Cardiovasc. Res. *89*, 794–804.

CHAPTER 6-APPENDICES

6.1 APPENDIX A- SUPPLEMENTARY CHAPTER 3

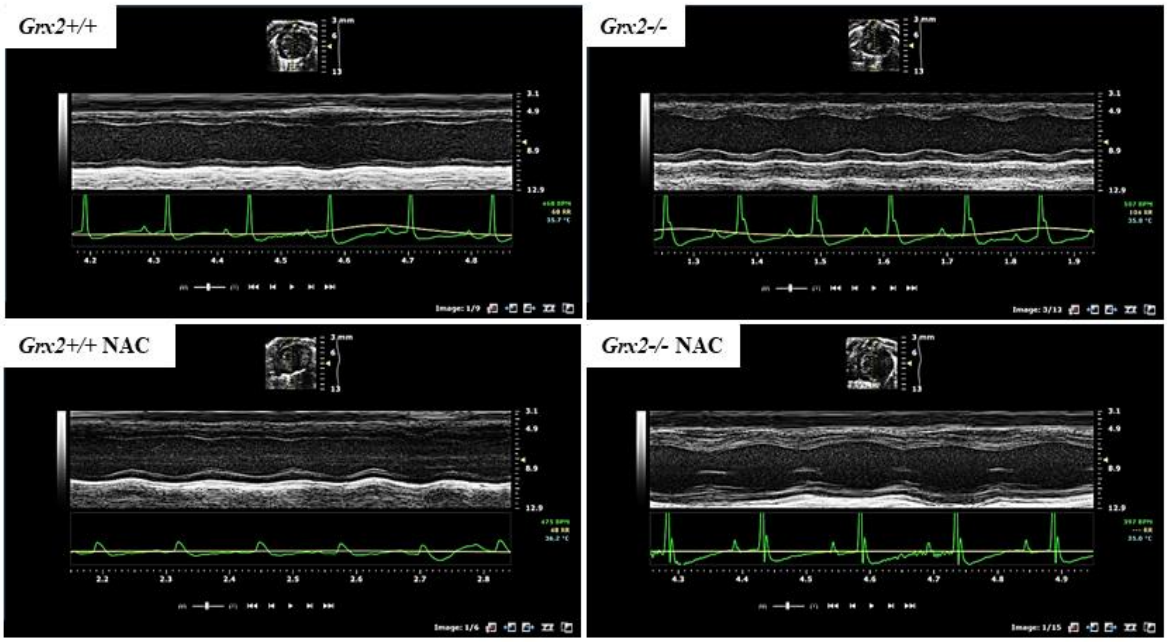


Figure 6.1: Representative M-mode images with the short-axis view of the echocardiographic analysis of the left ventricular.

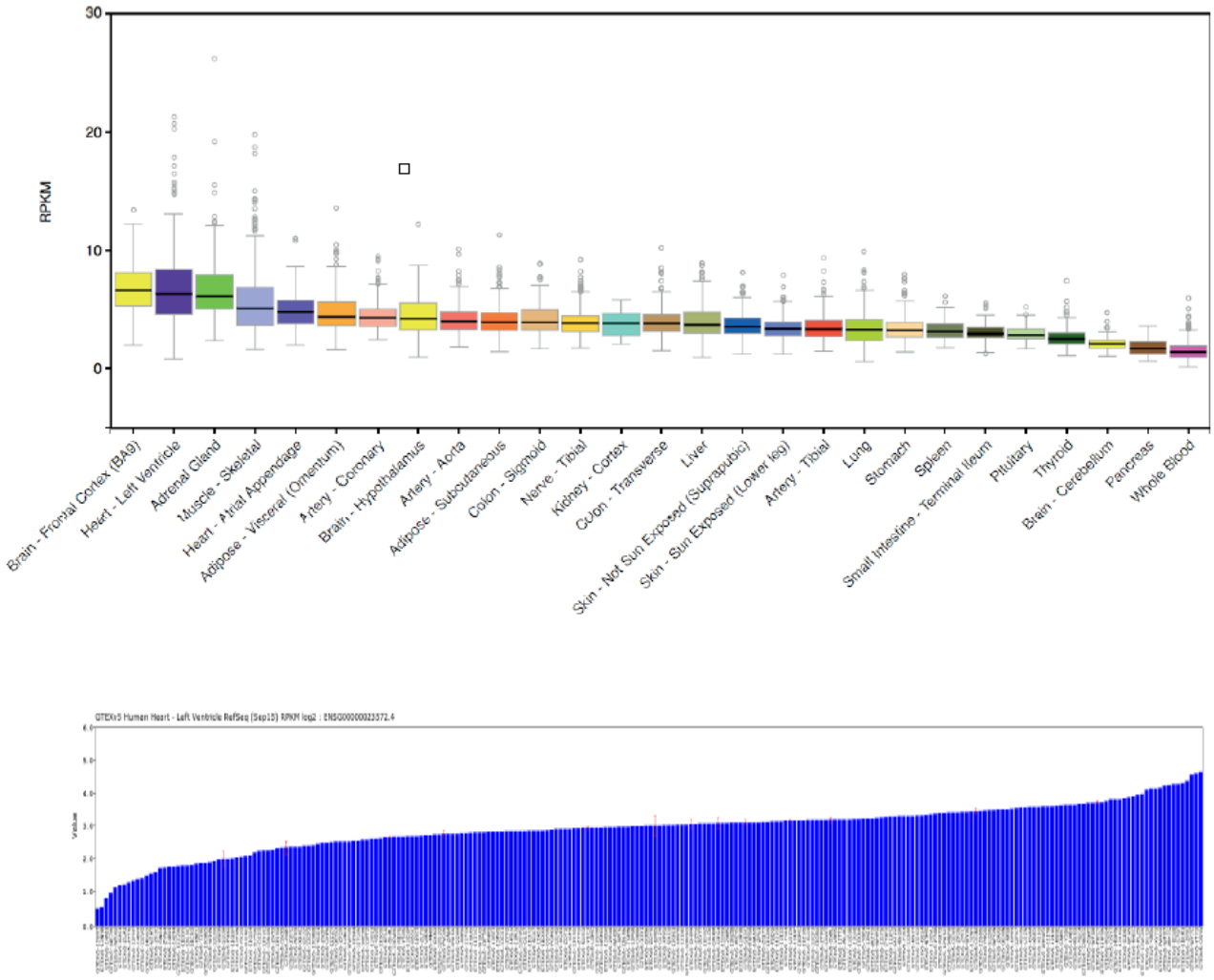


Figure 6.2: **Grx2 expression in tissues and samples.**

(A) The expression values of *GRX2* (also known as *GLRX2*) in 27 tissue types was plotted using GTEx Analysis Release v6 (dbGaP accession number: phs000424.v6.p1). Expression values were plotted as log(RPKM). Box plots are shown as median and 25th and 75th percentiles; points are displayed as outliers if they are above or below 1.5 times the interquartile range. (B) *GRX2* transcript expression data from all GTEx left ventricle tissues samples were downloaded from the GeneNetwork program and plotted as RPKM log2 values (n=246).

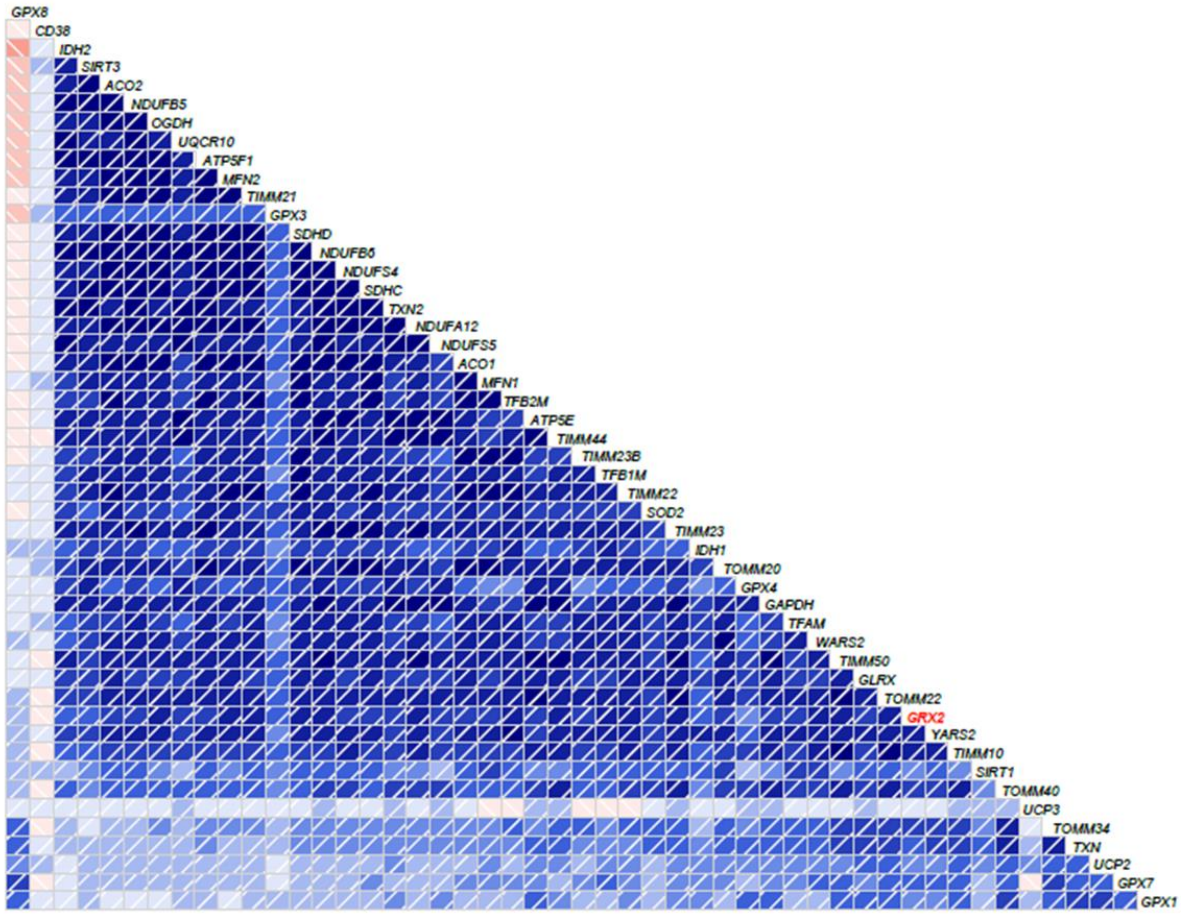


Figure 6.3: **Grx2 correlation with mitochondrial proteins.**

Transcript expression for *GRX2* (shown in red font) were positively correlated with an extensive list of mitochondrial-associated genes using custom-designed data sets derived from the GTEx left ventricle heart tissue transcriptome. As seen on the correlogram, blue correlations are positive (red correlations are negative—intensity of the colors correlates with level of significance).

6.2 APPENDIX B- REVIEW PAPER

Cellular redox dysfunction in the development of cardiovascular diseases

Georges N. Kanaan and Mary-Ellen Harper*

Department of Biochemistry, Microbiology and Immunology, and Ottawa Institute of Systems Biology, Faculty of Medicine, 451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5

* **To whom correspondence should be addressed:**

Dr. Mary-Ellen Harper, PhD
Professor
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine, University of Ottawa
451 Smyth Road, Ottawa, ON Canada, ON K1H 8M5
Email: mharper@uottawa.ca
Tel: +1-613-562-5800 Ext 8235

STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

6.2.1 STATEMENT OF MANUSCRIPT STATUS

The manuscript "Cellular redox dysfunction in the development of cardiovascular diseases" has been published in the journal *Biochimica et Biophysica Acta (BBA) - General Subjects*. PMID: 28778485

Biochimica et Biophysica Acta- General Subjects. 2017 November; 1861(11 Pt A):2822-2829. Doi:10.1016/j.bbagen.2017.07.027

6.2.2 CONTRIBUTION STATEMENT

GNK and MEH wrote and edited the manuscript.

6.2.3 ACKNOWLEDGMENTS AND FUNDING

Our laboratory is supported by funding from CIHR (FDN 143278).

6.2.4 CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

6.2.5 ABSTRACT

To meet its exceptionally high energy demands, the heart relies largely on fatty acid oxidation, which then drives the oxidative phosphorylation system in mitochondria. Each day, this system produces about 6kg of ATP to sustain heart function. Fatty acid oxidation is sometimes associated with high rates of mitochondrial reactive oxygen species (ROS) production. By definition, ROS are singlet electron intermediates formed during the partial reduction of oxygen to water and they include radical and non-radical intermediates like superoxide, hydrogen peroxide and hydroxyl radical. Superoxide can also interact with nitric oxide to produce peroxynitrite that in turn can give rise to other radical or non-radical reactive nitrogen species (RNS) like nitrogen dioxide, dinitrogen trioxide and others. While mitochondrial and cellular functions can be impaired by ROS if they accumulate, under normal physiological conditions ROS are important signaling molecules in the cardiovascular system. A fine balance between ROS production and antioxidant systems, including glutathione redox, is essential in the heart; otherwise the ensuing damage can contribute to pathogenic processes, which can culminate in endothelial dysfunction, atherosclerosis, hypertension, cardiac hypertrophy, arrhythmias, myocardial ischemia/reperfusion damage, and heart failure. Here we provide a succinct review of recent findings.

Keywords: mitochondria, oxidative phosphorylation, redox, glutathione, reactive oxygen species, oxidative stress, heart, cardiac pathologies.

6.2.6 REVIEW MANUSCRIPT

1. Introduction

Recent research has shown that mitochondria are much more than the cellular ‘powerhouses’ for ATP production, and are also important as hubs for cellular signaling, biosynthetic reactions and for the control of cell death. Nonetheless there have been significant advances in the field of mitochondrial bioenergetics and redox regulation. Indeed the innumerable redox reactions that feed into and control the production of ATP by oxidative phosphorylation (OXPHOS) are now known to involve a variety of protein post-translational control mechanisms, mitochondrial supercomplex formation, and mitochondrial dynamics (fusion and fission). Because the energy demands of the heart are unsurpassed by those of any other organ in the body, the control of oxidative phosphorylation is of central importance. The perpetually high energy requirements of the heart amount to approximately 6 kg ATP daily, which is 20 to 30 times the weight of the heart itself [1]. These large amounts of ATP are perhaps not surprizing given that the heart beats about 100,000 times daily to pump approximately 10 tons of blood delivering oxygen and nutrients to the entire body [1].

OXPHOS is a multifaceted and complex process in the mitochondrial inner membrane. More than 80 proteins are directly involved, and their control is only beginning to be understood. OXPHOS is responsible for the production of ~90% of a cell’s ATP. It is well known that OXPHOS includes five major multi-protein complexes, namely complexes I-V (CI-V), along with mobile electron carriers coenzyme Q and cytochrome c. Cellular catabolic reactions liberate electrons (or ‘reducing equivalents’) through a series of oxidation and reduction reactions that ultimately channel the reducing equivalents into OXPHOS at CI, CII and CIII. Recent research has shown that these complexes sometimes form ‘supercomplexes’ (SCs), which are apparently beneficial for electron flow and for decreasing reactive oxygen species (ROS) formation [2]. The term, ’redox’, is an abbreviation for the words reduction and oxidation, and refers to chemical reactions in which the oxidation state of atoms or molecules is changed. During cellular oxidative processes, a series of redox reactions occurs in a step-wise fashion involving increasingly powerful oxidizers. Oxygen is the ultimate oxidizer at CIV of the OXPHOS system. As described in more detail below, if redox reactions become imbalanced in the presence of oxygen, potentially damaging amounts of ROS can be produced.

2. Mitochondria in the heart

Considering the essential functions of mitochondria in OXPHOS and in other processes such as calcium homeostasis, apoptosis, and response to injury [3-6], it is logical that mitochondrial dysfunctions are associated with many cardiac pathologies, including hypertrophy, arrhythmias, myocardial ischemia/reperfusion injury, and heart failure. Moreover they are associated with other pathologies that are associated with subsequent development of cardiac diseases [7, 8]. Thus, a growing number of drugs are being targeted at mitochondrial processes to elicit cardioprotective mechanisms [9].

2.1 OXPHOS and ATP production

To sustain such high rates of ATP production, cardiomyocytes are packed with mitochondria, and this is visually obvious in electron micrographs of the heart. It has been estimated that mitochondria occupy between 23% and 32% of cardiomyocyte volume [10]. Allometric relationships exist, according to mass-specific metabolic rates; for example, mitochondrial volume increases with the greater heart rates and mass-specific oxygen demands in mice versus humans [10].

In healthy states, ATP production by OXPHOS in the heart relies primarily on fatty acid β-oxidation, supplying 60 - 90% of the ATP produced. Glucose metabolism through glycolysis or through the subsequent oxidation of glycolysis-derived pyruvate in the mitochondria contributes 10-40% of myocardial ATP synthesis [11]. The myocardium has a certain degree of metabolic flexibility allowing it to switch between these fuel sources as needed. For example, in the failing heart, this metabolic flexibility is impaired and in some cases there is a switch towards glucose oxidation instead of fatty acid oxidation [12-14]. However the opposite effect has been observed with diabetic hearts relying to a greater extent on lipid oxidation (instead of glucose oxidation) in high glucose environments [15, 16]. The synthesis and utilization of ATP are tightly balanced in the heart; the actual ATP content is extremely low. The entire ATP pool can be depleted in 10s in a normal heart with 60-70% of ATP fuelling the contraction process and the remaining 30-40% fuelling various ion pumps [11].

2.2 Mitochondrial dynamics

Mitochondria, by nature are dynamic organelles, and this structural plasticity provides additional functional attributes. Mitochondrial fusion and fission processes balance mitochondrial biogenesis and quality control by mitophagy or apoptosis. The major proteins involved in fusion are three large GTPases of the dynamin superfamily: mitofusin -1 and -2 (Mfn1, Mfn2) and optic atrophy-1 (Opa1). Being a double-membrane organelle, mitochondrial fusion occurs in two steps: the fusion of the outer membrane mediated by

Mfn1 and Mfn2 followed by the fusion of the inner membrane mediated by OPA1. Any genetic deletion in the fusion genes leading to the fragmentation of the mitochondrial network and impacting mitochondrial morphology can have severe consequences on mitochondrial function and ATP production [17, 18]. Another complexity relates to the tight localization of mitochondria in the intermyofibrillar spaces. Until recently little was known about the dynamics of the mitochondria that are packed between the dense parallel myofibrils. However, the very recent work of Eisner *et al.* demonstrates that the mitochondria do indeed fuse and exchange content in a robust manner, and that fusion mechanisms depend on calcium oscillations and contractile activity [19].

Mitochondrial fission on the other hand, an equally critical process for cellular physiology, is mainly mediated by the outer membrane GTPase dynamin related protein-1 (Drp1) with the participation of receptor proteins like fission protein 1 (Fis1) [4, 20]. Scission is mediated in a GTP-dependent manner affecting mitochondrial morphology, transport, mitophagy and apoptosis. A fused/ tubular mitochondrial reticulum is associated with efficient OXPHOS and low ROS emission, while fission is associated with mitochondrial degradation [18, 21].

2.3 ROS production

During OXPHOS, variable amounts of superoxide ($O2^{\cdot-}$), and peroxynitrite are formed, and these specific molecules can then be metabolized to form other types of ROS and RNS like hydrogen peroxide, hydroxyl radical, hypochlorite, carbonate radical and nitrogen dioxide (Figure 1) [22-25]. Although multiple processes within different cellular compartments can contribute to ROS generation, it is estimated that approximately 90% of cellular ROS is produced in the mitochondria [26]. In the OXPHOS system, electrons from $NADH+H^+$ or $FADH_2$ are shuttled through the different complexes of the electron transport chain (ETC). The free energy associated with electron transport drives the pumping of protons by CI, CIII and CIV out of the mitochondrial matrix into the intermembrane space, creating an electrochemical gradient, which is also referred to as the protonmotive force (PMF). The potential energy of PMF is then used by ATP synthase to phosphorylate ADP to ATP, as the protons return to the matrix. At several sites of the ETC, mainly complex I and III, electrons can react with oxygen and produce superoxide [27, 28]. Although it is estimated that the bulk majority of the mitochondrial ROS is produced by the ETC, other sources also exist. For example, recent research shows that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 (Nox4) preferentially produces hydrogen peroxide instead of superoxide anion, an observation also seen with the growth factor adaptor Shc ($p66^{Shc}$) and monoamine oxidase (MAO) [29]. Other mitochondrial enzymes also contribute to ROS production, including cytochrome b5 reductase, dihydroorotate dehydrogenase, glycerol-3-phosphate

dehydrogenase, succinate dehydrogenase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [30-35]. Low or moderate (physiological) concentrations of ROS can have beneficial effects such as protecting against infectious agents and participating in cellular signaling. Redox imbalance and subsequent overproduction of ROS and can cause oxidative stress, which is linked with a vast number of diseases including cardiac diseases [36-38]. The impact of ROS on the cardiac system is discussed in more detail below. However, cardiomyocytes are equipped with a sophisticated set of redox-sensitive mechanisms to protect the heart from oxidative or nitrosative damage. These include protective mechanisms, repair mechanisms and antioxidants [39].

3. Antioxidants and reactive species

3.1 Antioxidant systems.

After free radical exposure from different sources, living organisms developed a series of defence mechanisms against the oxidative stress including: preventative mechanisms, repair mechanisms, physical defenses and antioxidant defences. The antioxidant defence mechanisms can be divided into enzymatic and non-enzymatic defences. The enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin (Trx) and catalase (CAT). Ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids and many others constitute the non-enzymatic antioxidants [30, 40]. The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is thought to be the major thiol antioxidant and redox buffer within cells [41, 42]. It is synthesized in the cytosol by the two ATP requiring enzymes: γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS). In GSH synthesis, GSH itself provides feedback inhibition, thereby regulating cellular GSH concentration. Once synthesized, GSH is distributed in the endoplasmic reticulum, nucleus, and mitochondria. Almost 90% of glutathione is in the cytosol, ~ 10% in the mitochondria and a small percentage in the endoplasmic reticulum and the nucleus. Glutathione is mainly present (95-99%) in the reduced form (GSH) and GSSG content is usually between 1-5% of the total amount [42, 43]. Upon oxidation, GSH is transformed to GSSG which is reduced back to GSH by NADPH-dependent glutathione reductase (GR). During oxidative stress, many antioxidants systems work together to protect mitochondria from the deleterious effect of ROS. Within the mitochondrial matrix MnSOD converts superoxide radical anion into hydrogen peroxide (H_2O_2), which if not controlled, can diffuse to the cytosol and generate more free radicals like hydroxyl radical which can subsequently oxidize proteins, lipids and DNA. Thus a balance between the activity of MnSOD and GSH redox cycle in mitochondria can control H_2O_2 levels [44, 45]. To do so, many contributors come into action, including GPx, peroxiredoxin and GR. Gpx1 the main

isoform of GPx that detoxifies H_2O_2 is localised in the cytosol with a small fraction in the mitochondrial matrix. GSH will act through GSH transferases (GSTs) that exhibit modest GPx activity, and these include Gpx4, which is a membrane associated enzyme with an important role in protecting membranes against oxidative damage [46- 49]. While GSH is not the major player in eliminating electrophiles and xenobiotics on account of the high pKa of its thiol group, it does react with these compounds in a slow non-enzymatic manner [50]. Moreover, GSH supports the glutaredoxin system in which the GSH-dependent glutaredoxins (Grx) reduce GSH-protein mixed disulfides [51, 52]. Glutaredoxins have two isoforms: Grx1 in the cytosol and Grx2 in the mitochondrial matrix and nucleus [45]. Finally, there is the thioredoxin (Trx) system, which contributes to protein thiol maintenance through the actions of Trx and Trx-reductase (TrxR) (Figure 2) [53].
In the context of cardiovascular diseases, many studies have investigated the impact of exogenous supplementation of antioxidant vitamins such as vitamins C and E. Unfortunately, the studies have overall showed very limited benefits in the prevention and treatment of cardiovascular diseases [54].

3.2 Physiological roles of reactive species

Under normal conditions, fluctuations in ROS/RNS act as essential signaling molecules and thus control physiological functions in the cardiovascular system [53]. For instance nitric oxide, an important RNS in the cardiovascular system, regulates endothelium-dependent epicardial and microvascular vasodilation under metabolic stimulation. Disruption of aggregated platelets, inhibition of platelet aggregation and inhibition of platelet as well as leukocyte adhesion to the vascular endothelium are all important functions performed by nitric oxide [54-56]. Another example of the beneficial effects of ROS in the heart is associated with ischemic preconditioning. Ischemic preconditioning provides cardiac protection by decreasing the severity of arrhythmias, by reducing necrosis and by enhancing recovery after cardiac ischemia. Ischemic preconditioning refers to the effect of one or more relatively short phases of ischemia that precede short phases of reperfusion [57-59]. Notable also is that the protective effect is reduced in the presence of antioxidants, which demonstrates that ROS in low concentrations protect the heart against some of the detrimental effects of ischemia [60, 61].

Elegant empirical analyses have shown that the stretching of cardiomyocytes during systole activates Nox2 and ROS production increases. This mechano-chemo process is called “X-ROS signaling”. The increase in ROS activates the ryanodine receptors (RyR2) and triggers a calcium burst, which induces muscle contraction and normalization of X-ROS signaling.

When calcium concentrations return to basal levels, the muscle relaxes. This contraction and relaxation constitute one cycle [62-65]. With additional molecular components, this signaling pathway is also present in skeletal muscle. This sophisticated and delicate balance between contraction and relaxation can be affected by "hyperactive" X-ROS signaling leading to cardiomyopathy and contributing to Duchenne muscular dystrophy (DMD) [65-67]. Hydrogen peroxide is also an important signaling molecule. As a mild oxidant, it can react with cysteine residues in proteins, thereby affecting multiple pathways and networks that control various biological functions, e.g., cell cycle, cell migration and adhesion, cell contraction, stress response, redox homeostasis, ion channels and energy metabolism [16].

Epigenetic mechanisms can also be elicited by changes on metabolic flux and reactive species in cells, with outcomes including altered transcription factors, histone proteins and DNA. Nitric oxide metabolites are an important example; organic nitrate treatment of patients with cardiovascular disease was shown to cause post-translational and epigenetic regulation by downstream signaling pathways [68].

Of the hypotheses that link ROS production and mitochondrial respiration in the heart and cardiovascular system, two are widely mentioned. The "mild uncoupling" (MU) hypothesis and the recent "Redox-Optimized ROS balance" (R-ORB) hypothesis differ from each other mainly by the proposed intermediary between respiration and ROS, with mitochondrial membrane potential ($\Delta\Psi m$) in the former, and redox environment in the latter [69,70]. Within the MU hypothetical framework, a slight decrease in $\Delta\Psi m$ (due to mild uncoupling) should be followed by a decrease in ROS and this has been verified in isolated heart mitochondria but not in intact cardiac cells. On the other hand, the R-ORB hypothesis postulates that the net flux of mitochondrial ROS is the result of an imbalance between ROS production and scavenging. Moreover, while the MU hypothesis relies on a decrease in $\Delta\Psi m$ (the driving force for ATP synthesis) for decreased ROS production, the R-ORB hypothesis proposes that mitochondria minimize ROS production as they maximize ATP synthesis [69,70].

3.3 Pathophysiological roles of reactive species

3.3.1 In endothelial dysfunction

Regulation of vasodilation, inflammation, smooth muscle cell growth, platelet aggregation, and coagulation are all processes controlled by the endothelium. The dysregulation of these processes is termed "endothelial dysfunction" [71]. Nitric oxide secreted by endothelial nitric oxide synthase (eNOS) is key, and oxidative stress compounds its effects during endothelial

dysfunction. Superoxide reacts with nitric oxide and impairs the endothelial function especially when there are impairments in the exogenous antioxidant system, as in hypercholesterolemia [72, 73]. Two important elements in eNOS function are L-arginine and the cofactor tetra-hydrobiopterin (BH4); when either is absent there is increased superoxide production in a phenomenon called “eNOS uncoupling”. This uncoupling was reported in diabetes, pulmonary hypertension, ischemia-reperfusion, atherosclerosis and aging [74-78]. Atherosclerosis and ischemic heart disease share the occurrence of endothelial dysfunction as an initial event in their pathological progress [79]. Recently it was discovered that MitoQ, a mitochondria-targeted antioxidant, ameliorates age-related arterial endothelial dysfunction in mice and in human skeletal muscle arteries [80, 81].

Another important player in endothelial dysfunction caused by disturbed oscillatory flow is Trx1. It has been shown recently in a disturbed flow model that upon oscillatory shear, Trx1 translocates to the nucleus leading to an increase in lipid accumulation, protein oxidation and proinflammatory signaling. Similar observations were made in other cell lines, consistent with the notion that Trx1 plays a critical role in vascular disease processes [82].

3.3.2 In atherosclerosis

Atherosclerosis risk factors such as hypertension and hypercholesterolemia can induce the expression of cell adhesion molecules after endothelial dysfunction and activation. Molecules like vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin allow circulating T lymphocytes and monocytes to adhere to the endothelium [83, 84]. Furthermore endothelial activation increases the permeability of macromolecules like low-density lipoproteins (LDL). In addition, oxidant enzymes like NADP oxidases (Nox) and myeloperoxidase (MPO) can activate inflammatory cells by generating ROS and by oxidizing phospholipids and proteins leading to accumulation of oxidized LDL (oxLDL). OxLDL is an important effector in atherogenesis [85]. Other than oxidizing LDL, oxidative stress inhibits the cholesterol efflux of high-density lipoproteins (HDL). MPO causes the chlorination of the major protein component of HDL, apoA-I. This redox sensitive modification inhibits cholesterol efflux, which in turn, increases ROS production and inflammation turning HDL to an oxidized proinflammatory molecule [86-89]. A number of studies in the past decade also show the involvement of Nox1, Nox2 and Nox4 in increasing ROS levels in atherosclerotic lesions and plaques [90-94]. Overall, ROS are thought to play a role in the major events occurring in atherosclerosis by enhancing inflammation and contributing to arterial wall remodeling through smooth muscle proliferation [95, 96].

3.3.2 In hypertension

In addition to endothelial dysfunction and atherosclerosis, a role for ROS in hypertension was first described in 1991 by Nakazono and his colleagues [97]. Their findings in various animal models essentially revealed that an increase in the activity of NADPH oxidase leads to detrimentally high ROS levels. Nox1, Nox2 and Nox4 were shown to have a role in blood pressure control. ROS production by these enzymes decreases nitric oxide bioavailability and uncouples eNOS leading to impaired vasorelaxation and increased blood pressure [98-101].

3.3.3 In hypertrophy and heart failure

Impaired redox and ROS signaling also have implications in cardiac hypertrophy and heart failure. Left ventricular hypertrophy occurs initially as an adaptive response to situations in which increased pumping and decreased wall stress are needed [102]. However extended periods of high workload, due often to hypertension or ischemic heart disease, causes pathologic cardiac hypertrophy, and eventually chronic heart failure. This transition from hypertrophy to heart failure is mediated at least in part by redox-sensitive molecular mechanisms that involve ventricular remodeling and dilatation, a decrease in contractility, fibrosis and apoptosis [103]. Increased ROS production along with ERK1/2 pathway activation leads to cardiac hypertrophy after a low-amplitude stretch. High-amplitude stretches cause apoptosis via JNK pathway activation [104]. In addition, phosphorylation of class II histone deacetylases (HDACs) has been shown to promote hypertrophy and relocalization of this protein from the nucleus to the cytoplasm. Moreover the antioxidant enzyme, Trx, regulates this translocation and inhibits hypertrophy [105, 106]. Furthermore Nox2 and Nox4 were shown to be involved in cardiac remodeling and apoptosis in a failing heart, respectively [107, 108]. Reports also suggest that ROS generated by xanthine oxidase (XO) have a role in impairing energy metabolism in heart failure [109, 110]. More work should be done to investigate the full potential of mitochondrial-targeted chemical interventions in restoring physiological levels of ROS and normal energy production.

3.3.4 In ischemia-reperfusion injuries

ROS also play an important role in ischemia-reperfusion injury. During ischemia, ROS levels are low but these levels greatly increase with the restoration of oxygen supply during reperfusion, and this causes high levels of ROS and extensive damage to cardiac cells [111]. Antioxidant mechanisms play a crucial role in recovery after ischemia-reperfusion injury. For example the absence of glutathione peroxidase 1 (GPx1) results in an impaired cardiac

recovery; Trx1 and Trx2 also protect the heart by lowering ROS and the deletion of thioredoxin-interacting proteins in cardiac cells causes increased glycolysis to maintain ATP levels [112, 113]. It should also be noted that during hypoxia increased ROS production induces hypoxia-inducible factor 1 (HIF1), which activates genes involved in angiogenesis and vascular remodeling, vasomotor reactivity, vascular tone, erythropoiesis and energy metabolism as a protective mechanism [114-117]. Recently it was shown that SIRT6, a deacetylase and mono-ADP ribosyltransferase, provides protection from ischemia-reperfusion injury in the heart by inducing the expression of the antioxidant enzymes MnSOD and catalase, thus reducing oxidative stress [118].

4. Conclusions and perspectives

Altogether, mitochondria are of central importance in cardiovascular health and disease. Recent research demonstrates the importance of acute and precise control of cellular and mitochondrial redox signaling to prevent dysfunctional redox reactions and pathological levels of ROS and RNS. ROS have a crucial role in cellular signaling and cardioprotection, but this requires the complex balance between ROS production and antioxidant systems (Figure 3). A major ongoing challenge is the translation of this rapidly growing body of knowledge into effective preventative strategies and therapies for cardiovascular diseases. Specific approaches must balance the need for physiological levels of ROS for proper cell signaling processes with the need to prevent the deleterious effects of supraphysiological levels of ROS that have been associated with the development of cardiovascular diseases.

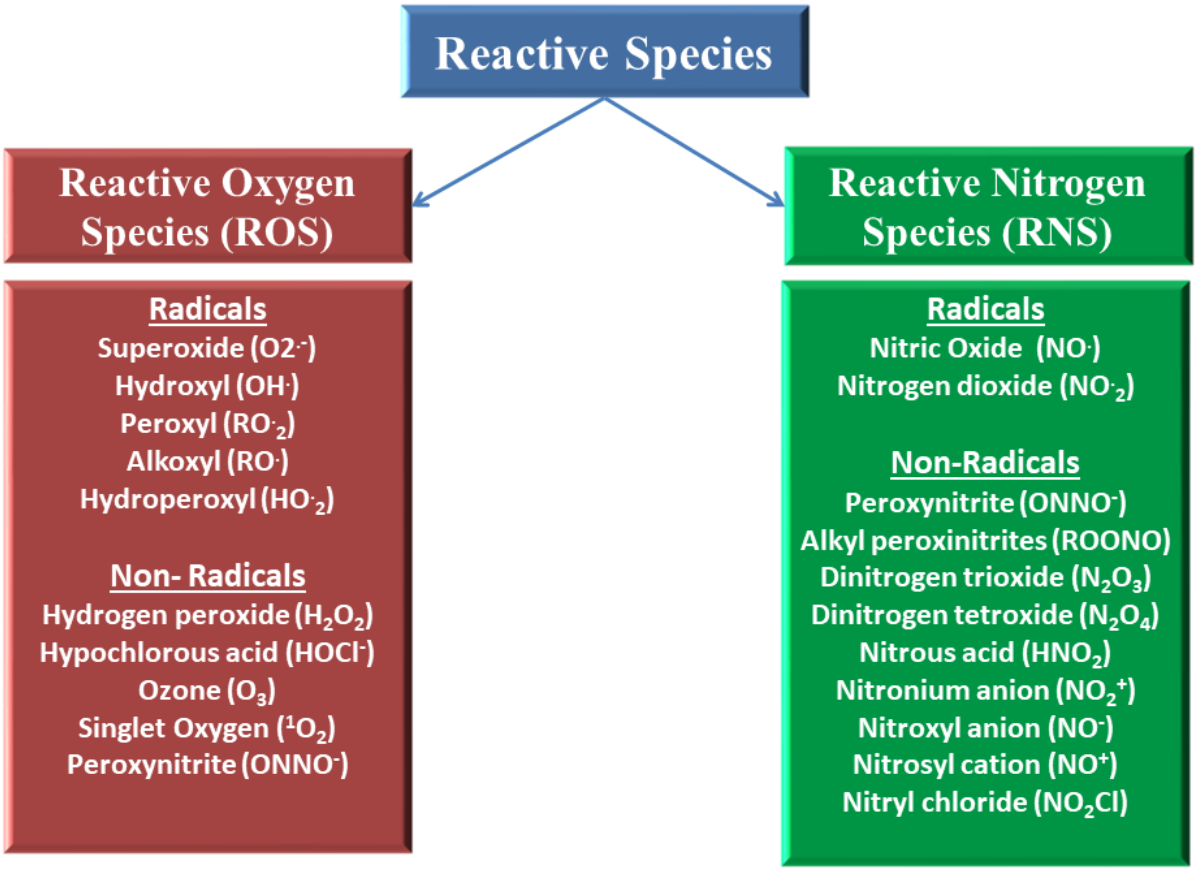


Figure R1: Reactive species

Radical and non- radical reactive oxygen species (ROS) and reactive nitrogen species (RNS).

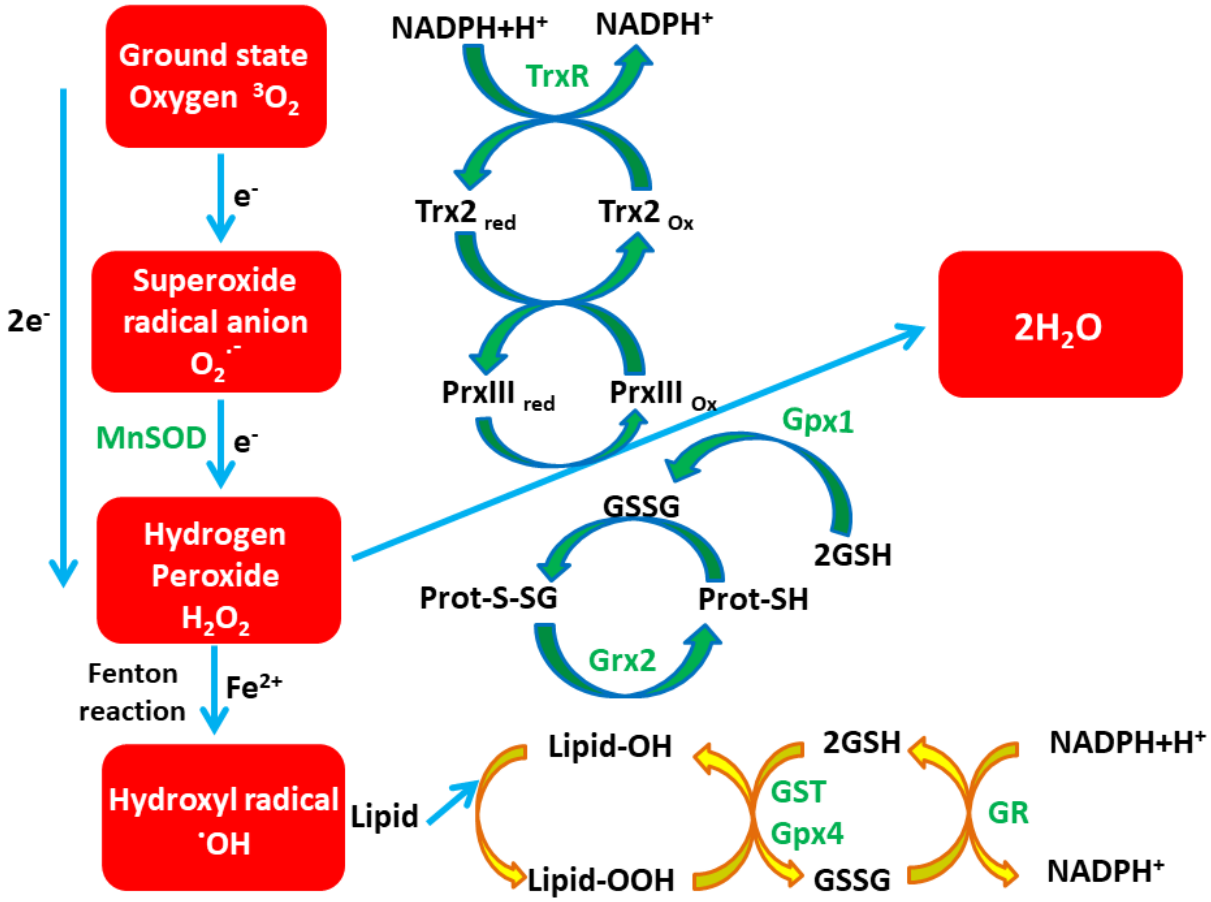


Figure R2: Mitochondrial control of oxidative stress.
The different mitochondrial reactions during oxidative stress induced by superoxide radical anion, hydrogen peroxide and hydroxyl radical. GSH peroxidase (Gpx); GSSG-reductase (GR); glutaredoxin (Grx); Mn-dependent superoxide dismutase (MnSOD); Trx-reductase (TrxR); peroxiredoxin III (PrxIII); red: reduced and ox: oxidized.

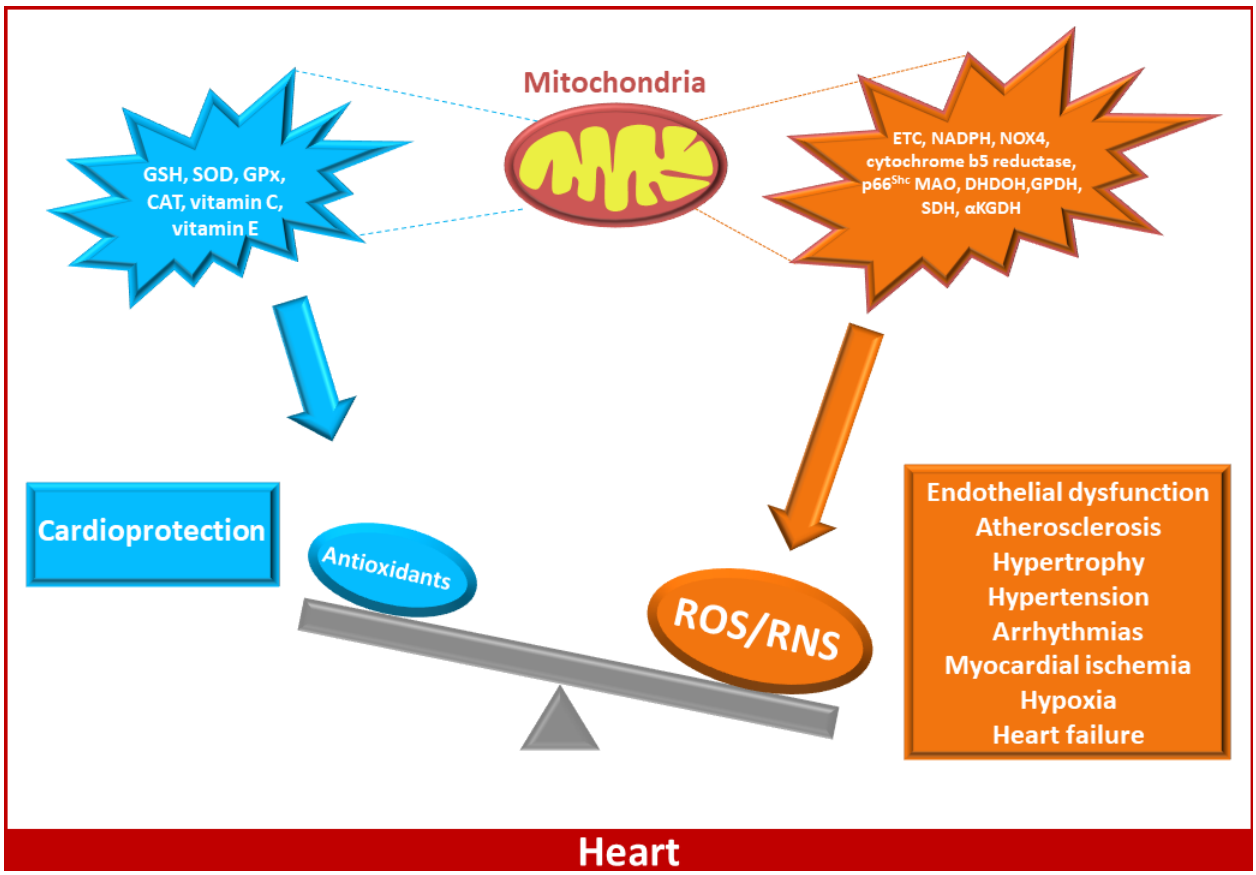


Figure R3: The delicate balance between the antioxidants and ROS in the heart.
A balance shifted towards more ROS production in the heart, overwhelming the antioxidant systems, has been shown to be involved in many pathophysiological cardiac diseases including endothelial dysfunction, atherosclerosis, cardiac hypertrophy, hypertension, cardiac arrhythmias, myocardial ischemia, hypoxia and heart failure.

6.2.7 REFERENCES

1. Neubauer, S. (2007). The failing heart--an engine out of fuel. *The New England Journal of Medicine*, *356*(11), 1140–1151. https://doi.org/10.1056/NEJMra063052
2. Moreno-Loshuertos, R., & Enríquez, J. A. (2016). Respiratory supercomplexes and the functional segmentation of the CoQ pool. Free Radical Biology & Medicine, 100, 5–13. https://doi.org/10.1016/j.freeradbiomed.2016.04.018
3. Territo, P. R., French, S. A., & Balaban, R. S. (2001). Simulation of cardiac work transitions, in vitro: effects of simultaneous Ca2+ and ATPase additions on isolated porcine heart mitochondria. Cell Calcium, 30(1), 19–27. https://doi.org/10.1054/ceca.2001.0211
4. Ong, S.-B., Kalkhoran, S. B., Cabrera-Fuentes, H. A., & Hausenloy, D. J. (2015). Mitochondrial fusion and fission proteins as novel therapeutic targets for treating cardiovascular disease. *European Journal of Pharmacology*, *763*(Pt A), 104–114. https://doi.org/10.1016/j.ejphar.2015.04.056
5. Disatnik, M.-H., Ferreira, J. C. B., Campos, J. C., Gomes, K. S., Dourado, P. M. M., Qi, X., & Mochly-Rosen, D. (2013). Acute inhibition of excessive mitochondrial fission after myocardial infarction prevents long-term cardiac dysfunction. Journal of the American Heart Association, 2(5), e000461. https://doi.org/10.1161/JAHA.113.000461
6. Dorn, G. W. (2016). Parkin-dependent mitophagy in the heart. Journal of Molecular and Cellular Cardiology, 95, 42–49. https://doi.org/10.1016/j.yjmcc.2015.11.023
7. Köhler, A. C., Sag, C. M., & Maier, L. S. (2014). Reactive oxygen species and excitation-contraction coupling in the context of cardiac pathology. *Journal of Molecular and Cellular Cardiology*, *73*, 92–102. https://doi.org/10.1016/j.yjmcc.2014.03.001
8. Walters, J. W., Amos, D., Ray, K., & Santanam, N. (2016). Mitochondrial redox status as a target for cardiovascular disease. *Current Opinion in Pharmacology*, *27*, 50–55. https://doi.org/10.1016/j.coph.2016.01.006
9. Dongworth, R. K., Hall, A. R., Burke, N., & Hausenloy, D. J. (2014). Targeting mitochondria for cardioprotection: examining the benefit for patients. Future Cardiology, 10(2), 255–272. https://doi.org/10.2217/fca.14.6
10. Schaper, J., Meiser, E., & Stämmler, G. (1985). Ultrastructural morphometric analysis of myocardium from dogs, rats, hamsters, mice, and from human hearts. Circulation Research, 56(3), 377–391.
11. Stanley, W. C., Recchia, F. A., & Lopaschuk, G. D. (2005). Myocardial substrate metabolism in the normal and failing heart. *Physiological Reviews*, *85*(3), 1093–1129. https://doi.org/10.1152/physrev.00006.2004
12. Rosca, M. G., & Hoppel, C. L. (2010). Mitochondria in heart failure. *Cardiovascular Research*, *88*(1), 40–50. https://doi.org/10.1093/cvr/cvq240
13. Rosca, M. G., & Hoppel, C. L. (2013). Mitochondrial dysfunction in heart failure. *Heart Failure Reviews*, *18*(5), 607–622. https://doi.org/10.1007/s10741-012-9340-0
14. Fillmore, N., Mori, J., & Lopaschuk, G. D. (2014). Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy.

British Journal of Pharmacology, *171*(8), 2080–2090. https://doi.org/10.1111/bph.12475
15. Heather, L. C., & Clarke, K. (2011). Metabolism, hypoxia and the diabetic heart. Journal of Molecular and Cellular Cardiology, 50(4), 598–605. https://doi.org/10.1016/j.yjmcc.2011.01.007
16. Aon, M. A., Tocchetti, C. G., Bhatt, N., Paolocci, N., & Cortassa, S. (2015). Protective mechanisms of mitochondria and heart function in diabetes. Antioxidants & Redox Signaling, 22(17), 1563–1586. https://doi.org/10.1089/ars.2014.6123
17. Friedman, J. R., & Nunnari, J. (2014). Mitochondrial form and function. Nature, 505(7483), 335–343. https://doi.org/10.1038/nature12985.
18. Mishra, P., & Chan, D. C. (2016). Metabolic regulation of mitochondrial dynamics. The Journal of Cell Biology, 212(4), 379–387. https://doi.org/10.1083/jcb.201511036
19. Eisner, V., Cupo, R. R., Gao, E., Csordás, G., Slovinsky, W. S., Paillard, M., … Hajnóczky, G. (2017). Mitochondrial fusion dynamics is robust in the heart and depends on calcium oscillations and contractile activity. Proceedings of the National Academy of Sciences of the United States of America, 114(5), E859–E868. https://doi.org/10.1073/pnas.1617288114
20. Archer, S. L. (2013). Mitochondrial dynamics--mitochondrial fission and fusion in human diseases. *The New England Journal of Medicine*, *369*(23), 2236–2251. https://doi.org/10.1056/NEJMra1215233
21. Westermann, B. (2012). Bioenergetic role of mitochondrial fusion and fission. *Biochimica Et Biophysica Acta*, *1817*(10), 1833–1838. https://doi.org/10.1016/j.bbabio.2012.02.033
22. Muller, F. (2000). The nature and mechanism of superoxide production by the electron transport chain: Its relevance to aging. *Journal of the American Aging Association*, *23*(4), 227–253. https://doi.org/10.1007/s11357-000-0022-9
23. Aon, M. A., Cortassa, S., Marbán, E., & O'Rourke, B. (2003). Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. The Journal of Biological Chemistry, 278(45), 44735–44744. https://doi.org/10.1074/jbc.M302673200
24. Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L., & Lesnefsky, E. J. (2003). Production of reactive oxygen species by mitochondria: central role of complex III. *The Journal of Biological Chemistry*, *278*(38), 36027–36031. https://doi.org/10.1074/jbc.M304854200
25. Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *The Biochemical Journal*, *417*(1), 1–13. https://doi.org/10.1042/BJ20081386
26. Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. Nature Reviews. Immunology, 4(3), 181–189. https://doi.org/10.1038/nri1312
27. Nemoto, S., Takeda, K., Yu, Z. X., Ferrans, V. J., & Finkel, T. (2000). Role for mitochondrial oxidants as regulators of cellular metabolism. Molecular and Cellular Biology, 20(19), 7311–7318.
28. Sas, K., Robotka, H., Toldi, J., & Vécsei, L. (2007). Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on

neurodegenerative disorders. Journal of the Neurological Sciences, 257(1–2), 221–239. https://doi.org/10.1016/j.jns.2007.01.033
29. Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiological Reviews, 94(3), 909–950. https://doi.org/10.1152/physrev.00026.2013
30. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology, 39(1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001
31. Gao, L., Laude, K., & Cai, H. (2008). Mitochondrial pathophysiology, reactive oxygen species, and cardiovascular diseases. *The Veterinary Clinics of North America. Small Animal Practice*, *38*(1), 137–155, vi. https://doi.org/10.1016/j.cvsm.2007.10.004
32. Campos, J. C., Gomes, K. M. S., & Ferreira, J. C. B. (2013). Impact of exercise training on redox signaling in cardiovascular diseases. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *62*, 107–119. https://doi.org/10.1016/j.fct.2013.08.035
33. Kalogeris, T., Bao, Y., & Korthuis, R. J. (2014). Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology*, *2*, 702–714. https://doi.org/10.1016/j.redox.2014.05.006
34. Fisher-Wellman, K. H., Lin, C.-T., Ryan, T. E., Reese, L. R., Gilliam, L. A. A., Cathey, B. L., … Neufer, P. D. (2015). Pyruvate dehydrogenase complex and nicotinamide nucleotide transhydrogenase constitute an energy-consuming redox circuit. The Biochemical Journal, 467(2), 271–280. https://doi.org/10.1042/BJ20141447
35. Holmström, K. M., & Finkel, T. (2014). Cellular mechanisms and physiological consequences of redox-dependent signalling. Nature Reviews. Molecular Cell Biology, 15(6), 411–421. https://doi.org/10.1038/nrm3801
36. Kovacic, P., Pozos, R. S., Somanathan, R., Shangari, N., & O'Brien, P. J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. Current Medicinal Chemistry, 12(22), 2601–2623.
37. Bartosz, G. (2009). Reactive oxygen species: destroyers or messengers? Biochemical Pharmacology, 77(8), 1303–1315. https://doi.org/10.1016/j.bcp.2008.11.009
38. Ray, J. G., Schull, M. J., Kingdom, J. C., & Vermeulen, M. J. (2012). Heart failure and dysrhythmias after maternal placental syndromes: HAD MPS Study. Heart (British Cardiac Society), 98(15), 1136–1141. https://doi.org/10.1136/heartjnl-2011-301548
39. Bartz, R. R., Suliman, H. B., & Piantadosi, C. A. (2015). Redox mechanisms of cardiomyocyte mitochondrial protection. Frontiers in Physiology, 6, 291. https://doi.org/10.3389/fphys.2015.00291
40. Cadenas, E. (1997). Basic mechanisms of antioxidant activity. BioFactors (Oxford, England), 6(4), 391–397.

41. Schafer, F. Q., & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biology & Medicine, 30(11), 1191–1212.
42. Marí, M., Morales, A., Colell, A., García-Ruiz, C., & Fernández-Checa, J. C. (2009). Mitochondrial glutathione, a key survival antioxidant. Antioxidants & Redox Signaling, 11(11), 2685–2700. https://doi.org/10.1089/ARS.2009.2695
43. Murphy, M. P. (2012). Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. Antioxidants & Redox Signaling, 16(6), 476–495. https://doi.org/10.1089/ars.2011.4289
44. Fernandez-Checa, J. C., & Kaplowitz, N. (2005). Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicology and Applied Pharmacology, 204(3), 263–273. https://doi.org/10.1016/j.taap.2004.10.001
45. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., … Holmgren, A. (2001). Cloning and Expression of a Novel Human Glutaredoxin (Grx2) with Mitochondrial and Nuclear Isoforms. Journal of Biological Chemistry, 276(28), 26269–26275. https://doi.org/10.1074/jbc.M011605200
46. Chen, J., Schenker, S., & Henderson, G. I. (2002). 4-Hydroxynonenal Detoxification by Mitochondrial Glutathione S-Transferase Is Compromised by Short-Term Ethanol Consumption in Rats. Alcoholism: Clinical and Experimental Research, 26(8), 1252–1258. https://doi.org/10.1111/j.1530-0277.2002.tb02664.x
47. Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2007). Mitochondrial Oxidative Stress: Implications for Cell Death. Annual Review of Pharmacology and Toxicology, 47(1), 143–183. https://doi.org/10.1146/annurev.pharmtox.47.120505.105122
48. Savaskan, N. E., Ufer, C., Kühn, H., & Borchert, A. (2007). Molecular biology of glutathione peroxidase 4: from genomic structure to developmental expression and neural function. Biological Chemistry, 388(10), 1007–1017. https://doi.org/10.1515/BC.2007.126
49. Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., … Prolla, T. A. (2003). The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. Free Radical Biology & Medicine, 34(4), 496–502.
50. Hayes, J. D., Flanagan, J. U., & Jowsey, I. R. (2005). Glutathione transferases. Annual Review of Pharmacology and Toxicology, 45, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857
51. Herrero, E., & Ros, J. (2002). [14] Glutaredoxins and oxidative stress defense in yeast. Methods in Enzymology, 348, 136–146. https://doi.org/10.1016/S0076-6879(02)48633-8
52. Koehler, C.M., Beverly, K., and Leverich, E.P. (2006). Redox pathways in the mitochondrion. Antioxidants and Redox Signaling 8: 813–822.
53. Pedrajas, J.R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J.A., Wright, A.P., and Spyrou, G. (1999). Identification and functional characterization of a novel

mitochondrial thioredoxin system in Saccharomyces cerevisiae. The Journal of Biological Chemistry 274: 6366–6373.
54. Go, Y.-M., & Jones, D. P. (2011). Cysteine/cystine redox signaling in cardiovascular disease. Free Radical Biology & Medicine, 50(4), 495–509. https://doi.org/10.1016/j.freeradbiomed.2010.11.029
55. Madamanchi, N. R., & Runge, M. S. (2013). Redox signaling in cardiovascular health and disease. Free Radical Biology & Medicine, 61, 473–501. https://doi.org/10.1016/j.freeradbiomed.2013.04.001
56. Kubes, P., Suzuki, M., & Granger, D. N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(11), 4651–4655.
57. Quyyumi, A. A., Dakak, N., Andrews, N. P., Gilligan, D. M., Panza, J. A., & Cannon, R. O. (1995). Contribution of nitric oxide to metabolic coronary vasodilation in the human heart. *Circulation*, *92*(3), 320–326.
58. Ahluwalia, A., Foster, P., Scotland, R. S., McLean, P. G., Mathur, A., Perretti, M., … Hobbs, A. J. (2004). Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(5), 1386–1391. https://doi.org/10.1073/pnas.0304264101
59. Murry, C. E., Richard, V. J., Reimer, K. A., & Jennings, R. B. (1990). Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circulation Research*, *66*(4), 913–931.
60. Liu, Y., & Downey, J. M. (1992). Ischemic preconditioning protects against infarction in rat heart. *The American Journal of Physiology*, *263*(4 Pt 2), H1107-1112.
61. Lasley, R. D., Anderson, G. M., & Mentzer, R. M. (1993). Ischaemic and hypoxic preconditioning enhance postischaemic recovery of function in the rat heart. *Cardiovascular Research*, *27*(4), 565–570.
62. Chen, W., Gabel, S., Steenbergen, C., & Murphy, E. (1995). A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. Circulation Research, 77(2), 424–429.
63. Forbes, R. A., Steenbergen, C., & Murphy, E. (2001). Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circulation Research*, *88*(8), 802–809.
64. Bers, D. M. (2002). Cardiac excitation-contraction coupling. *Nature*, *415*(6868), 198–205. https://doi.org/10.1038/415198a
65. Prosser, B. L., Khairallah, R. J., Ziman, A. P., Ward, C. W., & Lederer, W. J. (2013). X-ROS signaling in the heart and skeletal muscle: stretch-dependent local ROS regulates [Ca^{2+}]i. Journal of Molecular and Cellular Cardiology, 58, 172–181. https://doi.org/10.1016/j.yjmcc.2012.11.011
66. Prosser, B. L., Ward, C. W., & Lederer, W. J. (2011). X-ROS signaling: rapid mechano-chemo transduction in heart. *Science (New York, N.Y.)*, *333*(6048), 1440–1445. https://doi.org/10.1126/science.1202768

67. Kerr, J. P., Robison, P., Shi, G., Bogush, A. I., Kempema, A. M., Hexum, J. K., … Ward, C. W. (2015). Detyrosinated microtubules modulate mechanotransduction in heart and skeletal muscle. Nature Communications, 6, 8526. https://doi.org/10.1038/ncomms9526
68. Wende, A. R., Young, M. E., Chatham, J., Zhang, J., Rajasekaran, N. S., & Darley-Usmar, V. M. (2016). Redox biology and the interface between bioenergetics, autophagy and circadian control of metabolism. Free Radical Biology & Medicine, 100, 94–107. https://doi.org/10.1016/j.freeradbiomed.2016.05.022
69. Aon, M. A., Cortassa, S., & O'Rourke, B. (2010). Redox-optimized ROS balance: a unifying hypothesis. Biochimica Et Biophysica Acta, 1797(6–7), 865–877. https://doi.org/10.1016/j.bbabio.2010.02.016
70. Cortassa, S., O'Rourke, B., & Aon, M. A. (2014). Redox-optimized ROS balance and the relationship between mitochondrial respiration and ROS. Biochimica Et Biophysica Acta, 1837(2), 287–295. https://doi.org/10.1016/j.bbabio.2013.11.007
71. Hidalgo, C., & Donoso, P. (2011). Cell signaling. Getting to the heart of mechanotransduction. *Science (New York, N.Y.)*, *333*(6048), 1388–1390. https://doi.org/10.1126/science.1212183
72. Santos, C. X. C., Anilkumar, N., Zhang, M., Brewer, A. C., & Shah, A. M. (2011). Redox signaling in cardiac myocytes. *Free Radical Biology & Medicine*, *50*(7), 777–793. https://doi.org/10.1016/j.freeradbiomed.2011.01.003
73. Schulz, E., Gori, T., & Münzel, T. (2011). Oxidative stress and endothelial dysfunction in hypertension. *Hypertension Research: Official Journal of the Japanese Society of Hypertension*, *34*(6), 665–673. https://doi.org/10.1038/hr.2011.39
74. Mügge, A., Elwell, J. H., Peterson, T. E., Hofmeyer, T. G., Heistad, D. D., & Harrison, D. G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circulation Research*, *69*(5), 1293–1300.
75. Cai, H., & Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circulation Research*, *87*(10), 840–844.
76. Hein, T. W., Zhang, C., Wang, W., Chang, C.-I., Thengchaisri, N., & Kuo, L. (2003). Ischemia-reperfusion selectively impairs nitric oxide-mediated dilation in coronary arterioles: counteracting role of arginase. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *17*(15), 2328–2330. https://doi.org/10.1096/fj.03-0115fje
77. Zhang, C., Hein, T. W., Wang, W., Miller, M. W., Fossum, T. W., McDonald, M. M., … Kuo, L. (2004). Upregulation of vascular arginase in hypertension decreases nitric oxide-mediated dilation of coronary arterioles. *Hypertension*, *44*(6), 935–943. https://doi.org/10.1161/01.HYP.0000146907.82869.f2
78. White, A. R., Ryoo, S., Li, D., Champion, H. C., Steppan, J., Wang, D., … Berkowitz, D. E. (2006). Knockdown of arginase I restores NO signaling in the vasculature of old rats. *Hypertension*, *47*(2), 245–251. https://doi.org/10.1161/01.HYP.0000198543.34502.d7

79. Ryoo, S., Gupta, G., Benjo, A., Lim, H. K., Camara, A., Sikka, G., … Berkowitz, D. E. (2008). Endothelial arginase II: a novel target for the treatment of atherosclerosis. *Circulation Research*, *102*(8), 923–932. https://doi.org/10.1161/CIRCRESAHA.107.169573
80. Gioscia-Ryan, R. A., LaRocca, T. J., Sindler, A. L., Zigler, M. C., Murphy, M. P., & Seals, D. R. (2014). Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice. The Journal of Physiology, 592(12), 2549–2561. https://doi.org/10.1113/jphysiol.2013.268680
81. Park, S.-Y., Kwon, O. S., Andtbacka, R. H. I., Hyngstrom, J. R., Reese, V., Murphy, M. P., & Richardson, R. S. (2017). Age-related endothelial dysfunction in human skeletal muscle feed arteries: The role of free radicals derived from mitochondria in the vasculature. Acta Physiologica (Oxford, England). https://doi.org/10.1111/apha.12893
82. Go, Y.-M., Son, D. J., Park, H., Orr, M., Hao, L., Takabe, W., … Jones, D. P. (2014). Disturbed flow enhances inflammatory signaling and atherogenesis by increasing thioredoxin-1 level in endothelial cell nuclei. PloS One, 9(9), e108346. https://doi.org/10.1371/journal.pone.0108346
83. Romero, M. J., Platt, D. H., Tawfik, H. E., Labazi, M., El-Remessy, A. B., Bartoli, M., … Caldwell, R. W. (2008). Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circulation Research*, *102*(1), 95–102. https://doi.org/10.1161/CIRCRESAHA.107.155028
84. Vanhoutte, P. M. (2003). Endothelial control of vasomotor function: from health to coronary disease. Circulation Journal: Official Journal of the Japanese Circulation Society, 67(7), 572–575.
85. Madamanchi, N. R., Vendrov, A., & Runge, M. S. (2005). Oxidative stress and vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(1), 29–38. https://doi.org/10.1161/01.ATV.0000150649.39934.13
86. Hulsmans, M., & Holvoet, P. (2010). The vicious circle between oxidative stress and inflammation in atherosclerosis. *Journal of Cellular and Molecular Medicine*, *14*(1–2), 70–78. https://doi.org/10.1111/j.1582-4934.2009.00978.x
87. Stocker, R., & Keaney, J. F. (2004). Role of oxidative modifications in atherosclerosis. *Physiological Reviews*, *84*(4), 1381–1478. https://doi.org/10.1152/physrev.00047.2003
88. Bergt, C., Pennathur, S., Fu, X., Byun, J., O'Brien, K., McDonald, T. O., … Heinecke, J. W. (2004). The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(35), 13032–13037. https://doi.org/10.1073/pnas.0405292101
89. Shao, B., Oda, M. N., Bergt, C., Fu, X., Green, P. S., Brot, N., … Heinecke, J. W. (2006). Myeloperoxidase impairs ABCA1-dependent cholesterol efflux through methionine oxidation and site-specific tyrosine chlorination of apolipoprotein A-I. *The Journal of Biological Chemistry*, *281*(14), 9001–9004. https://doi.org/10.1074/jbc.C600011200

90. Peshavariya, H., Dusting, G. J., Di Bartolo, B., Rye, K.-A., Barter, P. J., & Jiang, F. (2009). Reconstituted high-density lipoprotein suppresses leukocyte NADPH oxidase activation by disrupting lipid rafts. *Free Radical Research*, *43*(8), 772–782. https://doi.org/10.1080/10715760903045304
91. Undurti, A., Huang, Y., Lupica, J. A., Smith, J. D., DiDonato, J. A., & Hazen, S. L. (2009). Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *The Journal of Biological Chemistry*, *284*(45), 30825–30835. https://doi.org/10.1074/jbc.M109.047605
92. Sorescu, D., Weiss, D., Lassègue, B., Clempus, R. E., Szöcs, K., Sorescu, G. P., … Griendling, K. K. (2002). Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation*, *105*(12), 1429–1435.
93. Vendrov, A. E., Madamanchi, N. R., Niu, X.-L., Molnar, K. C., Runge, M., Szyndralewiez, C., … Runge, M. S. (2010). NADPH oxidases regulate CD44 and hyaluronic acid expression in thrombin-treated vascular smooth muscle cells and in atherosclerosis. *The Journal of Biological Chemistry*, *285*(34), 26545–26557. https://doi.org/10.1074/jbc.M110.143917
94. Fenyo, I. M., Florea, I. C., Raicu, M., & Manea, A. (2011). Tyrphostin AG490 reduces NAPDH oxidase activity and expression in the aorta of hypercholesterolemic apolipoprotein E-deficient mice. *Vascular Pharmacology*, *54*(3–6), 100–106. https://doi.org/10.1016/j.vph.2011.03.006
95. Patel, R. S., Al Mheid, I., Morris, A. A., Ahmed, Y., Kavtaradze, N., Ali, S., … Quyyumi, A. A. (2011). Oxidative stress is associated with impaired arterial elasticity. Atherosclerosis, 218(1), 90–95. https://doi.org/10.1016/j.atherosclerosis.2011.04.033
96. Park, S., & Lakatta, E. G. (2012). Role of inflammation in the pathogenesis of arterial stiffness. Yonsei Medical Journal, 53(2), 258–261. https://doi.org/10.3349/ymj.2012.53.2.258
97. Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T., & Inoue, M. (1991). Does superoxide underlie the pathogenesis of hypertension? *Proceedings of the National Academy of Sciences of the United States of America*, *88*(22), 10045–10048.
98. Zimmerman, M. C., Lazartigues, E., Sharma, R. V., & Davisson, R. L. (2004). Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. *Circulation Research*, *95*(2), 210–216. https://doi.org/10.1161/01.RES.0000135483.12297.e4
99. Peterson, J. R., Burmeister, M. A., Tian, X., Zhou, Y., Guruju, M. R., Stupinski, J. A., … Davisson, R. L. (2009). Genetic silencing of Nox2 and Nox4 reveals differential roles of these NADPH oxidase homologues in the vasopressor and dipsogenic effects of brain angiotensin II. *Hypertension*, *54*(5), 1106–1114. https://doi.org/10.1161/HYPERTENSIONAHA.109.140087
100. Dikalova, A. E., Bikineyeva, A. T., Budzyn, K., Nazarewicz, R. R., McCann, L., Lewis, W., … Dikalov, S. I. (2010). Therapeutic targeting of mitochondrial superoxide in hypertension. *Circulation Research*, *107*(1), 106–116. https://doi.org/10.1161/CIRCRESAHA.109.214601

101. Dikalova, A. E., Góngora, M. C., Harrison, D. G., Lambeth, J. D., Dikalov, S., & Griendling, K. K. (2010). Upregulation of Nox1 in vascular smooth muscle leads to impaired endothelium-dependent relaxation via eNOS uncoupling. *American Journal of Physiology. Heart and Circulatory Physiology*, *299*(3), H673-679. https://doi.org/10.1152/ajpheart.00242.2010
102. Claycomb, W. C. (1983). Cardiac muscle cell proliferation and cell differentiation in vivo and in vitro. *Advances in Experimental Medicine and Biology*, *161*, 249–265.
103. Abate, C., Patel, L., Rauscher, F. J., & Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. *Science (New York, N.Y.)*, *249*(4973), 1157–1161.
104. Pimentel, D. R., Amin, J. K., Xiao, L., Miller, T., Viereck, J., Oliver-Krasinski, J., … Sawyer, D. B. (2001). Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circulation Research*, *89*(5), 453–460.
105. Yamamoto, M., Yang, G., Hong, C., Liu, J., Holle, E., Yu, X., … Sadoshima, J. (2003). Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy. *The Journal of Clinical Investigation*, *112*(9), 1395–1406. https://doi.org/10.1172/JCI17700
106. Ago, T., Liu, T., Zhai, P., Chen, W., Li, H., Molkentin, J. D., … Sadoshima, J. (2008). A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. *Cell*, *133*(6), 978–993. https://doi.org/10.1016/j.cell.2008.04.041
107. Doerries, C., Grote, K., Hilfiker-Kleiner, D., Luchtefeld, M., Schaefer, A., Holland, S. M., … Landmesser, U. (2007). Critical role of the NAD(P)H oxidase subunit p47phox for left ventricular remodeling/dysfunction and survival after myocardial infarction. *Circulation Research*, *100*(6), 894–903. https://doi.org/10.1161/01.RES.0000261657.76299.ff
108. Ago, T., Kuroda, J., Pain, J., Fu, C., Li, H., & Sadoshima, J. (2010). Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. *Circulation Research*, *106*(7), 1253–1264. https://doi.org/10.1161/CIRCRESAHA.109.213116
109. Ekelund, U. E., Harrison, R. W., Shokek, O., Thakkar, R. N., Tunin, R. S., Senzaki, H., … Hare, J. M. (1999). Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circulation Research*, *85*(5), 437–445.
110. Cappola, T. P., Kass, D. A., Nelson, G. S., Berger, R. D., Rosas, G. O., Kobeissi, Z. A., … Hare, J. M. (2001). Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy. *Circulation*, *104*(20), 2407–2411.
111. Murphy, E., & Steenbergen, C. (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological Reviews*, *88*(2), 581–609. https://doi.org/10.1152/physrev.00024.2007
112. Forgione, M. A., Cap, A., Liao, R., Moldovan, N. I., Eberhardt, R. T., Lim, C. C., … Loscalzo, J. (2002). Heterozygous cellular glutathione peroxidase deficiency in the mouse: abnormalities in vascular and cardiac function and structure. *Circulation*, *106*(9), 1154–1158.

113. Yoshioka, J., Chutkow, W. A., Lee, S., Kim, J. B., Yan, J., Tian, R., … Lee, R. T. (2012). Deletion of thioredoxin-interacting protein in mice impairs mitochondrial function but protects the myocardium from ischemia-reperfusion injury. *The Journal of Clinical Investigation*, *122*(1), 267–279. https://doi.org/10.1172/JCI44927
114. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., & Schumacker, P. T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(20), 11715–11720.
115. Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., & Schumacker, P. T. (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *The Journal of Biological Chemistry*, *275*(33), 25130–25138. https://doi.org/10.1074/jbc.M001914200
116. Agani, F. H., Pichiule, P., Chavez, J. C., & LaManna, J. C. (2000). The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. *The Journal of Biological Chemistry*, *275*(46), 35863–35867. https://doi.org/10.1074/jbc.M005643200
117. Giordano, F. J. (2005). Oxygen, oxidative stress, hypoxia, and heart failure. *The Journal of Clinical Investigation*, *115*(3), 500–508. https://doi.org/10.1172/JCI24408
118. Wang, X.-X., Wang, X.-L., Tong, M., Gan, L., Chen, H., Wu, S., … Jiang, W. (2016). SIRT6 protects cardiomyocytes against ischemia/reperfusion injury by augmenting FoxO3α-dependent antioxidant defense mechanisms. Basic Research in Cardiology, 111(2), 13. https://doi.org/10.1007/s00395-016-0531-z

6.3 APPENDIX C- PERMISSIONS

6.3.1 CHAPTER 3 AND APPENDIX B

From Elsevier publishing group's website: https://www.elsevier.com/about/our-business/policies/copyright

"**Authors sign an exclusive license agreement, where authors have copyright but license exclusive rights in their article to the publisher**. In this case authors have the right to:**

- Share their article in the same ways permitted to third parties under the relevant user license (together with **Personal Use** rights) so long as it contains a CrossMark logo, the end user license, and a DOI link to the version of record on ScienceDirect.
  - **Personal Use**: Authors can use their articles, in full or in part, for a wide range of scholarly, non-commercial purposes as outlined below:
    - Use by an author in the author's classroom teaching (including distribution of copies, paper or electronic)
    - Distribution of copies (including through e-mail) to known research colleagues for their personal use (but not for Commercial Use)
    - **Inclusion in a thesis or dissertation (provided that this is not to be published commercially)**
    - Use in a subsequent compilation of the author's works
    - Extending the Article to book-length form
    - Preparation of other derivative works (but not for Commercial Use)
    - Otherwise using or re-using portions or excerpts in other works"

- Retain patent, trademark and other intellectual property rights (including research data).
- Proper attribution and credit for the published work.