Investigating Novel Caveolar Protein Interactions in Cardiovascular Redox Signalling

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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November 2021

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, ***Seyed Mojtaba Moosavi,*** declare that this thesis is submitted in fulfilment of the requirements for the award of ***Doctor of Philosophy*** in the *School of Life Sciences/of the Faculty of Science* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

I certify that the work in this thesis has not previously been submitted for a degree, nor has it been submitted as part of the requirements for a degree at any other academic institution except as fully acknowledged within the text. This thesis is the result of a Collaborative Doctoral Research Degree program with Kolling Medical Research Institute, University of Sydney.

This research is supported by the Australian Government Research Training Program.

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Date: 07th November 2021

ACKNOWLEDGEMENTS

It is a genuine pleasure to express my deep sense of thanks and gratitude to all people who decided to make this PhD possible and assisted me along the journey.

I would like to begin by sincere thanking my supervisor Dr Kristen Bubb who has been tremendously supportive throughout the project, and this thesis will not happen to be possible without her assistance, guidance, mentorship, and encouragement. She created a welcoming work environment through her enthusiasm, patience, and positive attitude. Working under her guidance was motivating and helped me achieve my goals.

I would like to extend my gratitude to Professor Gemma Figtree, who allowed me to start my PhD in her laboratory and provided guidance and feedback throughout this project. Her knowledge and achievements in and outside of the world of academia are inspiring.

I owe a deep sense of gratitude to Dr David van Reyk who offered valuable input and assistance with submitting my thesis.

I would like to thank all the team members in the lab, Dr Belinda Di Bartolo for scientific and technical guidance, Dr Own Tang for their technical assistance, Dr Marie Besnier for her comments, Dr Zara Sharane Ali and Dr Luisa Osorio for their administrative assistant. Furthermore, I would like thanks to Tom, Kat, Steve, Woody, Elijah, and Meg.

I would like to thank Prof Stuart Cordwell, Dr Melanie White and Mr Alexander Rookyard for their assistance with collecting the proteomics data and analysis; I also express my deep gratitude to Prof Marc Wilkins and Dr Matt Padula for their excellent advice for proteomics analysis.

The financial support of the University of Technology Sydney and for awarding the UTSD scholarship is greatly acknowledged. Without their support, I would not have been able to complete this project.

At a personal level, I would like to express my deep and sincere gratitude to my wife, Dr Sussan Ghassabian, for her love and support.

I feel a deep sense of gratitude to my late father, mother and sister, who formed a part of my vision and taught me the good things that really matter in life. The happy memory of my father still provides endless inspiration for my journey in this life.

ABSTRACT

Background: Cardiovascular disease (CVD) is a chronic disorder developing perniciously during life and usually progressing to an advanced stage by the time symptoms occur. CVD has been listed as the most common non-communicable disease globally. Notwithstanding some decline, CVD remains the principal cause of death in both developing and developed countries. Despite some recent success, current therapeutic methods are not efficient enough to prevent CVD, so it is essential to look for a novel therapeutic approach to preclude mortality and morbidity caused by CVD. FXYD1 protein is abundant in the heart and is known to protect cardiac sodium-potassium ATPase from oxidative stress. Nevertheless, little is known about the interaction of FXYD1, which is localised in caveolae, with other caveolae resident proteins in the heart or the role of the FXYD1 in other cardiovascular tissues. Our lab has recently demonstrated that FXYD1 protein, which is located in the invaginations of the plasma membrane called caveolae, protects eNOS from dysregulated redox signalling in the vasculature, making it a potential therapeutic target for vascular diseases.

Methods and Results: In this project, we first aimed to investigate the role of FXYD1 in cardiac and vascular redox signalling in several models of cardiovascular disease, including atherosclerosis, diabetes, and hypertension. For this project's aim, FXYD1 knock out mice, which exhibit enhanced oxidative stress and are prone to subtle increased cardiac dysfunction under normal conditions, were used. In the first instance, I examined the cardiac and vascular expression of redox signalling proteins by immunoblotting. Overall, there appeared to be some protection from oxidative stress by the presence of FXYD1. Heart and vascular tissues were obtained from atherosclerosis-prone apolipoprotein knockout (ApoE KO) mice crossed with FXYD1 wildtype and knockout mice to examine the role of FXYD1. ApoE KO / FXYD1KO males had lower NOX2 protein expression, while females had higher eNOS. In hypertensive mice, which was induced by chronic angiotensin 2 infusion, the expression level of Prdx6 in mesentery vascular tissues in FXYD-1 KO mice was significantly decreased. In the diabetic mice, which was induced by injection of pancreatic beta-cell toxin, streptozotocin and a long-term high-fat diet, the expression level of glutaredoxin 1 (GLRX-1) and eNOS in heart tissues in FXYD-1 KO mice was significantly increased. The only pattern emerging from these three models was a propensity for modified eNOS expression. Taken together with findings from a parallel study in the laboratory (in appendix) indicating a functional

interaction of FXYD1 with eNOS, I proceeded to focus on the caveolae subcellular region, a known hotspot for both eNOS regulation and oxidative signalling.

Firstly, I examined the impact of FXYD1 on caveolae morphology using electron microscopy analysis of sections of the heart from FXYD1 +/+ and -/- mice. The results of electron microscopic images showed the caveolae were denser in FXYD1 KO heart tissue, and the diameter and circumferences significantly decreased. I next aimed to determine the interaction of FXYD1 with other caveolae resident proteins and compared this to whole heart preparations using proteomics analysis. The results of cell and molecular biology studies showed that the protein expression of FXYD1 in mouse hearts was highest in caveolae subfractions (4-6) compared with other sub-fractions, which agrees with those studies that demonstrated the FXYD1 protein localized in caveolae. The results of whole heart unbiased proteomics showed that 11 proteins were considerably upregulated, although none of these were typical redox signalling proteins; rather they fall mainly within the haemostasis, immune system, metabolism, and transportation of small molecules groups. In addition, 61 proteins were significantly down-regulated in whole hearts of FXYD1 KO mice, including peroxiredoxin 5 (Prdx5), which acts as a cytoprotective antioxidant enzyme in inflammation and Phospholamban (Pln) which has a vital role in calcium homeostasis in the heart muscle.

Remarkably, from the isolated caveolae sub-fractions (fractions 4 and 5 combined), 139 proteins were upregulated, and 39 proteins were significantly downregulated in FXYD1 KO mice compared with WT. That these 139 proteins were upregulated in the caveolae fractions suggests a potential accumulation or translocation of these proteins to the caveolae. Of these, the most common signalling pathway affected were complex I biogenesis and respiratory electron transport. On the other hand, 39 proteins were uniquely down regulated in FXYD1 KO mice's caveolae, which may have contributed to the disease phenotype. Within the caveolae subfractions, glutathione peroxidase 1(Gpx1), which is an antioxidant enzyme counteracting oxidative stress, and apolipoprotein A-I (Apoa1), which participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase, apolipoprotein C-I (ApoC I), which is an inhibitor of lipoprotein binding to LDL and catalase (Cat), which is involved in redox signalling, were significantly upregulated in FXYD1 KO mice. In addition, glutathione synthetase (GSS), a redox-signalling proteins, was upregulated in caveolae, whilst down-regulated

proteins included glutaredoxin-3 (Glrx3), a critical negative regulator of cardiac hypertrophy and thioredoxin (Txn), which has a critical role in the reversible S-nitrosylation of cysteine residues in target proteins thereby contributing to intracellular nitric oxide response.

Given the key changes to lipoprotein signalling proteins, I utilised our uniquely established mouse line of FXYD1 KO mice on the atherosclerosis-prone apolipoprotein E KO background to assess the functional impact on atherosclerosis development. Here I demonstrated that FXYD1 was involved in regulating body weight in male mice but had minimal effect on plaque development. Interestingly, FXYD1 appeared pro-inflammatory and detrimental to cholesterol metabolism, as FXYD1 KO mice had lower circulating total cholesterol and increased circulating IL-1ß. This pro-inflammatory phenotype was restricted to females, hinting at a potential unique mechanism involved in the gender differences seen in our clinics' as to cardiovascular adverse events. As inflammation is a critical driver of plaque rupture, I also examined the impact of FXYD1 absence on plaque stability using the established carotid artery tandem stenosis protocol. However, no changes were evident in either necrotic core or fibrous cap thickness in FXYD1$^{-/-}$ vs. $^{+/+}$ mice on ApoE KO background. Therefore, I conclusively showed that whilst FXYD1KO mice were prone to inflammation, they were not at increased risk of plaque development or rupture.

Conclusion: This thesis's overall findings are that FXYD1 appears to be protective against oxidative damage and proinflammatory. Overall, cardiovascular disease's effect appears to be balanced with no change in atherosclerosis plaque development or stability, whether FXYD1 was present or absent. Some of the changes in atherosclerotic mice were sex-dependent. Future studies may investigate targeting FXYD1 in specific sub-cellular regions such as the caveolae, which are redox hotspots, to lower oxidative stress without causing inflammation.

PUBLICATIONS

Lo, C. C. W., **Moosavi, S.M**., Bubb, K.J. (2018). "The Regulation of Pulmonary Vascular Tone by Neuropeptides and the Implications for Pulmonary Hypertension." Front Physiol 9: 1167.

Bubb, K.J., Tang, O., Gentile, C., **Moosavi, S.M.,** Hansen, T., Liu, C.C., Di Bartolo, B.A. & Figtree, G.A. 2021, 'FXYD1 Is Protective Against Vascular Dysfunction', *Hypertension*, p. HYPERTENSIONAHA12016884.

PRESENTATIONS

S.M.Moosavi, D.van Reyk, B.Di Bartolo, O.Tang, K.J.Bubb, G.A.Figtree, FXYD1 is associated with a female-specific pro-inflammatory and hypercholesterolemic environment: Implications for Atherosclerosis, American Heart Association's annual Scientific Sessions conference, Nov.13-17th 2020, Virtual (Poster Presentation)

Seyed Mojtaba Moosavi, Belinda Di Bartolo, Owen Tang, Kristen J Bubb and Gemma Figtree, FXYD1 may be a new signalling protein in cholesterol metabolism or handling in a mouse model of atherosclerosis, New Horizon 2019, Nov. 14-15th 2019, Sydney, Australia (Oral Presentation)

Seyed Mojtaba Moosavi, Belinda Di Bartolo, Owen Tang, Kristen J Bubb and Gemma Figtree, FXYD1 is associated with lower circulating cholesterol in a mouse model of atherosclerosis, Australian Society of Atherosclerosis Meeting, Oct. 16-19th 2019, Melbourne, Australia (Finalist) (Oral Presentation)

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ABBREVIATIONS

5-LO	5-lipoxygenase
ALA	Alpha-lipoic acid
AGEs	Advanced glycation end-products
Akt	Protein Kinases B
AngII	Angiotensin II
ANOVA	Analysis of variance
ApoE	apolipoprotein E
BH_4	Tetrahydrobiopterin
CAT	Catalase
Cav-1	Caveolin 1
Cav-2	Caveolin 2
Cav-3	Caveolin 3
CHD	Coronary Heart Disease
CKD	Chronic Kidney Disease
CoQ	Coenzyme Q
CoQ10	Coenzyme Q10
CVD	Cardiovascular Disease
DALYs	Disability Adjusted Life Years
DHLA	Dihydrolipoic acid
DNA	Deoxyribonucleic Acid
eNOS	endothelial Nitric Oxide Synthetase
EDRF	Endothelium-Derived Relaxing Factor
EGF	Epidermal growth factor
FDR	False Discovery Rate
FH	Familial Hypercholesterolaemia
GPX	Glutathione Peroxidase
GSH	Glutathione
GSS	Glutathione synthetase
HDL	High-Density Lipoprotein
H&E	Hematoxylin and eosin
HT	Hypertension
IP	Intraperitoneal
IS	Indoxyl sulphate
IHD	Ischemic Heart Disease
LDL	Low-Density Lipoprotein
LDL-c	Low-Density Lipoprotein cholesterol
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
Mn-SOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
MURC	Muscle-restricted coiled-coil
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
ox-LDL	Oxidized low-density lipoprotein
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor beta
TM	Melting Temperature
RAS	Renin-Angiotensin System

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SDPR	Serum deprivation-response protein
SFK	Src family kinase F
STZ	Streptozotocin
SOD	Superoxide dismutase
PCR	Polymerase chain reaction
PAH	Pulmonary arterial hypertension
PKA	Protein Kinases A
PKC	Protein Kinases C
PLM	Phospholemman
PLN	Phospholamban
PTRF	Polymerase I and transcript release factor
UA	Uric acid
VSMCs	Vascular smooth muscle cells
WHO	World Health Organisation
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

SYMBOLS

>	greater than
<	less than
α	alpha
β	beta
Δ	delta, change in
↑	increase
↓	decrease
↔	no change

UNITS OF MEASUREMENT

d	day
g	gram
hr	hour
L	litre
M	molar
m	milli
Min	minutes
mol	moles
n	nano
Pa	Pascal
Sec	second
V	volts
°C	degrees Celsius
%	percent
µ	micro

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Chapter One: General Introduction

1.1. Introduction

Cardiovascular diseases (CVD) are chronic disorders developing perniciously during life and usually progressing to an advanced stage by the time symptoms occur (Perk et al. 2012). CVD is an umbrella term for several diseases that share pathological symptoms and encompasses coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic and congenital heart diseases, and venous thromboembolism (Stewart, Manmathan & Wilkinson 2017). CVD has been listed as the most common non-communicable disease globally, responsible for 17.8 million deaths in 2017 and more than three-quarters of those deaths happened in low and middle-income countries (Feigin et al. 2018; WHO-Group 2019). It has been reported that 5.6% of Australian adults had conditions related to CVD (AIHW 2020). Despite some declines in ischaemic heart disease and stroke (Feigin et al. 2018; Grey et al. 2018; WHO-Group 2019), CVD remains the principal cause of death in both developing and developed countries (Rosamond et al. 2007; Roth 2018; WHO-Group 2019). Because CVD is generally progressive and, in most cases, associated with inter-related disease states, these conditions also represent significant social costs requiring years of therapy and extensive health care costs (Wattanapitayakul & Bauer 2001). CVD is not only the leading cause of mortality but also the leading cause of disability and loss of productivity (Van Camp 2014). The disability-adjusted life years estimate has been expected to rise from 85 million in 1990 to almost 150 million globally by the end of 2020 (Benjamin et al. 2019; Hay et al. 2017; Perk et al. 2012). The cost of CVD in Australia during 2015-2016 was 8.9% ($10.4 billion) of total disease expenditure (AIHW 2020). The World Health Organisation (WHO) stated that three-quarters of all CVD deaths could be avoided by adequate coordinated preventative actions (WHO-Group 2019). Despite some recent success, current therapeutic methods are not adequate to prevent CVD, so it is essential to look for a novel therapeutic approach to preclude mortality and morbidity caused by CVD.

ROS (Reactive oxygen species) is a collective term that includes highly reactive free radicals (Halliwell & Gutteridge 1984), whereby overproduction of these chemical molecules (that exceeds the antioxidant defence capacity) causes oxidative stress in cells and tissues (Zuo, Best, et al. 2015). Studies show that oxidative stress is the prominent feature of the atherothrombotic process involved in heart attacks, ischaemic strokes, and peripheral arterial disease (Goszcz et al. 2015). In addition, it has been reported that ROS

overproduction can cause atherosclerosis, hypertension, diabetes, myocardial infarction, hyperlipidaemia, obesity, and congestive heart failure (Araujo et al. 1995; Bakker et al. 2000; Biswas & de Faria 2007; Le Brocq et al. 2008; Paravicini & Touyz 2006; Ray, Huang & Tsuji 2012; Sugamura & Keaney 2011; West 2000).

The potential of dietary antioxidant vitamins in preventing CVD has evoked significant interest because different studies demonstrated that oxidative modification of LDL may promote atherosclerosis (Maiolino et al. 2013; Palinski et al. 1989; Steinberg et al. 1989). Also, it has been shown that the cytotoxic effect of LDL (low-density lipoprotein) is subsequent of their oxidation and internalisation (Parthasarathy et al. 2010; Steinberg et al. 1989).

The antioxidant characteristics of vitamins (Vitamin B, C, D and E) and their impacts on various diseases, including CVD, have been widely investigated (Kushi et al. 1996; Motiwala & Wang 2012; Mozos & Marginean 2015; Norman & Powell 2014; Sesso et al. 2008). *In vitro* study has demonstrated that vitamin E could block the toxicity of ox-LDL (oxidative-LDL) in cultured endothelial cells (Negre-Salvayre et al. 1995). In addition, clinical studies showed a strong reverse association between the level of vitamin E in plasma and ischaemic heart disease (IHD) (Gey et al. 1991) and angina (Riemersma et al. 1991). It has also been demonstrated that in volunteers who took vitamin E for more than 2 years, the risk of CHD was significantly reduced (Rimm et al. 1993). Further to this, different clinical and animal studies showed vitamin E and D were associated with the reduction of myocardial infarction (Boaz et al. 2000; Giovannucci et al. 2008; Motiwala & Wang 2012; Weishaar & Simpson 1987). Also different epidemiological studies showed that intake of fruit and vegetables rich in different vitamins and antioxidants reduce the risk of CVD (Myung et al. 2013). These laboratory and clinical studies and animal experiments suggest that oxidative modification of LDL-cholesterol has a crucial role in the early development of atherosclerotic lesions (Libby et al. 2019; Ness, Chee & Elliott 1997).

The consistency in different studies and the biologic plausibility led many to endorse an explanation to suggest that vitamin supplements may reduce the risk of CVD.

However, the Heart Outcomes Prevention Evaluation (HOPE) study showed that the condition of patients, who received 400 IU of vitamin E daily did not improve compared with the patients who received placebo (Sesso et al. 2008; Yusuf et al. 2000). In addition,

the meta-analysis of randomised placebo control trials of different dosage (50-800IU) of vitamin E supplements did not benefit CVD or other diseases (Eidelman et al. 2004; Losonczy, Harris & Havlik 1996; Vivekananthan et al. 2003). More importantly, it has been demonstrated that the risk of mortality increased in the clinical trial participant who received vitamin E supplements more than 150 IU/day (Miller et al. 2005). Therefore, it is important to investigate the reason for inconsistency in clinical trials, using *in vitro* and *in vivo* studies. Noteworthy, since in the previously outlined studies there have been significant improvements in therapeutic approaches for all disease including, CVD.

Due to the important role and function of plasma membrane caveolae in compartmentalized redox signalling and significant contribution of oxidative stress in development and progression of CVD and the role and function of the caveolae and their associate proteins that are linked to different diseases, including CVD (Rajab, Straub et al. 2010, Parton 2018), which may have protective effects associated with ameliorating oxidative stress. It has been shown that the FXYD1 protein, which is a caveolae resident protein (Park, Pavlovic & Shattock 2018), has protective role against redox dependent endothelial nitric oxide synthase (eNOS) (Bubb et al. 2016) and vascular dysfunction (Bubb et al. 2021).

FXYD1 is a 72-amino-acid membrane protein, belonging to the FXYD family of ion transport regulators. FXYD1 has been detected in the heart, aorta, stomach, liver, and skeletal muscle and plays an essential role in the regulation of potassium homeostasis, especially during exercise (Bogaev et al. 2001; Nielsen & Clausen 2000; Rembold et al. 2005). In the heart, FXYD1 regulates not only Na^+-K^+-ATPase but also regulates the Na^+-Ca^+ exchanger and L-type calcium channels (Cheung et al. 2013b; Pavlovic, Fuller & Shattock 2013; Zhang et al. 2015). FXYD1 has a prominent regulatory role in cardiac performance, and emerging evidence suggests that it may protect both cardiac and vascular cells from oxidative dysregulation. Bubb etal. (2016 & 2021) showed that FXYD1 protects from cardiac oxidative dysregulation and may have a protective role against endothelial dysfunction (Bubb et al. 2021; Bubb et al. 2016). In this thesis, I will investigate the role of FXYD1 in redox signalling and the impact of FXYD1 absence in several CVD states.

1.2. CVD Risk Factors:

Risk factors associated with CVD can be modifiable or non-modifiable (Qizilbash 1995; Tuomilehto 2004). Modifiable risk factors include inappropriate nutrition, high

alcohol intake, lack of physical activity, increased tobacco consumption, the high consumption of saturated fats, salt, refined carbohydrates, and low consumption of fruits and vegetables. In addition, modifiable metabolic risk factors include overweight/obesity and low cardiac-respiratory fitness. Non-modifiable risk factors include high blood pressure, diabetes, dyslipidaemia (Perk et al. 2012), increasing age and male sex (Jousilahti et al. 1999; Kovacic, Moreno, Hachinski, et al. 2011; Kovacic, Moreno, Nabel, et al. 2011; Woodward 2019).

1.2.1. Modifiable Risk factors

1.2.1.1. Cholesterol

Cholesterol, which was isolated for the first time from gallstones in 1789 (Maxfield & Tabas 2005), plays an important role in the plasma membrane's structural and functional characteristics (Benito-Vicente et al. 2018). Cholesterol is an indispensable contributor to cell barrier formation and cell signalling transduction (Cherezov et al. 2007; Ikonen 2008) and can regulate the function of many important proteins directly by interacting with those proteins (Grouleff et al. 2015) or indirectly by effects on membrane fluidity. Cholesterol also affects the plasma membrane's biophysical properties and, consequently, membrane permeability (Yang et al. 2016). Besides, cholesterol acts as the precursor of many steroid molecules such as bile salts (Russell 2003), steroid hormones (Midzak & Papadopoulos 2014) and vitamins (Wang 2016).

Due to cholesterol insolubility in plasma, it requires to be carried by molecular complexes called lipoproteins. The main cholesterol carrying lipoproteins are low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Hegele 2009). The LDL that carries cholesterol, can cross arterial walls, and be engulfed by macrophages, subsequently oxidation of accumulated LDL leads to cytokine production and chemotaxis of inflammatory cells. The LDL that is engulfed in macrophages in the arterial wall results in cytosolic cholesterol accumulation leading to the development of the “foam cell” phenotype which are central to the development of atherogenic plaques (Hegele 2009; Rader & Daugherty 2008). In addition, it has been established in human young adults that the smooth muscle cells (SMCs) have an essential role in “foam cells” formation in early stage of atherosclerosis (Wang et al. 2019).

Taken together, cholesterol acts as a double-edged sword, while the optimum level of it has a crucial role in cells, tissues and body functions, over-accumulation, and abnormal

deposition of cholesterol inside the body can be the detrimental cause of fatal diseases such as CVD.

1.2.2. Non-modifiable risk factors

1.2.2.1. Age is an Independent Risk Factor for Cardiovascular Disease

Age is a fundamental and significant risk factor affecting cardiovascular homeostasis (Kovacic, Moreno, Hachinski, et al. 2011). It has been shown that 40% of the deaths in people aged > 65 are caused by atherosclerotic disease and its complications (Heidenreich et al. 2011). However, the age as indicated by the multivariable models can be an expression of the strength and the length of exposure to other traditional CVD risk factors (Sniderman & Furberg 2008). Clinical trials showed that normalisation of cholesterol and blood pressure in elderly patients via prescription drugs and lifestyle intervention is less beneficial than in younger patients (Tuomilehto 2004). Different studies also showed that the burden of CVD risk linked with increasing age could be reduced partially by the adjustment of traditional coexisting CVD risk factors (Casiglia et al. 2003; Dhingra & Vasan 2012).. In addition, it has been shown that aging is associated with a low-grade chronic pro-inflammatory state, which participate in chronic degenerative disease including CVD (Franceschi et al. 2000; Frasca & Blomberg 2016; Liberale et al. 2020). Therefore, the rising age could be considered as an independent risk factor for CVD

1.2.2.2. The impact of sex on CVD

For many years, CVD has been considered as a “male” disease, owing to men’s higher sheer risk compared with women (Jousilahti et al. 1999). However, studies showed that the comparative risk of CVD morbidity and mortality in women is slightly higher than men (Garcia, Miller, et al. 2016; Garcia, Mulvagh, et al. 2016; Moller-Leimkuhler 2007; Nabel 2015; Wilmot et al. 2015). CHD has been reported to be the cause of most CVD deaths in women, and because it is a silent disease in two-third of cases and has high mortality rate, the prevention of CHD is vital (Baigent et al. 1998; Zhao et al. 2018). Similar to the CHD, atherosclerotic/thrombotic CVD, such as peripheral arterial disease, which is underdiagnosed and under-treated in women; should be considered and treated more seriously (Jelani et al. 2018; Mosca et al. 2004; Olinic et al. 2018). The prevalence of some of the CVD, such as CHD is greater in post-menopausal women than in men (Crandall & Barrett-Connor 2013; Mosca et al. 1999; Pathak et al. 2017; Woodward 2019). Whilst younger women are protected from CVD, in post-menopausal women, the risk of CVD increases (Cagnacci & Venier 2019), and this has been predominantly

attributed to lowering of circulating oestrogen. Evidence suggests that hormone replacement therapy may reduce the risk of heart disease and mortality if started immediately (Schierbeck et al. 2012) or within ten years of the onset of menopause in women under 60 years of age (Cagnacci & Venier 2019).

Until 2000, CVD in women had been underappreciated because of lower prevalence rates in younger age groups, and due to the image of CVD as a male disorder resulting in predominant under-diagnosis of these disorders in women. Therefore, for a better understanding of the impact of sex on CVD, in all studies, sex-disaggregated analyses are encouraged.

1.2.2.3. Genetics and CVD:

Over the past century, the improvement of molecular biology tools helped biomedical researchers correlate genotype with phenotype and provide researchers and clinicians with the ability to identify the specific genes and DNA sequence variants responsible for trait variation in humans. Applying these technologies has helped to discover the role of genetics in different diseases, including CVD.

The development of CVD is shaped by many factors including gene-gene and gene-environment interactions (Abhik, Souvick & Birendranath 2015) and the linkage between genetics and CVD has been demonstrated by the study of twins and families with CVD history (Abbate, Sticchi & Fatini 2008). To date, some genetically-based cardiac dysfunction and their associate genes, such as long QT syndrome (Alders, Bikker & Christiaans 1993), short QT syndrome (Templin et al. 2011), Brugada syndrome (Grant et al. 2002), familial atrial fibrillation (Lin et al. 2014), catecholaminergic polymorphic ventricular tachycardia (Napolitano & Priori 2002; Priori et al. 2002) and Wolff-Parkinson-White syndrome (Vega et al. 2009); have been identified.

One of the key focus areas of this thesis is atherosclerotic vascular disease, for which there is a strong hereditary component. Lloyd-Jones et al. (2004) showed that in the general population, a history of premature atherosclerotic CVD in a parent results in a 3.0-fold increase in CVD risk to offspring (Lloyd-Jones et al. 2004). However; the precise magnitude of the inheritance function changes by disease, age of disease onset, and disease subtype (Kathiresan & Srivastava 2012).

One of the most frequent CVD hereditary diseases is familial hypercholesterolemia (FH) (Benito-Vicente et al. 2018), which is an autosomal co-dominant disorder mainly

characterised by high plasma levels of low-density lipoprotein cholesterol (LDL-C) (Brown & Goldstein 1986). A mutation in the LDL receptor gene is responsible for producing FH (Hobbs, Brown & Goldstein 1992) and it has been demonstrated that untreated FH leads to premature atherosclerotic cardiovascular disease (Ference et al. 2017).

1.3. Reactive oxygen species (ROS)

ROS is a collective term that includes highly reactive free radicals that contain one or more unpaired electrons (Halliwell & Gutteridge 1984). ROS are produced in all aerobic cells, involved in an oxidising biological molecule such as DNA, proteins, carbohydrate, and lipids. Oxidative stress in cells and tissues happens when ROS production exceeds the antioxidant defence capacity (Zuo, Best, et al. 2015). Different studies in the last few decades show how oxidative stress and redox-regulated mechanisms are involved in endothelial dysfunction (Incalza et al. 2018), pathological remodelling (Schiattarella & Hill 2017), profibrotic function (Iglarz et al. 2004) and inflammation (Federico et al. 2007). These processes contribute to the pathogenesis of many diseases, including CVD (e.g., peripheral vascular disease, coronary heart disease, stroke, and myocardial infarction) and a significant correlation has been reported between oxidative stress and CVD (Blomhoff 2005; Bubb et al. 2017; Giordano 2005; Nojiri et al. 2006). Also, it has been reported that oxidative stress, disrupts redox- dependant signalling processes in arterial walls and permanently damages the macromolecules present (Stocker & Keaney 2005).

Some of the most important ROS are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) (Bubb et al. 2017; Zuo et al. 2000; Zuo, Zhou, et al. 2015), which have a crucial role in promoting and generating oxidative stress in biological systems. Additionally, the term of ROS is often used to cover reactive nitrogen species (RNS), and both species, ROS and RNS, are typical metabolic by-products (Doshi et al. 2012; Valko et al. 2007). It is also worth knowing that the low ROS concentrations are required in many cellular processes, and excess ROS needs to be neutralised by endogenous antioxidants (Labunskyy & Gladyshev 2013).

A better understanding of ROS's role and function in the pathogenesis of vascular disease may provide an insight into the development of novel therapeutic methods for treating cardiovascular disease via targeting ROS production and increasing the

antioxidant defences which lead to reduction of CVD risk (Leopold 2015; Senoner & Dichtl 2019).

1.3.1. What is the cause of oxidative stress?

Oxidative stress occurs due to the imbalance between the generation of ROS and the ability to neutralise these ROS by endogenous antioxidant systems, whereby ROS levels exceed the antioxidant capacity. Because the endogenous antioxidant systems fail to neutralise all the ROS, the irreversible damages to the cell membranes, DNA and other cellular components happen by oxidising lipids, proteins, and nucleic acids. Oxidative stress plays a crucial role in the pathogenesis of many diseases, including cardiovascular diseases related to hypoxia, cardiotoxicity, and ischaemia reperfusion (Burtenshaw et al. 2017; Daugherty et al. 1994; Ellulu et al. 2016; Mehrabian et al. 2002; Rao & Berk 1992; Sugamura & Keaney 2011; Zalba et al. 2001). The study of ROS and its effects on CVD is one of the central parts of this study.

1.3.2. Different types and Sources of ROS

ROS can be formed by a number of different cellular enzymes, including NAD(P)H oxidases (Seshiah et al. 2002), xanthine oxidase (XO), and cytochrome P450. (Griendling, Sorescu & Ushio-Fukai 2000; Sawyer et al. 2002; Xia, Roman, et al. 1998; Xia, Tsai, et al. 1998; Xiao et al. 2002). Production of ROS also occurs through autooxidation of catecholamines (Bindoli, Rigobello & Deeble 1992; Miller, Selhub & Joseph 1996; Niu et al. 2012).

1.3.2.1. NADPH oxidases (NOX)

NOX are a family of enzymes that generate reactive oxygen species that are involved in the pathology of cardiac vascular disease (Lassegue & Griendling 2010; Robert & Robert 2014). NOX catalyses ROS production, leading to the elimination of invading microorganisms by macrophages and neutrophils and thereby functions as an inflammatory mediator (Franchini et al. 2013). NOX are a family of isoenzymes of which four of those (NOX1, NOX2, NOX4, and NOX5) are expressed in the vasculature (Lassegue & Griendling 2010). NOX2 and NOX4 are two of the NOX isoforms, which are most abundantly expressed in cardiomyocytes (Santillo et al. 2015). NOX2 is localised in the cardiomyocytes' plasma membrane and is regulated by angiotensin II (Ang II) (Byrne et al. 2003). NOX4 is localised in intracellular compartments and has been shown to protect mouse hearts from chronic load-induced stress by enhancing angiogenesis (Zhang et al. 2010) and also by modulating cardiomyocyte differentiation

(Murray et al. 2013). However, it has been demonstrated that overexpression of NOX4 in the heart increases O_2^- production and induce cardiac dysfunction (Ago et al. 2010).

NOX facilitates electron transfer from the cytosol to different parts of cells across the membrane (Lassegue & Griendling 2010). That is, the transmembrane proteins expressed by the NOX gene are responsible for transporting electrons across biological membranes and reducing oxygen to superoxide (Cross & Segal 2004). Specifically, NOX enzymes transfer electrons from NADPH to O_2 (molecular oxygen) to generate superoxide ($O_2^{\bullet-}$). Because superoxide cannot cross the membranes and is transitory, it affects only the surrounding area. However, in some cases, superoxide dismutase quickly converts superoxide to hydrogen peroxide, a relatively stable molecule compared with superoxide which and can diffuse across the membrane (Bedard & Krause 2007; Lassegue & Griendling 2010).

1.3.2.2. Mitochondria

Mitochondria are the primary source of intracellular ROS production and the electron transport chain is known as one of the essential sources of ROS (Wattanapitayakul & Bauer 2001). Any leakage of electrons, from the electron transport chain to oxygen, may result in physiological ROS production. However, the endogenous antioxidants superoxide dismutase (SOD2) and glutathione quickly degrade $O_2^{\bullet-}$ to halt the reactivity (Labunskyy & Gladyshev 2013).

Under normal physiological conditions, mitochondria use oxygen for oxidative phosphorylation and ATP production, and the ROS are the by-products of these reactions (van Hameren et al. 2019). Mitochondrial ROS has been reported as the causes of many metabolic conditions and disease, including ischemia-reperfusion injury, inflammation, and pulmonary hypertension syndrome (Bottje 2019).

In mitochondria $O_2^{\bullet-}$ are converted to H_2O_2 by manganese superoxide dismutase (Mn-SOD/SOD2) (Chance, Sies & Boveris 1979). The importance of Mn-SOD, which is a free radical scavenging enzyme, in CVD, has been well studied. The seminal finding showed that cardiomyopathy was developed in Mn-SOD knockout mice, and all mice died within ten days after birth (Koyama et al. 2013; Lebovitz et al. 1996; Li et al. 1995).

1.3.2.3. Oxidised low-density lipoprotein (ox-LDL)

Ox-LDL is not only a circulating biomarker related to CVD (Trpkovic et al. 2015), but also has an important role in causing proatherogenic effects, such as endothelial

dysfunction and smooth muscle proliferation in the vascular wall (Sawamura et al. 1997; Yoshimoto et al. 2011). ox-LDL causes oxidative stress in a pathophysiologic setting and promotes atherogenesis (Verhoye, Langlois & Asklepios 2009). Cominacini et al. (1997) showed the ox-LDL increases ROS formation in human umbilical vein endothelial cells (HUVECs) (Cominacini et al. 1997).

1.3.2.4. Angiotensin II (AngII) - Indoxyl sulphate (IS)

AngII and IS are known as other sources for increasing the ROS levels and causing cellular dysfunction (Nakano et al. 2019; Yang et al. 2012).

Angiotensinogen, which is found mainly in the liver, is the source of AngII (Biancardi et al. 2017). Although, it has been shown that AngII is produced by other organs such as cardiovascular system, kidney and central nervous system (Paul, Poyan Mehr & Kreutz 2006). Ang II is one of the renin-angiotensin system's major elements and modulates various physiological and pathological processes, including fluid homeostasis, aldosterone production, vasomotor tone, and renal function. Ang II is not only an autacoid with haemodynamic and renal actions but also a biologically active intermediator that acts directly on endothelial cells (Yang et al. 2012). Different studies have demonstrated that Ang II stimulates NADPH oxidase-dependent superoxide production in endothelial cells and that Ang II is the primary inducer of ROS in these cells (Alvarez et al. 2010; Shuvaev et al. 2009).

Throughout chronic kidney disease (CKD) progression, kidney-specific risk factors for cardiovascular disease come into play. Indoxyl sulphate is the end-stage product of dietary tryptophan metabolism and is significantly elevated in the plasma of patients with CKD. This has been attributed its high-affinity binding to albumins preventing it from being efficiently removed by haemodialysis (Kaminski et al. 2017; Niwa 2010). It has been demonstrated that indoxyl sulphate induced ROS generation by upregulating NOX4 and subsequently induced the expression of osteoblast-specific proteins including Cbfa1, ALP and osteopontin in human aortic smooth muscle cells (Muteliefu et al. 2009). It has been shown that indoxyl sulphate induced ROS generation can occur by down-regulation of uncoupling protein 2, a protector of cardiomyocytes against oxidative stress and hypertrophy (Yang et al. 2015).

Thus, a high level of indoxyl sulphate in the plasma of CKD patients might lead to a high risk of CVD via the mechanism related to haemostatic system disturbances (Kaminski et al. 2017).

1.3.2.5. Tetrahydrobiopterin (BH_4)

BH_4 is an essential cofactor for nitric oxide (NO) formation from eNOS (Channon 2004). eNOS presents as a dimer to generate NO in the presence of enough levels of BH_4. In the process of generating NO from eNOS, BH_4 is changed into 7,8-dihydrobiopterin (BH_2) and is recycled back to BH_4 in a two-step process. If the level of BH_4 is not adequate, this process will be impaired and leads to the generation of superoxide from the ferrous-dioxygen complex instead of NO. The superoxide anion, which is generated due to lack of BH_4, then reacts with free NO to form peroxynitrite, which can subsequently react with other molecules and generate different ROS (Schmidt & Alp 2007). In this process, eNOS presents as uncoupled monomers, and this leads to superoxide production (Oelze et al. 2000; Ozkor & Quyyumi 2008).

1.3.2.6. The Lipoxygenase (LOX) family

LOXs are iron-containing enzymes that catalyse the peroxidation of polyunsaturated fatty acids. LOX products and their metabolites, eicosanoids, are potent lipid mediators that invoke various biological reactions. These eicosanoids, derived from arachidonic acid, play significant roles in inflammation pathways and inflammation resolution (Kobe & Newcomer 2013). Notably, it has been shown that 5-lipoxygenase (5-LO) was found in atherosclerotic plaque and abdominal aortic aneurysms (Mehrabian et al. 2002).

1.3.2.7. Myeloperoxidase (MPO)

MPO is another source of ROS and is a haem-containing peroxidase present in neutrophils, monocytes, and macrophages. MPO generates hypochlorous acid (HOCl), which is a damaging ROS utilising hydrogen peroxide (H_2O_2) and chloride (Cl^-) (Davies et al. 2008). MPO produced ROS, contribute to lipid oxidation in atherosclerosis (Daugherty et al. 1994).

1.3.2.8. Xanthine oxidoreductase (XOR)

XOR is an enzyme that is a member of the “molybdoenzyme” family (Berry & Hare 2004). XOR is a homodimer with a molecular weight of 150 KDa (Krenitsky, Spector & Hall 1986). Two forms of XOR have been detected that are interconvertible, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). While XO can only reduce oxygen,

XDH can reduce either oxygen or NAD^+(Berry & Hare 2004). XOR is known for its catalytic role in purine degradation and metabolising hypoxanthine to xanthine and subsequently xanthine to uric acid. Hyperuricaemia (high level of circulating uric acid) is considered as an independent atherogenic risk factor (Feig et al. 2006), and the close relationship between hyperuricaemia and CVD has been demonstrated (Li et al. 2014). Due to its role and function in ROS production and the severe impact on CVD, XOR has attracted much attention from scientists during the last two decades (Feig, Kang & Johnson 2008; Feoli et al. 2014).

1.3.2.9. Glucose

The metabolic abnormalities of hyperglycemia cause tissue damage through the four following mechanisms:

1. The glucose and other sugars increase flux through the polyol pathway. The polyol pathway involves two enzymes. The first enzyme, aldose reductase (AR), reduces glucose to sorbitol with its co-factor NADPH, and the second enzyme, sorbitol dehydrogenase (SDH), with its co-factor NAD^+, changes sorbitol to fructose. Aldose reductase competes with glutathione reductase for their co-factor NADPH, leading to a decrease in GSH, which can lead to oxidative stress (Horakova et al. 2013).
2. The formation of advanced glycation end-products (AGEs) increases inside the cells and damages the cells by three different mechanisms; a) modification of intracellular proteins that regulate the gene transcription (Shinohara et al. 1998), b) AGE diffuse out of the cells and alter the molecules in the extracellular matrix around the cells (McLellan et al. 1994) and c) AGE precursors diffuse out of the cell and modify circulating blood proteins such as albumin, which can bind to AGE receptors resulting in the production of inflammatory cytokines and growth factors (Li, Mitsuhashi, et al. 1996; Li, Steffes, et al. 1996; Makita et al. 1996).
3. Hyperglycemia elevates diacylglycerol levels which is a vital activating cofactor for protein kinase C (PKC) (Koya & King 1998). Activation of PKC impacts the expression of a variety of the genes, for instance, reducing the expression level of eNOS (Kuboki et al. 2000).
4. High level of glucose increases the activity of the hexosamine pathway. One of the by-products of this pathway is uridine diphosphate N-acetyl glucosamine, which may get put onto serine and threonine of transcription factors and result in pathologic changes in gene expression (Wells & Hart 2003).

All these four mechanisms are activated by mitochondrial overproduction of ROS (Brownlee 2005).

Interestingly, a high level of glucose can generate ROS through additional pathways, such as mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Brownlee 2005; Giacco & Brownlee 2010), and also glucose increases the ROS formation via the sorbitol pathway, the insulin pathway and glycation (Prasad & Dhar 2014).

1.3.2.10. The major source of ROS in CVD

Some of the ROS sources that were explained in previous sections are heavily implicated in CVD induction. It has been shown that overexpression of mitochondrial respiratory chain components (Nojiri et al. 2006), NADPH oxidase (Chen et al. 2004; Judkins et al. 2010), xanthine oxidase (Schroder et al. 2006), lipoxygenase (Zhao & Funk 2004; Zhao et al. 2004), nitric oxide synthases (Laursen et al. 2001), and myeloperoxidase (Eiserich et al. 2002) correlate to the development and progression of CVD.

1.4. Antioxidants

While oxygen is a vital element for aerobic living organisms; its volatile nature means that oxygen reactions can lead to toxic effects (Halliwell 1999a). To overcome this potential, aerobic organisms have developed sophisticated antioxidant defence systems. Antioxidants can be defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Gutteridge & Halliwell 2018; Halliwell & Gutteridge 1995; Halliwell 2007). Antioxidants can be produced endogenously or ingested from fruits, vegetables, and grain (Halliwell 1999b). Some examples of naturally occurring sources of antioxidants are herbal plants (Saluk-Juszczak et al. 2010), grape seeds (Leifert & Abeywardena 2008a, 2008b), black chokeberry (Hwang et al. 2014) and nuts (Blomhoff et al. 2006). The critical roles and function of vitamin E and ascorbic acid, as antioxidants *in vivo* have been well studied and appear to have biological benefits (Diplock 1997; Levine 1986a, 1986b). In contrast, utilising these antioxidants as oral supplements in large-scale, strictly controlled clinical trials to improve cardiovascular health indicators have been unsuccessful (Bubb, Drummond & Figtree 2020).

Currently, two types of endogenous antioxidant have been identified, protein antioxidants and non-protein antioxidants.

1.4.1. Non-protein endogenous antioxidants

1.4.1.1. Glutathione ((L-γ-glutamyl-L-cysteinyl-glycine) (GSH))

GSH is a tripeptide non-protein thiol antioxidant that protects cells against reactive oxygen/nitrogen species and is present in high concentrations in living organisms (Schafer & Buettner 2001). GSH can scavenge free radicals by donating electrons and exhibits antioxidant activity (Mezyk 1996). That is, this electron denoting abilities enable GSH to neutralise free radicals and ROS, which are the electron acceptors.

In an environment of relatively high concentrations of GSH, the S-glutathionylation mechanism promotes and reverses the modification of oxidised cysteine, which leads to reduction of oxidative stress (Liu & Hannun 1997; Lundberg et al. 2001; Zitka et al. 2012). GSSG is an oxidised form of GSH, which contains two GSH with disulphide bonds, and the ratio of GSH vs GSSG is an indicator of the redox state of cells (Jeong & Joo 2016; Lillig et al. 2005). While in normal physiological conditions, the GSH/GSSG ratio exceeds 100 (Gladyshev et al. 2001), under oxidative stress conditions the ratio may approach one (Shelton, Chock & Mieyal 2005). Thus, any molecule or substance that can modify cysteine residues' oxidative capacity and maintain the normal GSH/GSSG ratio could be utilised as a therapeutic agent to treat oxidative stress.

1.4.1.2. Alpha-lipoic acid (ALA)

ALA is a coenzyme involved in mitochondrial metabolism pathways. It is a potent mitochondrial antioxidant agent that acts through multiple mechanisms also elevating anti-inflammatory and anti-thrombotic capacity and positively influencing NO-mediated vasodilation (Tromba et al. 2019). ALA can deliver the antioxidant activity in both oxidised (ALA) and reduced (DHLA [dihydrolipoic acid]) forms (Liu 2008). The cellular reaction between ALA and DHLA is carried out by NAD(P)H- dependent enzymes (Figure 1.1), thioredoxin reductase, lipoamide dehydrogenase, and glutathione reductase (Rochette et al. 2015). ALA can recycle other antioxidants, including vitamin C, E, and coenzyme Q (CoQ) and may chelate iron and copper (Moini, Packer & Saris 2002). The protective role of ALA has been studied and demonstrated in diseases associated with oxidative stress and metabolic disorders such as diabetes mellitus, hypertension and hyperhomocysteinaemia (Aguilar 2016; Akbari et al. 2018; Baydas et al. 2002; Estrada et al. 1996; Vasdev et al. 2000a, 2000b).

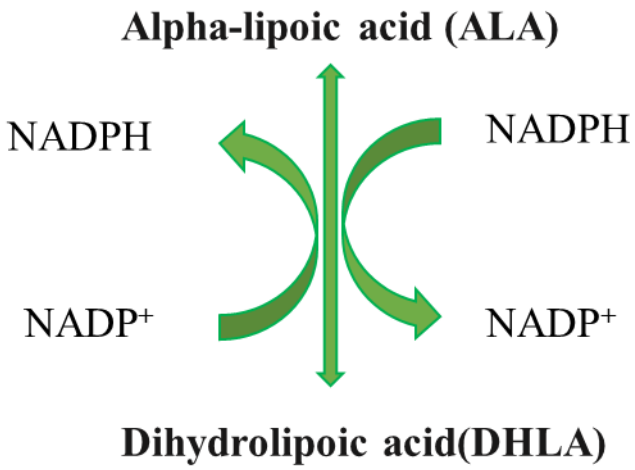


Figure 1.1: ALA oxidisation process, in an interconvertible process NADPH reduces ALA to DHLA by giving an electron and $NADP^+$oxidase DHLA to ALA by taking an electron

1.4.1.3. Coenzyme Q (CoQ)

CoQ or ubiquinone is localised in the cell membranes and is involved in electron transportation participating in many redox reactions, including bioenergetics, nucleotide biosynthesis and antioxidant mechanisms (Alcazar-Fabra, Navas & Brea-Calvo 2016; Gille & Nohl 2000). It is essential to know that the CoQ family members "ubiquinol" (CoQH2), which is a hydrogen donor, is the major endogenous lipid-soluble antioxidant in mammals that prevents the generation of lipid peroxidation, as well as DNA and protein oxidations mediated by lipid hydroperoxides (Ayer, Macdonald & Stocker 2015; Stocker & Keaney 2004). CoQ's protonmotive role has been proposed as one of the most crucial components of mitochondrial energy production (Mitchell 1975). Also, the impact of CoQ deficiency on heart disease has been studied and reported (Folkers et al. 1970). Rosenfeldt et al. (2005) investigated the effect of a daily 300 mg dose of Coenzyme Q10 (CoQ10) in patients before cardiac surgery. They showed that in patients who received CoQ10, the level of mitochondrial CoQ10 in myocardial and cardiac cells increased and was associated with the improved mitochondrial efficiency and myocardial tolerance to *in vitro* hypoxia-reoxygenation stress (Rosenfeldt et al. 2005). Because CoQ10 has a crucial role in cell metabolism and acts as an effective anti-inflammatory agent exerting endothelial function improvement, it has therapeutic potential for treatment of heart failure patients (Di Lorenzo et al. 2020)

1.4.1.4. Ferritin

Iron is necessary for normal mammalian cell growth and proliferation. Nevertheless, free iron can participate in oxygen-free radical formation via the Fenton reaction (Figure 1.2) (Linn 1998). Therefore, the cell must balance the beneficial and deleterious effects of iron to survive.

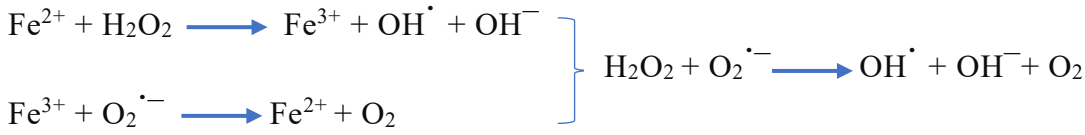


Figure 1.2: Oxygen free radical formation via Fenton Reaction

Ferritin is an iron-binding protein, which plays a central role in maintaining intracellular iron balance and controls the Fe (II) availability and thus generating of ROS (Balla, Jacob, Balla, Rosenberg, et al. 1992; Balla, Jacob, Balla, Nath, et al. 1992). The protective role of ferritin against oxygen free radical damage has been shown by several studies (Bresgen & Eckl 2015; Orino et al. 2001).

1.4.1.5. Bilirubin

Reduction of biliverdin by biliverdin reductase generates bilirubin, which is the end product of haem degradation (Aguilar 2016). The antioxidant activity of bile pigments, of which bilirubin is a component, has been studied (Baranano et al. 2002; Stocker, Glazer & Ames 1987). However, Maghzal et al. 2009 showed that bilirubin oxidation does not usually generate biliverdin and that biliverdin is not a significant cellular protector via the antioxidant redox cycle (Maghzal et al. 2009). Many studies have supported the beneficial role of bilirubin. Bilirubin prevents ischaemic injury of isolated hearts (Clark et al. 2000) and has beneficial action against cardiovascular disease development (Hopkins et al. 1996). Bilirubin attenuates the oxidative damage in cell culture (Foresti et al. 1999) and defends neurons against hydrogen peroxide damages (Dore & Snyder 1999).

1.4.1.6. Uric acid (UA)

UA, which is an end product of purine metabolism in humans and great apes (Figure 1.3), and also acts as an antioxidant (Davies et al. 1986; Maiuolo et al. 2016; Ndrepepa 2018). It has been demonstrated that urate is the strongest water-soluble antioxidant, and the resistance of plasma lipids to oxidation is due to its action (Nyyssonen et al. 1997).

UA's protective role in cardiac, vascular and neural cells against oxidative injury has been well studied; for instance, urate can bind transition metals that could be relevant for LDL lipid peroxidation in the vascular wall (Stocker & Keaney 2004). Despite UA's antioxidant activity, that it is optimally active in the hydrophilic environment limits its antioxidants function (Aguilar 2016).

Different studies showed that hyperuricaemia is associated with a significant increase in the risk of CHD events and mortality, specifically in females (Li et al. 2016); however, this topic remains controversial (Ndrepepa 2018).

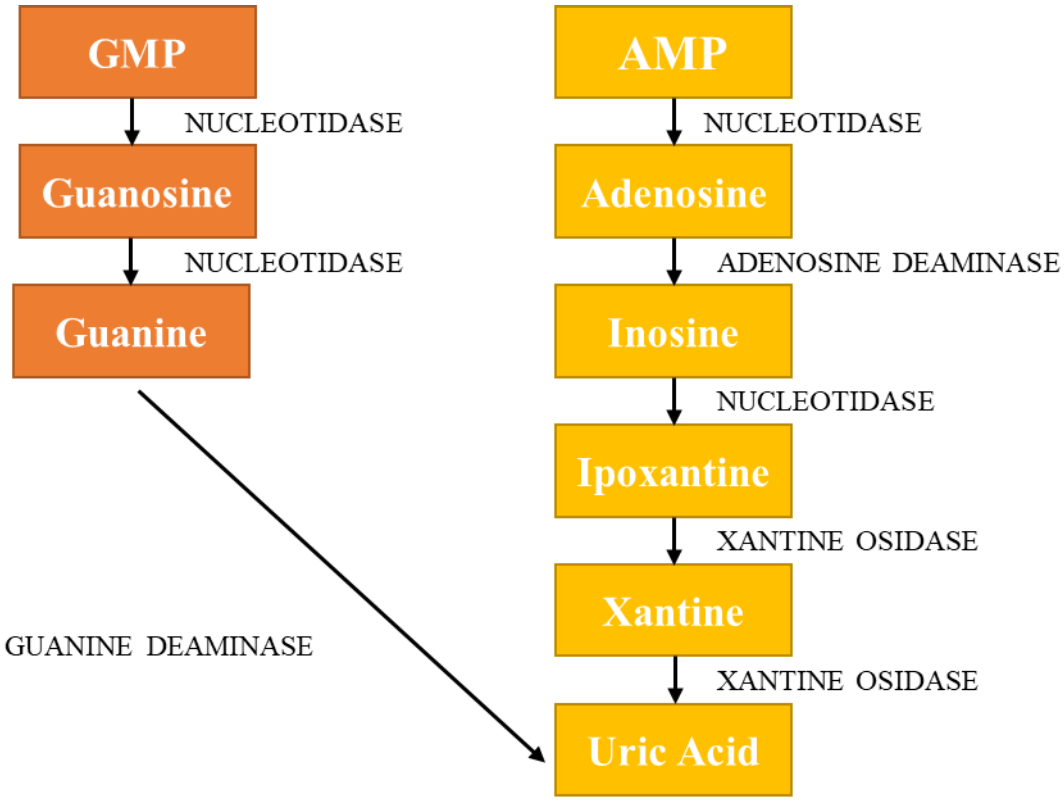


Figure 1.3: Enzymatic degradation of purine in humans (Maiuolo et al. 2016)

1.4.2. Endogenous protein antioxidants

Endogenous protein antioxidants are the antioxidant enzymes, which are the front line of defence against oxidative stress on the body. Three major ones are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), which will be briefly explained.

1.4.2.1. Superoxide dismutase (SOD)

SODs are a group of critical enzymes converting reactive superoxide radicals into hydrogen peroxide and molecular oxygen (dismutation) (Wang et al. 2016). In other words, SODs are the major antioxidant defence systems against $O_2^{\bullet-}$ (Fukai & Ushio-Fukai 2011). Figure 1.4 shows the dismutase reaction.

Figure 1.4: Dismutation reaction

Four isozymes of superoxide dismutase had been identified and studied; these isozymes are located in different parts of cells and protect cells against oxidative stress (Laukkanen 2016).

1.4.2.2. Catalase

Catalase is an important enzyme that is mainly detected in peroxisomes and is able to catalyse the conversion of H_2O_2 to water and molecular oxygen in two steps (Figure 1.5) (Amir Aslani & Ghobadi 2016; Chaudiere & Ferrari-Iliou 1999; Olson et al. 2017; Valko et al. 2006).

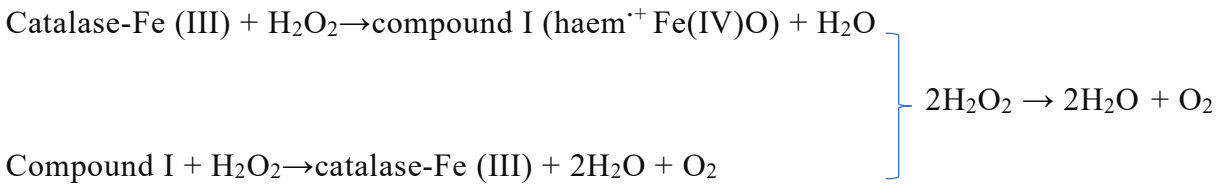


Figure 1.5: The figure shows how catalase converts H_2O_2 to water and molecular oxygen

As with other antioxidant enzymes, catalase is also present in animal cells such as erythrocytes, renal cells, and hepatic cells (Jenkins & Tengi 1981; Olson et al. 2017). Based on catalase's antioxidant activity, it has been reported as the second most abundant enzymatic antioxidant (after superoxide dismutase), by which it plays a major role in the attenuation of the levels of reactive oxygen species (Vendemiale, Grattagliano & Altomare 1999).

1.4.2.3. Glutathione peroxidase (GPx)

GPx is another endogenous protein antioxidant, which catalyses the reduction of H_2O_2 or organic peroxide (ROOH) to water or alcohol (Figure 1.6). This reaction occurs in the presence of GSH, which is oxidised and converted to GSSG (Dickinson & Forman 2002; Valko et al. 2006).

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{GPX} 2ROH + GSSG$$

Figure 1.6: GPx reduction process

Figure 1.6 illustrates the reactions that can protect polyunsaturated fatty acids, which are located inside the cell membranes (Forsberg, de Faire & Morgenstern 2001). Four isomers of GPx have been identified, and their antioxidant's role and functions in different tissues, including heart has been studied (Forsberg, de Faire & Morgenstern 2001; Jan et al. 2015).

1.5. Role and impacts of ROS in cardiovascular disease (CVD):

The majority of CVD is accompanied by an imbalance between formation of ROS and detoxification of ROS by low molecular weight antioxidants or ROS degrading enzymes (Griendling & FitzGerald 2003a, 2003b). The role and impacts of ROS and oxidative stress on CVD have been studied over the last decades, and the results of studies showed that ROS were important characteristics of CVD including atherosclerosis, hypertension, and congestive heart failure (Sugamura & Keaney 2011). The function of dietary antioxidant vitamins in preventing CVD has evoked significant interest because different studies demonstrate that oxidative modification of LDL may promote atherosclerosis (Maiolino et al. 2013; Palinski et al. 1989; Steinberg et al. 1989). Also, it has been shown that the cytotoxic effect of LDL is subsequent to its oxidation and internalisation (Parthasarathy et al. 2010; Steinberg et al. 1989). The outcome of some the antioxidant clinical trials were very promising (Losonczy, Harris & Havlik 1996; Schwingshackl et al. 2017; Stephens et al. 1996). However, other clinical trials results were very

disappointing (Miller et al. 2005; Yusuf et al. 2000). Table 1.1 demonstrates the summary of some of the published clinical trials that shows the impact of vitamins and mineral intake on CVD.

From the physiological point of view, ROS are formed in low concentrations and play as signalling molecules regulating vascular smooth muscle cell (VSMC)contraction and relaxation (Rao & Berk 1992). Whereas from a pathophysiological point of view the ROS act notably in developing disorders, including atherosclerosis, arrhythmia, cardiomyopathy, congestive heart failure, ischaemic heart disease and diabetes (Zalba et al. 2001).

Due to inconsistency of the role and function of antioxidant vitamins on CVD, it is important to study and find the cause of this inconsistency, which is the core object of this study.Table 1.1: Clinical trials Summary

Study ID	Vitamin E	Vitamin C	B-Carotene	Is there any **Benefit?**	Ref.
The Epidemiology, Demography and Biometry Program (n=11178)	✓	✓	×	YES	(Losonczy, Harris & Havlik 1996)
CHAOS	✓	×	×	YES	(Stephens et al. 1996)
ATBC (n=21930)	✓	×	×	NO	(Pietinen et al. 1997)
T HE H EART O UTCOMES P REVENTION E VALUATION S TUDY IVESTIGATORS (n=9541)	✓	×	×	NO	(Yusuf et al. 2000)
Meta-analysis of 19 clinical trials (n=135967)	✓	In 9 trials only vitamin E tested and in 10 vitamin E combined with other vitamins and minerals.		NO	(Miller et al. 2005)
Woman's Health (n=39876)	✓	×	×	NO	(Lee et al. 2005)
HOPE (n=4000)	✓	×	×	NO	(Lonn et al. 2005)
GISSI (n=11324)	✓	×	×	NO	(Marchioli et al. 2006)
Physician's health (n=14641)	✓	✓	×	NO	(Sesso et al. 2008)

Meta-analysis of 50 randomised controlled trials (n=294478)	vitamin A, vitamin B6, vitamin B12 vitamin C, vitamin D, vitamin E, β carotene, folic acid, and selenium		NO	(Myung et al. 2013)
Meta-analysis of 26 trials (n=324653)	✓	Different vitamins and minerals	NO	(Fortmann et al. 2013)
Meta-analysis of 49 trials (n=287304)	vitamin E, vitamin D, vitamin C vitamin K, β carotene, Zinc, folic acid, pre and probiotics		YES	(Schwingshackl et al. 2017)

1.5.1. Atherosclerosis

Atherosclerosis, which is leading cause of death worldwide, is a progressive inflammatory disease of large and medium-sized arteries that causes CVD and is characterised by the accumulation of fibrous elements, lipid retention and inflammation in arterial walls which leads to the formation of the atherosclerotic plaques (Kobiyama & Ley 2018; Lusis 2000; Murray & Lopez 2013). Atherosclerotic plaques mainly form in the areterila tree, where the blood flow has the maximum disturbance (Libby et al. 2019). Lipid, lipid-engoraed, macrophages from blood monocytes, T lymphocytes, and smooth muscle cell accumulate in the atheroscrolic plaques, in addition extracellular matrix of plaques comprises elastin, collagen, proteoglycans and glycosaminoglycans (Bennett, Sinha & Owens 2016; Libby et al. 2019). One of the main causes of myocardial infarction is the rupture of atherosclerotic plaques that leads to acute thrombosis of coronary arteries (Bentzon et al. 2014; Libby 2013). The ruptured atherosclerotic plaque often have large lipid core which covered by thin fibrous cap and called “vulnerable plaques”, in contrast, plaques with thicker fibrous caps and limited lipid accumulation are referred to as “stable plaques” that do not rupture (Bentzon et al. 2014).

Population studies and observational evidence support that the elevation of low-density lipoprotein (LDL) cholesterol level has a crucial role in the atherosclerotic plaque formation (Gidding & Allen 2019; Linton et al. 2000; Lusis 2000) and it has been demonstrated that atherosclerosis is always associated with an autoimmune response (Kobiyama & Ley 2018; Lundberg & Hansson 2010).

The current approaches for the treatment of atherosclerosis in patients are targeting the control of the cholesterol level (Ray & Cannon 2005; Tousoulis et al. 2016) and inflammatory cascades (Ridker & Luscher 2014; Soeki & Sata 2016).

Statin therapy is the most commonly prescribed medication to reduce the risk of CVD by lowering LDL cholesterol blood concentrations (Ray & Cannon 2005; Ward et al. 2018; Weber & Noels 2011). However, despite the great efficacy of statin family on lowering LDL level in atherosclerosis patients (Ference et al. 2017), adverse effects such as muscle-related side effects (Ward et al. 2018), new-onset type 2 diabetes mellitus (T2DM), renal toxicity, hepatotoxicity, and adverse neurological and neurocognitive effects has limited their applications (Bitzur et al. 2013). In addition to statin therapy, anti-inflammatory therapy is another approach for the treatment of CVD, considering both innate immunity and adaptive immunity (Hermansson et al. 2010; Nilsson, Bjorkbacka & Fredrikson 2012; Ridker & Luscher 2014).

1.5.1.1. Oxidative stress and inflammatory basis of plaque development

Oxidative stress and inflammation play a crucial role in developing the atherosclerotic plaques (Mury et al. 2018) and studies have demonstrated that inflammation can increase oxidative stress in cells, tissues and the body (Guzik & Touyz 2017; McGarry et al. 2018). Animal studies support the role of oxidative stress in plaque development and progression of atherosclerosis through oxidation of low-density lipoprotein (LDL) (Steinberg 2009).

1.5.1.2. Plaque instability

About 60% of acute coronary syndrome (ACS) cases occur due to plaque ruptures (Davies 1996; Soeki & Sata 2016). Major clinical events seen as a result of atherosclerosis, such as myocardial infarction or stroke, happen due to activation of platelets and thrombus formation secondary to rupture or erosion (Bennett, Sinha & Owens 2016).

Badimon and Vilahur showed that plaque composition determines the degree of plaques vulnerabilities (Badimon & Vilahur 2014). The unstable plaques have specific morphological characteristics such as a thin fibrous cap (Pelisek, Eckstein & Zernecke 2012; Redgrave et al. 2008), a large lipid-rich necrotic core (Mughal et al. 2011) and intraplaque haemorrhage (Michel et al. 2011).

1.5.1.3. ROS in Endothelium

The inner surface of vascular arteries is covered by endothelial cells that form a monolayer. For some time this monolayer was considered as a mere physical barrier between underlying tissues and circulating blood (Sena, Pereira & Seica 2013). However, it has been shown that this monolayer operates as a selectively semi-permeable barrier

between blood and underlying tissues and contributes to the maintenance of the vascular homeostasis under physiological conditions (Bonetti, Lerman & Lerman 2003). The unique position of endothelial cells exposes them to the hormonal effects of vasoactive substances, as well as physical or mechanical stimuli such as pressure and shear stress from the blood, surrounding tissue and cells. In response to these stimuli, endothelial cells release chemicals to regulate vascular tone, inflammation, thromboresistance, cellular adhesion and smooth muscle proliferation and phenotype (Boulanger 2016; Endemann 2004; Lundberg & Hansson 2010). Endothelial dysfunction, which leads to a nitric oxide (NO) deficiency, is a well-established risk factor in the initiation and progression of some diseases including cardiovascular and metabolic diseases (Incalza et al. 2018) pulmonary arterial hypertension (Suresh et al. 2018), and diabetes (Odegaard et al. 2016). An increased accumulation of ROS leads to oxidative stress and reduces the amount of bioactive NO (Endemann 2004; Yuyun, Ng & Ng 2018), which has a crucial impact on CVD health and diseases (Cipollone, Fazia & Mezzetti 2007; Farah, Michel & Balligand 2018).

1.5.1.4. Influence of ROS in smooth muscle cells

VSMCs are located in the medial layer of healthy arteries and have a vital role in arterial contraction and extracellular matrix production (Basatemur et al. 2019). The proliferation pace in normal vessel walls is very low; however, VSMCs proliferation increases during early atherogenesis of vascular lesions as well as in aged VSMCs (Johnson 2014; Moon, Cha & Kim 2003). It has been shown that ROS induces the proliferation of VSMCs *in vitro* (Shimokawa 2013) and any deviation in the proliferation of VSMCs elevates atherosclerotic plaque formation (Bennett, Sinha & Owens 2016). In addition, it has been demonstrated that ROS contributes to the migration of VSCMs out of the media and thus affects formation of neointimal after arterial injury (Jagadeesha et al. 2012; Johnson 2014).

1.5.2. Hypertension

Hypertension is a complex condition, which is recognised as a major risk factor leading to atherosclerosis and contributes to CVD mortality and morbidity (Dinh et al. 2014; Messerli, Williams & Ritz 2007; Weber & Noels 2011). Hypertension is classified as essential or secondary hypertension. Essential hypertension, which consists of almost 90% of cases, has an unknown cause (Messerli, Williams & Ritz 2007). Secondary hypertension is less common and is associated with conditions including primary

aldosteronism, obstructive sleep apnoea, and renovascular disease (Onusko 2003). Hypertension is associated with vascular aging (Lakatta 2007) and increases VSMCs stiffness and VSMC adhesion (Sehgel et al. 2015). Oxidative stress and inflammation, which have a key role in endothelial dysfunction and arterial damage, leading to vascular disease, arterial stiffness, and aging (Guzik & Touyz 2017).

1.5.2.1. ROS in the development of hypertension

Elevation of ROS levels is associated with hypertension and often with impairment of endogenous antioxidant mechanisms (Lassegue & Griendling 2004). The renin-angiotensin-aldosterone system has a vital role in regulating blood pressure and thus the pathophysiology of hypertension (Kang et al. 1994; Ogihara et al. 1993). Ang II presents one of the main vasoactive peptides required to regulate and activate NAD(P)H oxidase, together with cytokines and growth factors (Harrison & Gongora 2009). The activation of NAD(P)H oxidase subunits induces vascular ROS generation (Touyz et al. 2003). In vivo studies shows that the administration of an NAD(P)H oxidase inhibitor in AngII infused hypertensive rats which have high expression level of NAD(P)H, reduces vascular $O_2^{\bullet -}$ production (Rey et al. 2001).

1.5.2.2. Endothelial dysfunction

The endothelium has a vital function in the body's homeostasis (Sena, Pereira & Seica 2013). Endothelial dysfunction, which may happen due to inflammation, causes an aberration in NO production (Chrissobolis et al. 2011; Dinh et al. 2014). It has also been demonstrated that IL-1β, which is a proinflammatory cytokine, causes endothelial dysfunction that is dependent on the exposure time to the cytokine (Vallejo et al. 2014; Wimalasundera et al. 2003).

NO has a variety of key roles dependent on the site of generation such as vasodilation, as mediated by eNOS (Baylis, Mitruka & Deng 1992) and facilitating sodium elimination when produced by kidney epithelial cells (Stoos, Garcia & Garvin 1995). It has been demonstrated that endothelial dysfunction is linked to several pathophysiological conditions such as atherosclerosis and diabetes (Sena, Pereira & Seica 2013). Also, it has been shown that endothelial dysfunction could increase systemic vascular resistance, which leads to the development of hypertension (Chrissobolis & Faraci 2008).

1.5.3. Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterised by chronic hyperglycaemia and the development of diabetic micro and macrovascular complications (Cole & Florez 2020). Diabetes mellitus is one of the fastest-growing disorders and is expected to exceed 693 million cases by 2045, which imposes a huge healthcare and economic burden in Australia and worldwide (Cho et al. 2018; Lee et al. 2018). The linearity of the blood glucose concentration with cardiovascular risk and heart failure has been fully studied (Gilbert & Krum 2015; Gu et al. 2003; Schmidt 2019). The increasing evidence showing the connection between diabetes and heart failure led to the term "diabetic cardiomyopathy", which encompasses the functional and structural abnormalities in the cardiovascular system due to diabetes (De Blasio et al. 2020). Diabetic cardiomyopathy increases oxidative stress, inflammation, hypertrophy, interstitial fibrosis and apoptosis (Marwick et al. 2018).

1.5.3.1. ROS in diabetic vascular disease

Hyperglycaemia and insulin resistance are major factors in progression of atherosclerosis and its complications. It has been shown that metabolic abnormalities cause overproduction of ROS that leads to endothelial dysfunction (Creager et al. 2003; Luscher et al. 2003). Two types of diabetes have been identified, type 1 diabetes mellitus (T1DM),which is characterised by an absolute insulin deficiency caused by T-cell-mediated autoimmune destruction of pancreatic β cells and T2DM, which is characterised by relative insulin deficiency caused by pancreatic β-cell dysfunction and insulin resistance (American Diabetes 2010). T2DM explicates 90% to 95% of all DM cases (American Diabetes 2014). Both T1DM and T2DM are heterogenous diseases in which clinical presentation and disease progression may vary substantially. It has been shown that the risk of heart failure in patients with DM was over twice than patients without DM (Dei Cas et al. 2015; Nichols & Brown 2003).

In the DM patients the immune system destroys pathogens and includes the inflammatory response and production of ROS/RNS. Excessive oxidative stress, which happens due to overproduction of ROS/RNS, seems to have a crucial role in the development and progression of type 2 diabetes mellitus (Gerrits et al. 2014; Tiwari et al. 2013). In the organs and tissues such as liver, skeletal muscle and adipose tissues, inflammation caused by ROS, leads to a failure in cells to react adequately to insulin level

(Keane & Newsholme 2014; Newsholme et al. 2014). This is the basis of insulin resistance and key determinant in the pathogenesis of diabetes.

It has been shown that oxidative stress contributes to diabetic cardiomyopathy (Bugger & Abel 2014; Huynh et al. 2014). Also, different studies showed that the hyperglycaemia plays a crucial role in cardiac dysfunction and remodelling (Huynh et al. 2012; Lebeche, Davidoff & Hajjar 2008).

Endothelial dysfunction has a crucial role in vascular complications (Sabayan et al. 2014). Vascular complications are considered one of the highest causes of mortality and morbidity in the diabetic nephropathy and it has been also shown that endothelial dysfunction and oxidative stress, that happens due to hyperglycaemia, contribute to vascular complications (Domingueti et al. 2016).

1.6. Redox signalling in subcellular regions

The plasma membrane of eukaryotic cells is not a simple sheet of lipids and proteins but is instead a highly organised structure providing a bilayer barrier for the cells that protect them from the outside environment (Edidin 2003; Hommelgaard et al. 2005). Due to its ability to localise, amplify and direct signals, the plasma membrane plays a crucial role in cell signalling (Nordzieke & Medrano-Fernandez 2018).

The plasma membrane consists of several classes of lipid and membrane associated and transmembrane proteins (Yang & Hinner 2015). The lipids include glycerophospholipids, sphingolipids and cholesterol as the three major lipids classes, which are distributed asymmetrically across the bilayer (Engelman 2005). The molar ratio of protein to lipid in the plasma membrane is almost 40 considering the average molecular weight of 550 Da for the lipids and 50 KDa for plasma membrane proteins (Di et al. 2012; Engelman 2005; Goni 2014; Jacobson, Mouritsen & Anderson 2007; Quinn, Griffiths & Warren 1984). This ratio varies between different cell types and has direct relation to the metabolic activity of the membrane (Yang & Hinner 2015). Membrane proteins can form specific and nonspecific interactions with lipids in their proximity and influence the plasma membrane organisation (Lee 2003).

1.7. Caveolae as a centre of cell signalling

Caveolae are omega or flask-shaped, protein-coated, invaginations of the plasma membrane that are found in many tissues and organs and are one of the most abundant features of the plasma membrane (Palade 1953; Parton 2018; Yamada 1955). These

structures are 50-100 nanometres (nm) in diameter (Rothberg et al. 1992) and enriched in sphingolipids (Schnitzer et al. 1995), cholesterol (Pike et al. 2002), proteins collectively referred to as either caveolins (Kurzchalia et al. 1992; Parton, Tillu & Collins 2018; Way & Parton 1995) or cavins (Parton, Tillu & Collins 2018).

While caveolae are not detectable in many tissues such as the liver, neurons and the proximal kidney tubules (Parton, Tillu & Collins 2018; Zhuang et al. 2011), they can be present in almost 50% of the plasma membrane surfaces in adipose tissue and endothelial cells (Thorn et al. 2003). Caveolae are linked to a significant number of signalling pathways (Parton, Tillu & Collins 2018). The high number of caveolae in endothelial cells indicates the possibility of an essential role of caveolae in endothelial function (Tse & Stan 2010).

The presence and importance of caveolae have been studied in the cardiovascular system (Insel et al. 2005; Parton 2018; Williams & Lisanti 2004) and it has been shown that the number of caveolae increases notably when cardiac ischemia occurs (Tsutsumi et al. 2008).

Caveolins are the most abundant proteins of caveolae. Three isoforms of caveolin have been isolated and identified. Caveolin 1 (Cav-1) is the first member of caveolins that was identified (Kurzchalia et al. 1992; Rothberg et al. 1992). The second isoform, caveolin-2 (Cav-2), was identified in 1995 (Scherer et al. 1996), followed by the third isoform, caveolin-3 (Tang et al. 1996; Way & Parton 1995). Interestingly all three isoforms contain a family signature of 8 amino acids (Phe-Glu-Asp-Val-Ile-Ala-Glu-Pro), which are localised in the N-terminal cytosolic oligomerisation domain (Tang et al. 1996). When caveolin isomers were identified, it was first thought that Cav-1 and Cav-3 were enough for caveolae morphogenesis (Fra et al. 1995). However, different studies in the last two decades showed that the caveolin-associated cavin proteins are also critical for normal caveolae formation. These include cavin 1 (also known as Polymerase I and transcript release factor (PTRF)), which is required for caveolae formation along with caveolin 1 (Hill et al. 2008; Liu et al. 2008); cavin 2 (called as Serum deprivation-response protein (SDPR), which is co-localised with caveolin -1 and induces membrane tubulation (Hansen et al. 2009); cavin 3 (SRBC), that regulates caveolae function (McMahon et al. 2009); and cavin 4 (Muscle-restricted coiled-coil (MURC)), which is associated with caveolae and also regulates caveolae formation (Bastiani et al. 2009).

Studies showed that a wide variety of signalling receptors, growth factors, enzymes and kinases are co-localised with Cav1. Examples include eNOS (Garcia-Cardena et al. 1997), insulin (Nystrom et al. 1999), transforming growth fact (TGF)-β (Strippoli et al. 2015) and P2X7 receptors (Gangadharan et al. 2015), epidermal growth factor (EGF) (Couet, Sargiacomo & Lisanti 1997), Src tyrosine kinase (Li, Couet & Lisanti 1996), haem oxygenase (Taira et al. 2011) and H-Ras and K-Ras (Song, Li, et al. 1996). Different studies support the idea that dysfunction of caveolae is linked to human diseases including cardiac disease, lipodystrophy, muscular dystrophies, infection, cancer, and osteoporosis (Hayashi et al. 2009; Rajab et al. 2010). Cav-1 deficient mice show alterations in continuous endothelial permeability (Schubert et al. 2001). They tend to have pulmonary hypertension and dilated cardiomyopathy (Cruz et al. 2012), are intolerant to highly physical activity (Drab et al. 2001) and show impaired tumour angiogenesis (Chang et al. 2009). Cav-1 also plays a critical role in cholesterol transport (Murata et al. 1995).

Cav-2, in many respects, is closely co-regulated with Cav-1 (Scherer et al. 1996). However, Cav-2 deficient mice do not show caveolae disruption but demonstrate pulmonary dysfunction (Razani et al. 2002). Cav-3 is also involved in the formation of caveolae and is expressed in heart, skeletal and smooth muscle (Couchoux et al. 2011; Gazzerro, Bonetto & Minetti 2011; Tang et al. 1996). Cav-3 deficient mice show significant cardiac myocyte hypertrophy, dilation and a progressive cardiomyopathic phenotype (Park et al. 2002). While it has been proven that caveolae and different types of caveolins are responsible for many cellular processes, it is clear that the caveolae and caveolins physiological roles are immensely different depending on the cell types and organs (Razani, Woodman & Lisanti 2002).

1.8. The role of Caveolae in diseases

After the discovery of the caveolin proteins and their role in structure and function of caveolae, the association between caveolin proteins loss or mutation and variety of disease has been investigated (Garg & Agarwal 2008; Hayashi et al. 2009; Kim et al. 2008; Parton, Tillu & Collins 2018; Rajab et al. 2010). Molecules such as: eNOS, p42/p44 mitogen-activated protein kinase (MAPK), protein kinase A (PKA), protein kinase B (Akt) PKC, Src family kinase F (SFK) and glycogen synthase kinase-3β bind to caveolins' scaffolding domain that enable caveolins to modulate important signalling pathways including eNOS, MAPK, tyrosine kinases and G-protein-coupled receptor pathways (Engelman et al. 1998; Garcia-Cardena et al. 1996; Tian et al. 2020; Wan et al. 2017),

which are associated with inflammation and phenotypic changes in myocardium and vascular wall (Engelman et al. 1998; Garcia-Cardena et al. 1996).

The essential role of caveolae and associate proteins has been investigated, and it has been shown that caveolae and caveolins play crucial roles in variety of cellular process associated with atherosclerosis, such as cholesterol homeostasis, inflammation, oxidative stress vascular smooth muscle cell proliferation and endocytosis (Pavlides et al. 2012; Pavlides, Gutierrez-Pajares, Iturrieta, et al. 2014; Pavlides, Gutierrez-Pajares, Katiyar, et al. 2014).

Diseases such as lipodystrophy and pulmonary arterial hypertension (PAH) are associated with Cav1, and cardiomyopathies and skeletal muscle disorders that are linked to Cav 3 (Hayashi et al. 2009; Rajab et al. 2010; Rodriguez et al. 2011) are examples of those associations.

Also, it has been suggested that changes in the expression of cavins and caveolins have a strong link to cancer. The absence of Cav1 leads to tumorigenesis, which shows the potential role of Cav1 as a tumour suppressor (Cerezo et al. 2009; Lee et al. 1998). Also, the lack of Cav 3 expression in breast and lung tumours (Xu et al. 2001) and reduction of cavin 1 and 2 in breast cancer have been reported (Bai et al. 2011; Jab et al. 2016). However, the tumour-promoting role of Cav 1 without cavin 1 in prostate cancer shows the Cav 1 in cells without caveolae behaves differently (Moon et al. 2014).

1.8.1. eNOS and Caveolae

Thus, it has been shown that caveolins, which are the major caveolae proteins, are linked to numerous signalling pathways, and also it has been demonstrated that caveolins interact with numerous proteins within caveolae (Parton 2018; Parton, Tillu & Collins 2018). The interaction between caveolin and eNOS has been extensively studied, and the results show that eNOS has direct interaction with Cav1 (Garcia-Cardena et al. 1997), which is the main structural coat protein of caveolae (Rothberg et al. 1992). Due to the crucial role and function of eNOS in the cardiovascular system (Heitzer et al. 2001; Schachinger, Britten & Zeiher 2000) eNOS represents an attractive therapeutic target. Therefore, it is important to investigate all proteins and pathways that interact with eNOS to understand their impact on the function of eNOS fully.

1.9. Inflammation

Inflammation is one of the adaptive reactions to tissue injury due to physical, chemical, and biological agents and is the primary immune response to eliminate pathogens and repair damaged tissues (Chaplin 2010). Neutrophils and macrophages, which are endogenous immune cells, secrete ROS such as superoxide and hydrogen peroxide in order to eliminate pathogens as the prelude to the repair of damaged tissues (Crowley 2014). However, as these species are as equally reactive with host components as pathogenic ones, there is the possibility of ROS-mediated damage to host cells and tissues during the inflammatory response, leading to pathological changes such as endothelial dysfunction (Yang & Lian 2020).

It has been well documented that inflammation plays a crucial role in the development of atherosclerosis (Hassan 2018; Libby et al. 2019; Ross 1999; Soeki & Sata 2016). Notably, the elevation of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 as well as the presence of infiltrating macrophages and T cells in plaques are considered characteristics features of atherosclerosis (Danesh et al. 2008; Waehre et al. 2004). Despite the extensive evidence for a key role of inflammation in atherosclerosis, therapeutic targeting of this has only recently achieved success in humans (Ridker 2019). It has been shown that the IL-1 family in general, and particularly the IL-1β isoform, is mainly linked to acute and chronic inflammation (Dinarello 2011). Recent studies have been demonstrated that Canakinumab, which is a monoclonal antibody that neutralizes IL-1β, has a significant impact on reducing the rate of perennial CV events in high-risk patients with prior myocardial infarction and reveals new anti-inflammatory treatment options for atherosclerosis (Hassan 2018; Ridker et al. 2017; Weber & von Hundelshausen 2017).

Interestingly, Lynch et al. showed that in healthy people, the level of IL-1 family proteins is much higher in women's blood than in men in the absence of any stimulant (Lynch, Dinarello & Cannon 1994). The differences in male and female responses indicate that the therapeutic approaches should be optimised for each sex precisely.

In addition, it has been shown that Hydroxychloroquine, a derivative of chloroquine and used for treatment of inflammatory disease, significantly reduced the risk of vascular events in patients with systemic lupus erythematosus (Fasano et al. 2017; Hsu et al. 2017). Methotrexate, which is a drug for management inflammatory disease such as rheumatoid arthritis and psoriatic arthritis, reduces the risk of cardiovascular disease (Misra &

Shenoy 2017; Popkova et al. 2015; Ridker et al. 2019). Besides, it has been suggested that Colchicine, which predominantly acts on neutrophils, monocytes and macrophages by decreasing their chemotaxis and release of various inflammatory cytokines and has been used for treatment of gout, familial Mediterranean fever and Behcet's disease for decades, is a cardioprotective agent (Fujisue et al. 2017; Gasparyan et al. 2015; Martinez, Celermajer & Patel 2018).

1.10. FXYD family:

FXYD family proteins are so named because of a sequence signature in their structure (Proline (F), X is usually tyrosine (Y) but could be glutamate (E), threonine (T) or histidine (H); Tyrosine (Y) and aspartic acid (D)) (Sweadner & Rael 2000). At present, seven members of this family have been identified and studied (Table 1.2).

Table 1.2: The details of 7 FXYD family members

Protein Name	Abbreviation	Tissue	Function	Ref
Phospholemman	PLM-FXYD1	Heart, aorta, stomach, oesophagus, skeletal muscle, and liver	Na, K-ATPase regulator	(Palmer, Scott & Jones 1991)
γ-subunit of the Na, K-ATPase	FXYD-2	Kidney	Na, K-ATPase regulator	(Forbush, Kaplan & Hoffman 1978; Mercer et al. 1993)
Mammary tumour marker 8	MAT-8, or FXYD3	Breast, lung, stomach, colon	Regulation of transepithelial transport	(Morrison et al. 1995)
Corticosteroid hormone-induced factor	CHIF, or FXYD4	Kidney collecting duct and distal colon surface cells	Plays an indirect role as a modulator and is a regulator of Na + /K+-ATPase in the ion transport mechanism	(Attali et al. 1995)
Related to ion channel	RIC, or FXYD5	Cancerous tissues	Tumour progression and metastasis	(Fu & Kamps 1997)
Phosphohippolin	FXYD-6	Cerebellum, muscle tissue, blood cells and transporting tissues such as colon, kidney, liver, and lung	Developmental function in the cerebellum	(Kadowaki et al. 2004)
FXYD-7	FXYD-7	Brain	brain-specific regulator of Na+/K+-ATPase and may play a role in the neuronal excitability	(Beguin et al. 2002)

1.10.1. Phospholemman (PLM-FXYD1):

The Na/K pump or Na/K ATPase is a member of the P-type ion-transport ATPases (P-ATPase) protein family (Skou 1957) that is located in the plasma membrane and has a vital role in cellular homeostasis (Cornelius, Turner & Christensen 2003). Na/K ATPase is an active transport system which regulates the gradient of Na^+ and K^+ across the plasma membrane of all animal cells, for each ATP hydrolysed, the enzyme pumps three Na^+ ions out and two K^+ ions into the cells (Figure 1.7) (Geering 2008; Horisberger 2004; Kopec et al. 2014). The Na/K pump in cardiac muscle cells maintains the transsarcolemmal Na^+ and K^+ gradients involved in different electrophysiological processes such as transport processes that are crucial for cell function, cell volume control or Ca^{2+} extrusion via the sodium-calcium exchanger (Ramirez et al. 2011). It has been shown that impairment of the Na/K pump activity can cause disease conditions including heart failure (Bossuyt et al. 2005), hypertension (Dostanic-Larson et al. 2005) and diabetes (Suhail 2010). Also the function of FXYD1 as a novel treatment of redox-dependent vascular disease has been shown (Bubb et al. 2021; Bubb et al. 2016).

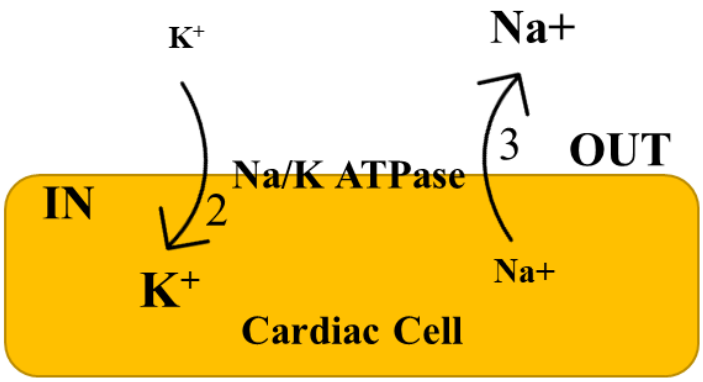


Figure 1.7: Mechanism of Na/K ATPase, the enzyme pumps three Na^+ ions out and two K^+ ions into the cells

FXYD1 is a 72-amino-acid membrane protein, belonging to the FXYD family of ion transport regulators, which was discovered in 1985 as a 15 KDa sarcolemmal protein, that was phosphorylated by isoproterenol (Presti, Jones & Lindemann 1985), α-adernergic agonists (Lindemann 1986) and protein kinases C (Presti, Scott & Jones 1985). FXYD1

can be detected in heart, aorta, stomach, liver, and skeletal muscle and plays an essential role in the regulation of potassium homeostasis especially during exercise (Bogaev et al. 2001; Nielsen & Clausen 2000; Rembold et al. 2005). Notably, Benziane et al. (2011) showed that in human skeletal muscle, the phosphorylation of ser63 and ser68 increased during acute exercise (Benziane et al. 2011). It is established that FXYD1 has a crucial role in regulating Na/K ATPase activity in the heart and skeletal muscles (Pavlovic, Fuller & Shattock 2013). In the heart, FXYD1 regulates not only Na, K-ATPase, but also it regulates the Na^+- Ca^+ exchanger and L-type calcium channels (Cheung et al. 2013b; Pavlovic, Fuller & Shattock 2013; Zhang et al. 2015). It has been proposed that the degree of FXYD1 phosphorylation regulates the activity of Na^+-K^-pumps in the muscle (Ingwersen et al. 2011). Na/K-ATPase α subunit, which is co-localised (Cheung et al. 2010; Silverman et al. 2005) and co-immunoprecipitated (Bossuyt et al. 2006; Crambert et al. 2002; Wang et al. 2010) with FXYD1, interacts with the caveolin binding motif (CBM) (Cai et al. 2008).

It has been shown that FXYD1 is the main sarcolemmal substrate for protein kinases A and C (PKA and PKC) (Presti, Jones & Lindemann 1985; Presti, Scott & Jones 1985), which are the regulators of biological properties of cells by inducing phosphorylation to the serine, threonine, and tyrosine residues of their substrate (Pavlovic, Fuller & Shattock 2013). Specifically, FXYD1 is a primary sarcolemmal substrate for PKA at Ser68 and PKC at residues Ser63, Ser68 and Ser/Thr69 (Fuller et al. 2009; Walaas et al. 1994) (Table 1.2). Table 1.2 shows a total of 14.1% of all amino acids in FXYD1 are Ser and Thr, which is quite a high percentage. It has been well established that the unphosphorylated FXYD1 suppresses the cardiac Na pump, whereas phosphorylation by either PKA or PKC stimulates it (Despa et al. 2005; Fuller et al. 2004; Lifshitz et al. 2006).

Table 1.3: Mouse FXYD1 amino acid compostions (Gasteiger E. 2005)

Amino Acid	No.	Percentage
Ala (A)	6	6.5%
Arg (R)	10	10.9%
Asn (N)	1	1.1%
Asp (D)	3	3.3%
Cys (C)	4	4.3%
Gln (Q)	4	4.3%
Glu (E)	6	6.5%
Gly (G)	7	7.6%
Hid (H)	2	2.2%
Ile (I)	9	9.8%
Leu (L)	11	12%
Lys (K)	2	2.2%
Met (M)	2	2.2%
Phe (F)	4	4.3%
Pro (P)	5	5.4%
Ser (S)	8	8.7%
Thr (T)	5	5.4%
Tyr (Y)	2	2.2%
Val (V)	1	1.1%

In humans, the FXYD1 gene is located on the long arm of chromosome 19 band 13.1 (Chen et al. 1997) and in mice, on the long arm of chromosome 7, B1 (Information 2004).

Figure 1.8 shows the nucleotide sequence alignment of the human, mouse, and rat FXYD1 coding regions; the comparison shows some differences between human and other species, which needs to be considered, being that in the current project, mouse models were utilised for the study of FXYD1.

```
SP|O00168|PLM_HUMAN MASLGHILVFCVGLLTMAKAESPKEHDPFTYDYQSLQIGGLVIAGILFILGILIVLSRRC 60
SP|Q9Z239|PLM_MOUSE MASPGHILALCVCLLSMASAEAPQEPDPFTYDYHTLRIGGLTIAGILFILGILIILSKRC 60
SP|O08589|PLM_RAT   MASPGHILIVCVCLLSMASAEAPQEPDPFTYDYHTLRIGGLTIAGILFILGILIILSKRC 60
                    *** **** ::** **:**.**:*:* *******::*:****.***********:**:**

SP|O00168|PLM_HUMAN RCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR 92
SP|Q9Z239|PLM_MOUSE RCKFNQQQRTGEPDEEEGTFRSSIRRLSSRRR 92
SP|O08589|PLM_RAT   RCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR 92
                    ***************************:***
```

*Figure 1.8: Nucleotide sequence alignment of the human, mouse, and rat PLM coding regions(.= one species sequence different from human sequence, := two species sequence different from human sequence, *=No deviation from human sequence.*

From the results of studies summarised herein, it can be concluded that FXYD1 has an essential role in Na^+/K^+-pump function in the heart, which can be used as a potential therapeutic target for the treatment of cardiovascular disease, which signifies the importance of studying FXYD1 protein and its impact on the cardiovascular disease in details.

1.10.2.FXYD1 in CVD

Several studies have investigated the regulation of FXYD1 in CVD (Park, Pavlovic & Shattock 2018) and its vital role in modulating Na^+/K^+ ATPase, which is colocalized with FXYD1, as regards to the maintenance of cellular homeostasis (Clausen & Poulsen 2013).

It has been shown that the stimulation of β_3 adrenergic receptors (β_3ARs), which are expressed in endothelial cells (Dessy et al. 2004) and associated with Cav1 in caveolae (Sato et al. 2012), reverses oxidative inhibition of the cardiac Na^+/K^+ pump (Bundgaard et al. 2010), and this event was regulated by reduction of glutathionylation in the Na^+/K^+ pump's β_1 subunit (Bundgaard et al. 2010).

It has been also shown that FXYD1 phosphorylation at Ser68 leads to increased Na/K pump activity during β-adrenergic receptor (β-AR) stimulation and protects myocytes from calcium overload, which could trigger arrhythmias (Despa, Tucker & Bers 2008; Pavlovic, Fuller & Shattock 2007). It has also been demonstrated that the FXYD1 Ser68 phosphorylation is reduced in mouse models of heart failure (Boguslavskyi et al. 2014). Reduced FXYD1 phosphorylation has been demonstrated to contribute to heart disease pathology, including arrhythmias, contractile dysfunction, and maladaptive hypertrophy.

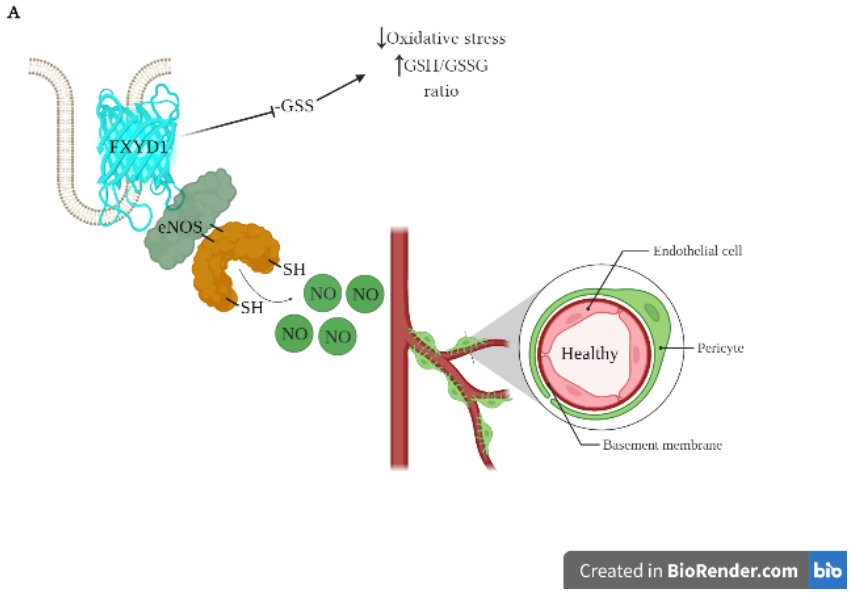
The preliminary evidence (Bubb 2016) from our laboratory suggests a role for FXYD1 in oxidative stress regulation, and this will be investigated further in this thesis.

1.11. Summary:

This work presented initially set out to examine the role and function of FXYD1 protein in the structure and protein components of caveolae in heart tissues and investigate the impact of FXYD1 protein. This investigation was stimulated by the need to understand the complex signalling by ROS to induce CVD and develop novel antioxidant strategies following the disappointing clinical trials (Lehr, Frei & Arfors 1994; Lonn et al. 2002; Yusuf et al. 2000). This thesis has evaluated the impact of FXYD1 protein on redox signalling in pre-clinical CVD models and utilised an unbiased approach to uncover novel targets related to FXYD1 within the subcellular redox signalling hotspot of the caveolae.

In addition to investigating the role and function of FXYD1 on the structure and proteomics of caveolae, the protective function of FXYD1 against atherosclerotic plaque formation has also been examined. This investigation was based on the protective role of FXYD1 against redox-dependent endothelial dysfunction (Bubb et al. 2021; Bubb et al. 2016).

Figure 1.9 summarizes the role and function of FXYD1, a protein which is resident of caveolae plasma membrane, it has been shown in the absence of FXYD1 (B) the disease was developed.



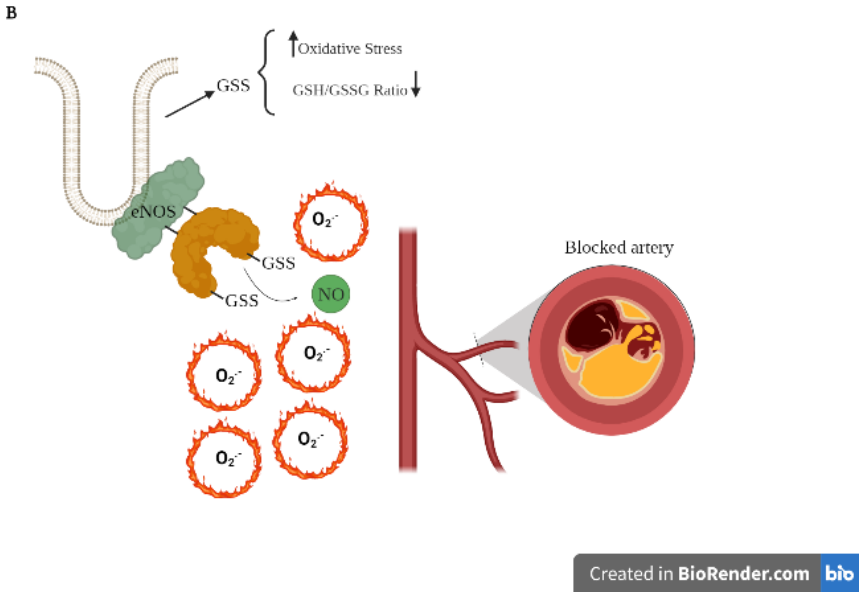


Figure 1.9: In healthy condition FXYD1 blocks the GSS, increases the GSH/GSSG ratio and decreases the oxidative stress (A), in the absence of FXYD1 (Unhealthy condition) the GSH/GSSG ratio decreases and the oxidative stress increases.

1.12. Aims and Hypotheses

The aim of this project was to determine the contribution of FXYD1 to cardiac protein interactions and redox signalling in cardiovascular disease. The specific aims were:

1. Determine the effect of FXYD1 knockout on expression of redox-sensitive protein in type 2 diabetes and hypertension
2. Compare the impact of FXYD1 on whole heart and cavolae proteome and on caveolae morphology
3. Examine the role of FXYD1 in atherosclerosis plaque formation and stability, inflammation and redox signalling in an atherosclerosis mouse model

I hypothesize that FXYD1 protein housed within the caveolae, has a co-ordinating role in the formation of caveolae and is a critical signalling intermediate that can reduce oxidative inhibition of eNOS. This may lead to a better understanding of its protective role against redox-dependent endothelial function and facilitating exogenous antioxidants into the cells. Also, I predict that FXYD1, through its protection of eNOS will inhibit plaque formation and improve plaque stability in atherosclerosis-prone mice, making FXYD1 knockout mice at greater risk of high plaque burden rupture.

CHAPTER TWO: GENERAL METHODS

2.1. Mouse models

2.1.1. FXYD1 Knock out (FXYD1KO) mice

Male FXYD1KO and wild type (WT) mice were bred as littermates from heterozygote parents within the Kearns Animal Facility at the Kolling Institute of Medical Research (University of Sydney). The original breeding pairs were obtained from Prof Kathleen Sweadner (Harvard Medical School, USA) and their generation has been previously described (Jia et al., 2005). The mice were weaned at 6-8 weeks of age and housed individually or in groups of up to 5 under standard laboratory conditions (07:00-19:00 hour (hr) light, 19:00-07:00 hr dark at a constant temperature (21°C) and humidity (40%)). Food and tap water were available *ad libitum.* All animal studies performed were approved by the Northern Sydney Local Health District Animal Ethics Committee (approval numbers: RESP 14/273; 16/263; 17/96; 17/56; 17/88) in accordance with the National Health and Medical Research Council of Australia's *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.1.2. Apolipoprotein E (ApoE) Knock out mice and FXYD1KO out/ApoE Knock out mice

Apolipoprotein E (ApoE) is an element of very-low-density lipoprotein (VLDL) produced by the liver. It acts mainly as a transporter of both triglycerides, from the liver to peripheral tissues. It is also an element of a subclass of high-density lipoproteins (HDLs) required for cholesterol transportation among cells (Mahley 1986, 1988). ApoE is also responsible for the ApoE-containing lipoprotein particles' cellular uptake by mediating high affinity of LDL receptors for the ApoE-containing lipoprotein particles (Hui, Innerarity & Mahley 1981). Knock-out of ApoE in mice has been developed as a powerful experimental model for the study of atherosclerosis (Piedrahita et al. 1992). For this part of the study, ApoE KO mice were used to determine the role and function of the FXYD1 gene in an atherosclerosis mouse model.

For generating FXYD1Knock out/ApoE Knock out mice, male ApoE KO mice were obtained from Australian Bioresources and bred with female FXYD1 KO mice. The F1 generation was selectively interbred to obtain a colony of ApoE KO, FXYD1 heterozygote breeders. ApoE KO / FXYD1 KO and ApoE KO / FXYD1 WT littermates (6 weeks of age) were then obtained from these mice. The bodyweight of mice was recorded, and they were placed onto a diet of standard chow, mixed with a high-fat and

high cholesterol diet (23.5% fat, 0.15% cholesterol;46% energy from lipids, 20% energy from protein Cat#SF04-027, Specialty Feeds, WA, Australia) at the ratio of 50/50, during a three-day acclimatisation period, after which the remainder of standard chow was removed and replaced with the high-fat diet chow.

2.1.3. Streptozotocin (STZ) Induced C57BL/6 wild type and FXYD-1 KO mice

Six to eight weeks old FXYD1 WT and KO mice were obtained and rendered diabetic or control as described previously (Prakoso et al. 2020; Ritchie et al. 2012) with some changes. Briefly, the mice were weighed and administered 55 mg/kg of streptozotocin in 0.1 mol/L citrate buffer, pH 4.5 (CAS#18883-66-4, Sigma Aldrich, Sydney, Australia) injected intraperitoneally, daily for the first three days. Matched control mice were injected with buffer alone. After the last injection, the food was changed to the high fat diet (23.5 % fat, 43% energy from lipids, 21% energy from proteins, Cat#SF04-001, Specialty Feeds, WA, Australia) for thirty weeks.

STZ was shown to induce beta-cell necrosis in the rat (Like & Rossini 1976). Multiple small injections of streptozotocin in mice produce pancreatic insulitis, which almost destroys the majority of the insulin-producing beta -cells, therefore producing a low-insulin diabetic phenotype (Ganda, Rossini & Like 1976; Lenzen 2008). Thus, injecting STZ to the mice is an ideal approach to create diabetic mice model for this part of the project.

2.1.4. Angiotensin II (Ang II) Induced C57BL/6 wild type and FXYD-1 KO mice

Both wild-type and FXYD1$^{-/-}$ mice (males, 3-4 months) received either vehicle (saline, product code: SC3050, BRIEMARPAK, 0.9% v/v sodium chloride for irrigation) or AngII (2.1 mg/kg per day) for 28 days via mini-osmotic pumps (Alzet Model 2002, ALZET® Osmotic Pumps, USA, CA). These were implanted beneath the skin behind the scapula while the animal was under isoflurane (1.5/0.5%isoflurane/air) anaesthesia.

Animals also underwent surgery to implant high fidelity pressure catheters and implantable radio telemeters (Data Sciences International, St Paul, MI, USA: HDX11) The pressure-sensing region (4 mm) of the catheter was situated in the aortic arch via catheterisation of the left or right carotid artery in isoflurane-anesthetised mice. The transmitter body was placed in a subcutaneous pocket, created with blunt scissors, in the right flank. Recordings of central arterial pressure and daily variability were started after the animals had completely recovered from surgery and continued for 28 days.

2.2. Anaesthesia

2.2.1. Anaesthesia for recovery

Anaesthesia was induced by placing the mice in a Perspex anaesthetic chamber filled with isoflurane (isoflurane 1mL/mL, Veterinary Companies of Australia PTY LTD, Australia). The mice were transferred to a bench heat pad, and anaesthesia was maintained via a cone attached to an anaesthetic pump (Cyprane, Advanced Anaesthesia Specialist, Australia) for delivery of isoflurane (2-3% in Oxygen). The absence of pedal reflexes was used to assess the success of anaesthesia before performing the surgery. After the surgery, the mouse was transferred to a cage and constantly checked until the full recovery.

2.2.2. Endpoint Anaesthesia

Anaesthesia was induced by placing the mice in a Perspex anaesthetic chamber filled with isoflurane. The mice were transferred to a bench pad, and anaesthesia was maintained via a cone attached to the aforementioned anaesthetic pump for the delivery of isoflurane (2-3% in Oxygen). As with anaesthesia for recovery, the absence of pedal reflexes assessed the success of anaesthesia.

2.3. Tissue and Organ collection

After inducing endpoint anaesthesia, the abdomen was cut, and the intestines were gently removed. The liver was pushed forward, and the posterior vena cava (between the kidneys) was identified. A 23G needle was then inserted to collect blood from the posterior vena cava into a 1mL heparin (20IU) (heparin Sod., Pfizer, Sydney Australia) coated syringe. The collected blood was transferred to the 1.5mL tubes, which contained heparin (20 IU/ml, a total of 0.4 IU of heparin sodium), and thoroughly mixed. The samples were spun down at 10000 RPM (10621 ×*g*) for 5 minutes at 4°C, followed by transferring to the fresh 1.5 mL tubes and storing at -80°C.

Heart, kidney, liver, and mesentery were collected and transferred in pre-labelled 1.5mL tubes and snap frozen in liquid nitrogen. Afterwards, the tissues were stored at -80 ºC until used for further experiments.

2.4. Detection of Proinflammatory and anti-inflammatory interleukins

Atherosclerosis was initially considered a lipid storage disease of the wall of medium and large-sized arteries. However, over the last three decades, the role of inflammation in atherosclerosis has been acknowledged (Galkina & Ley 2009; Libby et al. 2009; Libby, Ridker & Maseri 2002). To investigate the role and function of the FXYD-1 gene on the immune system, the levels of several cytokines in the plasma were determined.

2.4.1. Proinflammatory Cytokines

2.4.1.1. Blood sample collection:

As explained in section 2.2, anaesthesia was induced, and blood was collected (see section 2.3).

2.4.1.2. Interleukin 1-β (IL-1β):

Sample preparation: Plasma samples from mice were stored at -80°C and were subsequently thawed on ice. Samples were diluted 10 times in assay reagent diluent immediately before conducting the assay.

Assay preparation: To measure the level of circulating interleukin 1β, an ELISA (Enzyme-linked immunoassay) kit (Cat# DY401-05, Lot# P219907) and a DuoSet® Ancillary Reagent Kit 2 (Cat # DY008) were purchased from R&D Systems (Sydney, Australia). The capture antibody (Part # 840134, Lot # VI1318091) was prepared based on the manufacturer's instructions to a final concentration of 4 µg/mL in ELISA plate-coating buffer (Part # 896036) and aliquots of 100 µl were dispensed into 96 well flat-bottom polystyrene microplates. The microplates were stored at room temperature (RT) overnight. The capture antibody solution was aspirated, and wells were washed with wash buffer (Part # 895003 6) three times. Next, the plates were blocked using 300 µl of reagent diluent (Part # 841380) and incubated at RT for an hour. The reagent diluent used for blocking the plates was aspirated, and the wells were washed with wash buffer three times. A serial dilution of IL-1β standard (1000pg/mL to 15.6pg/mL) (Part # 840136-lot#1358945) in reagent diluent was prepared (Figure 2.1). Aliquots of diluted samples (100 µl) and standards were transferred into the plate, which was then incubated at RT for two hours.

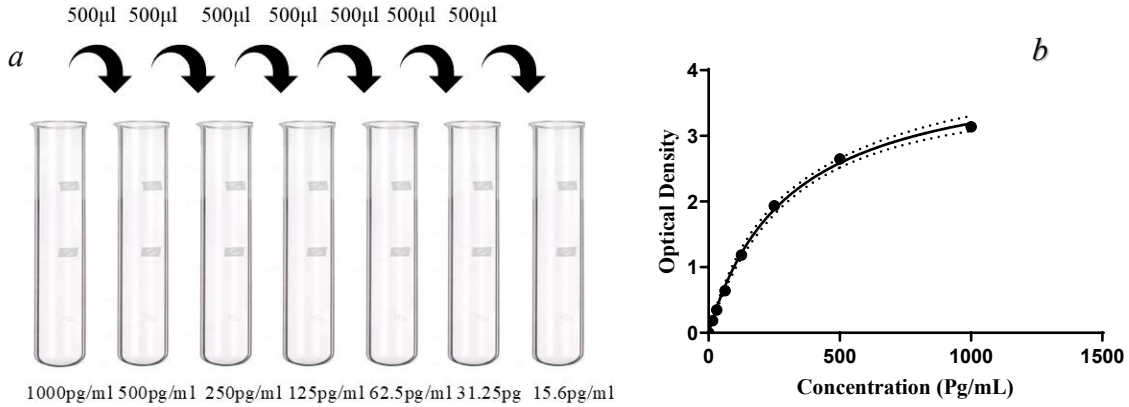


Figure 2.1: a) Serial dilution of IL-1β standards for standard curve, b) IL-1β standard curve obtained

Detection: Samples and standards were aspirated after two hours, and then washed three times with the wash buffer. The detection antibody (Lot# XN4717051) was diluted 60 times in reagent diluent, and 100 µl was transferred to the wells. The plate was covered with an adhesive strip and incubated at RT for 2 hours. After 2 hours, the detection biotinylated antibody was aspirated, and wells were washed three times with wash buffer. Streptavidin- horseradish peroxidase (HRP) (Cat # 893975, Lot # P216085) was diluted 40 times in reagent diluent, and 100 µl was transferred to the wells then incubated at RT in the dark for 20 min. The streptavidin-HRP solution was aspirated, and wells were washed three times with wash buffer. The substrate working solution was prepared by mixing of 5 mL of color reagent A (H_2O_2) with 5 mL of color reagent B (tetramethylbenzidine) (R&D Systems, Catalog # DY999). Then, the substrate solution (100 µL) was transferred to each well, and the plate was incubated for 20 min in the dark**.** After 20 min, stop solution (2N sulfuric acid, 50 µL) (Part # 895926,) was added. The samples' optical density was determined using a plate reader (Synergy 2, BioTek, Australia) at 450 nm with 540 nm as the correction wavelength.

2.4.1.3. IL-6

Samples were collected and prepared as previously explained (See section 2.3 and 2.4).

Assay preparation: To measure the level of circulating interleukin IL-6 in the mice plasma, an ELISA kit (Cat # DY406-05, Lot # P215618) and a DuoSet® Ancillary Reagent Kit 2 (Cat# DY008) were purchased from R&D Systems. The capture antibody (Part # 840171, Lot # AHV2317082) was prepared based on the manufacturer's instruction to a final concentration of 2 µg/mL in ELISA plate-coating buffer (Part # 896036) and 100 µl

was dispensed into 96 well flat-bottom polystyrene microplates. The microplates were stored at RT overnight. The capture antibody solution was aspirated, and wells were washed with wash buffer (Part # 8950036) three times. Next, the plate was blocked using reagent diluent (300 µL, Part # 841380) and incubated at RT for an hour. The reagent diluent used for blocking the plates was aspirated, and wells were washed with wash buffer three times. A serial dilution of IL-6 standard (1000pg/mL to 15.6pg/mL) (Part # 840173-lot #1373637) in reagent diluent was prepared (Figure 2.2), diluted samples and standards (100 µL) were transferred into the plate and the plate was incubated at RT for two hours.

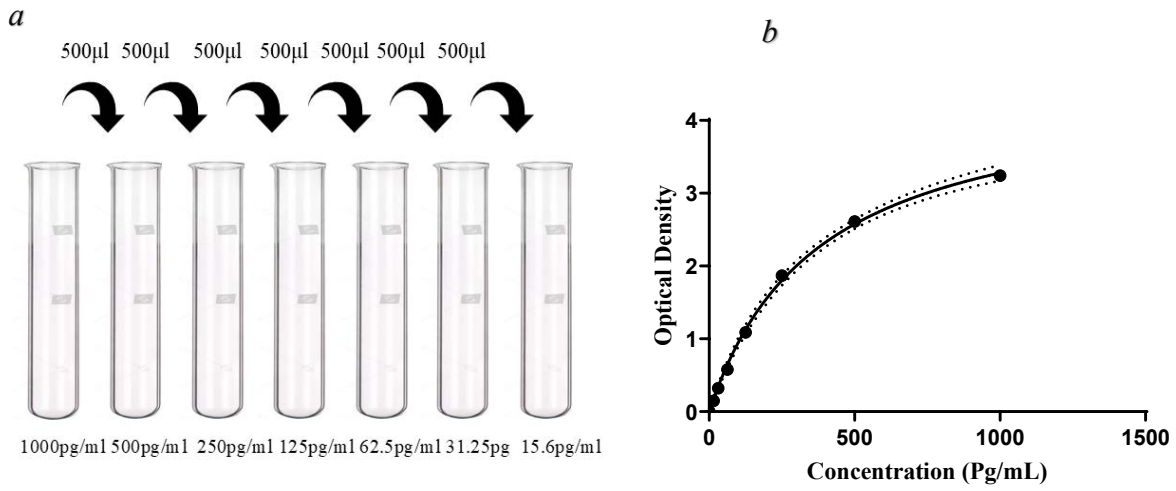


Figure 2.2: a) Serial dilution of IL-6 standards for standard curve, b) IL-6 standard curve obtained

Detection: After 2 hours, samples and standards were aspirated, then wells were washed three times with wash buffer. The detection antibody (Part # 840172, Lot # WC2317091) was diluted 60 times in reagent diluent, and 100 µl was transferred to the wells. The plate was covered with an adhesive strip and incubated at RT for 2 hours. After 2 hours, the detection antibody solution was aspirated, and wells were washed three times with wash buffer. Streptavidin-HRP (Cat # 893975, Lot # P208559) was diluted 40 times in reagent diluent, and aliquots of 100 µl were transferred to the wells of the plate and incubated at RT in the dark for 20 min. The streptavidin-HRP solution was aspirated, and wells were washed three times with wash buffer. The substrate working solution was prepared by mixing 5 mL of color reagent A (H_2O_2) with 5 mL of color reagent B (tetramethylbenzidine) (R&D Systems, Catalog # DY999). Substrate solution (100 µL) then was transferred to each well, and the plate was incubated for 20 min in the dark.

After 20 min, stop solution (50 µL, 2N sulfuric acid) (Part # 895926,) was added. The optical density of samples was determined using the Synergy 2 plate reader at 450 nm with 540 nm as the correction wavelength.

2.4.1.4. TNF-α

To measure the level of circulating TNF-α in the mice plasma samples, an ELISA kit (Cat# DY410-05, Lot#P219694) and a DuoSet® Ancillary Reagent Kit 2 (Cat # DY008) were purchased from R&D Systems. The capture antibody (Part # 840143, Lot # NQ2519061) was prepared based on the manufacturer's instruction to a final concentration of 800 ng/mL in ELISA plate-coating buffer (Part#896036) and 100 µl was dispensed in 96 well flat-bottom polystyrene microplates. The microplate was stored at RT overnight. The capture antibody solution was aspirated, and wells were washed with wash buffer (8950036) three times. Next, the plate was blocked using 300 µl of reagent diluent (Part # 841380) and incubated at RT for an hour. The reagent diluent, which was used for blocking the plates was aspirated, and wells were washed with wash buffer three times. A serial dilution (Figure 2.3) of TNF-α standard (2000 pg/mL to 31.2 pg/mL) (Part # 840145, lot#1354728) in reagent diluent was prepared. Diluted samples (100 µL) and standards were transferred into the plate and the plate was incubated at RT for two hours.

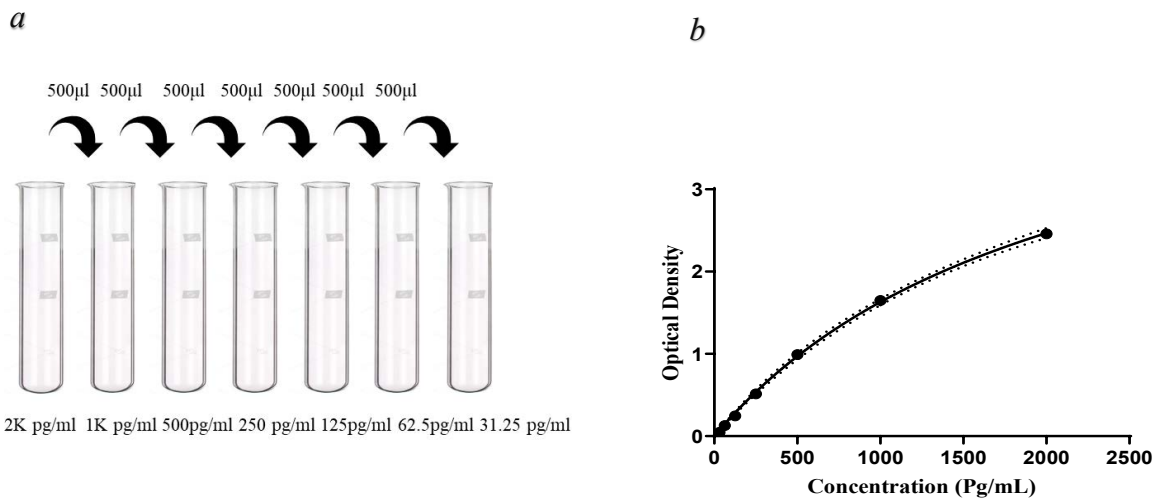


Figure 2.3: a) Serial dilution of TNF-α standards for standard curve, b) TNF-α standard curve obtained

Detection: After two hours, samples and standards were aspirated, and wells were washed three times with the wash buffer. The detection antibody (Part # 840144-Lot# XN4717051) was diluted 60 times in reagent diluent (100 µL) and transferred to the wells. The plates were covered with an adhesive strip and incubated at RT for 2 hours. The detection antibody solution then was aspirated, and wells were washed three times with wash buffer. The streptavidin-HRP (Cat # 893975, Lot # P208559) was diluted 40 times in reagent diluent, and aliquots of 100 µL were transferred to the wells, followed by the incubation of the plate at RT in the dark for 20 min. The streptavidin-HRP solution was aspirated, and wells were washed three times with wash buffer. The substrate working solution was prepared by mixing color reagent A (H_2O_2, 5 mL) color reagent B (tetramethylbenzidine, 5 mL) (R&D Systems, Catalog # DY999). Then, the substrate solution (100 µL) was transferred to each well and incubated for 20 minutes in the dark. After 20 minutes, stop solution (2N sulfuric acid, 50 µL) (Part # 895926,) was added. The optical density of samples was determined using the Synergy 2 plate reader at 450nm with 540 nm as the correction wavelength.

2.4.2. Anti-inflammatory cytokines

2.4.2.1. IL-10

To measure the level of circulating interleukin IL-10 in the mice plasma samples, an ELISA kit (Cat# DY417-05, Lot#P209010) and a DuoSet® Ancillary Reagent Kit 2 (Cat # DY008) were purchased from R&D Systems. The capture antibody (Part # 840125, Lot # AHZ1719042) was prepared based on the manufacturer's instruction to a final concentration of 4 µg/mL in ELISA plate-coating Buffer (Part # 896036) and 100 µl was dispensed into 96 well flat-bottom polystyrene microplates. The microplate was stored at RT overnight. The capture antibody solution was aspirated, and wells were washed with wash buffer (Part # 8950036) three times. Next, the plate was blocked using 300 µl of reagent diluent (Part # 841380) and incubated at RT for an hour. The reagent diluent, which was used for blocking the plates was aspirated, and wells were washed with wash buffer three times. A serial dilution (Figure 2.4) of IL-10 standard (2000 pg/mL to 31.2 pg/mL) (Part # 840127-lot#1471594) in reagent diluent was prepared. Aliquots of diluted samples and standards (100 µL) were transferred into the plate, which was incubated at RT for two hours.

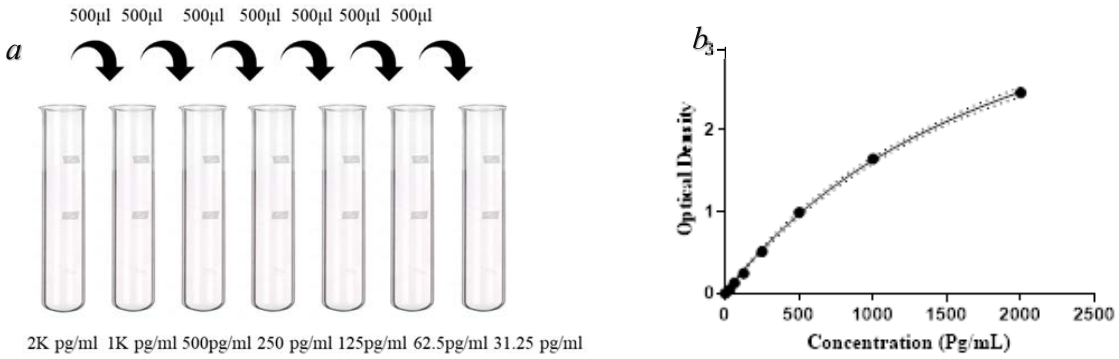


Figure 2.4: a) Serial dilution of IL-10 standards for standard curve, b) IL-10 standard curve obtained

Detection: After two hours, samples and standards were aspirated, then wells were washed three times with wash buffer. The detection antibody (Part # 840126-Lot # WD2719041) was diluted 60 times in reagent diluent, and 100 µl was transferred to the wells. The plate was covered with an adhesive strip and incubated at RT for 2 hours. After 2 hours, the detection antibody solution was aspirated, and wells were washed three times with wash buffer. The streptavidin-HRP (Cat # 893975, Lot # P202818) was diluted 40 times in the reagent diluent, transferred to the wells (100 µL) then incubated at RT in the dark for 20 min. The streptavidin-HRP solution was aspirated, and wells were washed three times with wash buffer. The substrate working solution was prepared by mixing color reagent A (H_2O_2, 5 mL) with color reagent B (Tetramethylbenzidine, 5 mL) (R&D Systems, Catalog # DY999). Then, the substrate solution (100 µL) was transferred to each well, and the plate was incubated for 20 min in the dark. After 20 minutes, stop solution (2N sulfuric acid, 50 µL) (Part # 895926,) was added. The optical density of samples was determined using the Synergy 2 plate reader at 450 nm with 540 nm as the correction wavelength.

2.5. Genotyping

2.5.1. Crude extraction of DNA:

Two millimetres of mouse tail was removed from the tail tip at post-mortem and placed into a 1.5 mL microcentrifuge tube containing 40 µL of lysis reagents ((0.2 M NaOH (Ajax Finechem, Cat #1823) and 1 mM EDTA (Sigma-Aldrich, Cat # E6758-500g)

solutions). The samples were heated to 95°C for 10 min and vortexed for 2 min while still hot to release the genomic DNA. Then 40 µL of neutralisation buffer (Tris-HCl (0.33 M-pH=8.5) (Astral, Cat # BIO3094T-1) and citric acid (0.2 M) (Sigma-Aldrich, Cat # 251275-500G) solutions) was added, and the samples were vortexed for 2 min. Samples were then centrifuged at maximum speed for 2 minutes to pellet the tissue debris. The supernatants were transferred to the fresh tubes, and 1 µL of each sample was used for polymerase chain reaction (PCR), and the remainder was stored at -20°C.

2.5.2. DNA quantitation

A NANODROP 1000 (Thermo SCIENTIFIC, Sydney, Australia) was used to quantify the extracted DNA.

2.5.3. Primer design

The primers for PCR were designed for the mouse using a web-based program available at https://primer3plus.com/. Briefly, the target sequence was entered on the webpage, followed by inputting required parameters, including the position of the point mutation in the sequence, the length of the PCR products, melting temperature (TM), primer size, and the percentage of GC content. The software generated several primers sets in the output window. Table 2.1 shows the details of primers which were used in this study.

Table 2.1: Details of primers that were used for genotyping of the mice

	Sequences	Tm °C	Concentration (µg/ml)	Supplier
Primer 1	CTT AAG TCC TTA GGC CGT CC	61.6	545	Sigma
Primer 2	GAT CAT CGC GAG CCA TGC	68.2	552	Sigma
Primer 3	CCG TAG GTG AAT GGA TCC	60.4	533	Sigma

Primers 1 and 2 were used to determine the FXYD1 KO samples and primers 1 and 3 were used to find out the FXYD1 wild type samples.

2.5.4. Polymerase Chain Reaction (PCR) and Gel Electrophoresis:

The PCR samples comprised 1µL of genomic DNA, 4µL of 5XHotFIRE 01 (Blend Master mix RTL with 12.5mM $MgCl_2$; Lot# B125BG0180), 2µL of primer master mix (4 µM of final concentration) and 13µL of deionised water.

The target DNA was amplified in a thermocycler machine (Veriti 96 well, Applied Biosystems, Australia), with initial denaturation at 95 °C for 8 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and a final elongation step at 72 °C for 5 min. The PCR products were electrophoresed on a 2% w/v SYBR® Safer DNA gel stain (Invitrogen, REF; S33102) agarose (Bioline, Cat # BIO-41025) gel in 1X TAE buffer at 100 volts for 40 min. An ImageQuant LAS 4000 luminescent image analyser gel and blot imager were used to take the images of the gel.

2.6. Metabolic Cage Measurements

At the age of 22-24 weeks, the ApoE KO/FXYD1 KO and ApoE KO/ FXYD1 WT mice were placed in metabolic cages (Image 2.1) for the measurement of parameters including food and water intake and faecal and urinary excretion throughout a 24hr period. Before the collection period, each mouse's bodyweight and the mass of food and water were determined. After the collection period, the mice, the remaining food, and water (including the water in excess water collector), the faeces and the urine were weighed. This experiment was repeated three times. From these measurements, a comparison of the metabolic parameters between the groups was made, and an indication of the impact of FXYD1 gene knock-out on mouse metabolism determined.

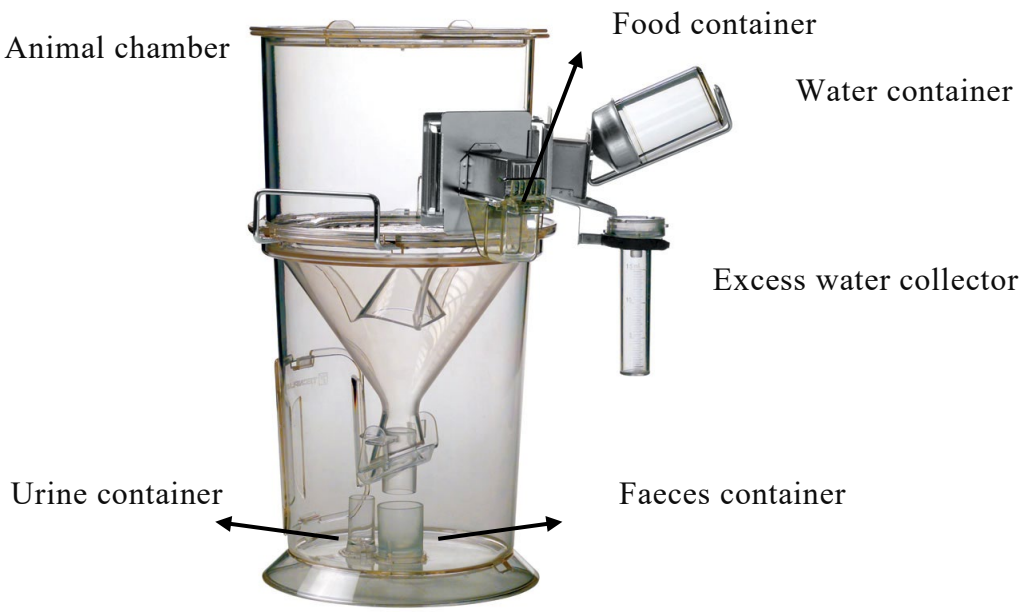


Image 2.1: Metabolic Cage, Image adapted from "TECNPLAST"

2.7. Blood glucose

2.7.1. Endpoint, non-fasted blood glucose

Anaesthesia was induced as explained in section 2.2, the tail was pricked, and the blood glucose was determined using FreeStyle Lite Blood Glucose Monitoring System and recorded (Abbott, Australia).

2.7.2. Regular blood glucose

Every two weeks light anaesthesia was induced, as explained in section 2.3, the tail was pricked, and the blood glucose was determined using FreeStyle Lite Blood Glucose Monitoring System and recorded (Abbott, Australia).

2.8. Total cholesterol, HDL, LDL, and triglyceride

2.8.1. Determination of the total cholesterol concentration:

Anaesthesia was induced (See section 2.2), and blood was collected (See section 2.3). Plasma samples from mice, which were stored at -80°C thawed on ice and total cholesterol, were determined by LabAssay ™ Cholesterol kit (Cholesterol Oxidase. DAOS method) (CAT # 294-65801, Novochem, Australia) following the manufacturer's protocol.

2.8.2. Determination of the HDL and LDL/VLDL concentration

The HDL and LDL/VLDL were separated using 2X LDL/VLDL precipitation buffer (ab105138, Abcam, Australia). Then the concentration of HDL and LDL/VLDL cholesterol were determined as detailed in Section 2.8.1.

2.8.3. Determination of the triglyceride concentration

The triglyceride concentration in mice plasma was determined using LabAssay ™ triglyceride kit following the supplier instructions (GPO.DAOS method) (CAT # 290-637011, Novochem, Australia).

2.9. Aorta collection

After 16 weeks on a high fat/cholesterol diet, anaesthesia was induced as detailed in section 2.2. Subsequently, the aorta and its arch were removed, and then all the attached adipose around aorta were cut and discarded. The aorta was immersed in formalin solution, neutral buffered, 10% v/v (Cat # HT501640, Sigma-Aldrich) and stored at 4°C.

2.10. Tandem Stenosis (TS) Surgery

At 16-22 weeks of age, nine weeks after commencement of high-fat diet (HFD) and high cholesterol diet, ApoE KO/ FXYD1 WT and ApoE KO / FXYD1 KO mice were anaesthetised (See section 2.2). An incision was then made in the neck, and the right common carotid artery was dissected from circumferential connective tissues. A TS with 150 µm (or 450 µm) outer diameters was introduced with the distal point 1 mm from the carotid artery bifurcation and the proximal point 3 mm from the lateral stenosis. Two silk ligatures were placed approximately 3 mm apart and tightened around a 150 µm spacer needle. The 150 µm spacer needle was subsequently removed to create stenosis. The distal was approximately 1 mm from the carotid artery bifurcation and the proximal 0.3 mm from the lateral stenosis, as described previously (Chen et al. 2013). Animals were euthanised at 7 weeks after surgery. The carotid arteries were dissected out and carefully cut into the five sections (Image 2.2) (Chen et al. 2013) and immersed in tissue-Tek OCT compound (Product code: IA018, ProSciTech). The sections and OCT compound were placed on dry ice to freeze and stored at -80°C.

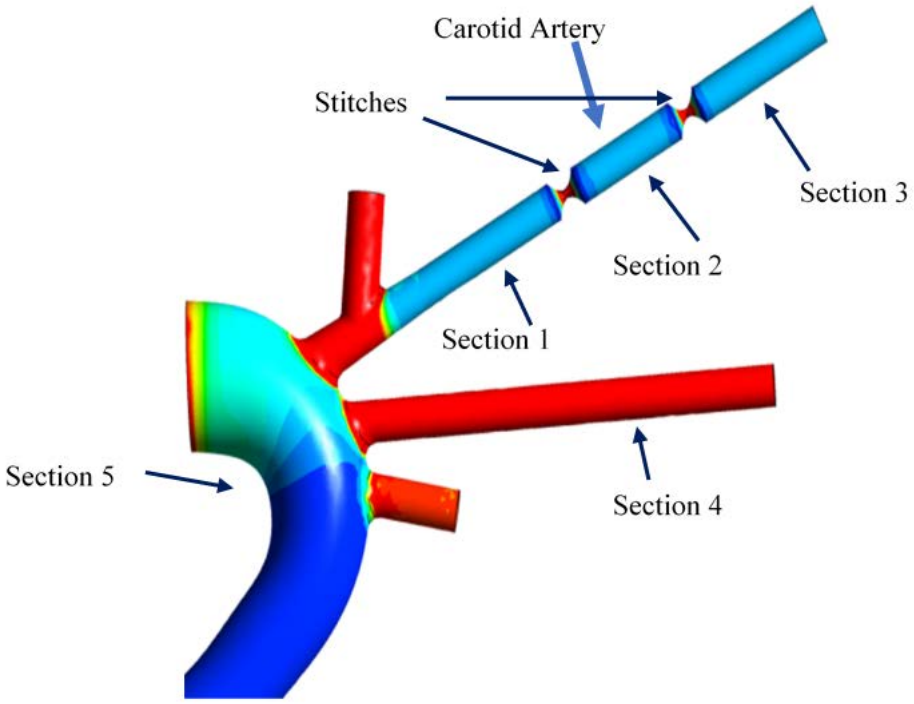


Image 2.2: Carotid Artery Tandem Stenosis (TS) Model (Chen, Y.C. et al., 2013)

2.11. Histochemistry

2.11.1. Sectioning:

The frozen tissues, which were collected (Section 6), were cut, using a Cryostate microtome (Lecia, CM3050 S), into 5-7 µM sections and transferred onto glass slides. The slides were placed in slide holders and immersed in ice-cold acetone (Cat # 179124-2.5L, Sigma Aldrich) for 10 minutes. The slides were then air-dried in the fume hood overnight.

2.11.2. Staining:

2.11.2.1. Hematoxylin and Eosin (H&E) staining

Slides were briefly rinsed in water for several seconds to remove the excess OCT and were then immersed in Mayer's Haematoxylin (Cat # H9627-100G, Sigma-Aldrich) for 5 min. Slides were washed with tap water until the water ran clear. The slides were then immersed in Scott's blueing solution (Cat # S5134, Sigma-Aldrich) for 1 minute, followed by 1 minute wash with tap water. The slides were then immersed in Eosin B solution (Cat

212954, Sigma-Aldrich) for 5 min, then washed with tap water to remove excess Eosin. The sections were then dehydrated and cleared, and slides were mounted.

2.11.2.2. Pico Sirius Red Stain (PSR)

Pico Sirius Red Stain Kit (Cat # ab150681) was purchased from Abcam (Sydney, Australia). The slides were briefly rinsed in water to remove the excess OCT, followed by immersion in PSR solution for 60 minutes. They were then rinsed twice in 0.5% v/v acetic acid (Cat#320099, Sigma-Aldrich), dehydrated, cleared, and mounted.

2.11.2.3. Trichrome Stain

Trichrome Stain kit (Product # ab 150686) was purchased from Abcam (Sydney, Australia). The slides were briefly rinsed in water for several seconds to remove the excess OCT. Then, the slides were placed in Bouin's Fluid overnight. The slides were then rinsed with tap water until the excess Bouin's Fluid was removed, followed by a brief wash with distilled water. Then slides immersed in the Weigert's Iron Hematoxylin working solution (prepared by mixing the equal volumes of Weigert's (A) and Weigert's (B) for 5 min), followed by rinsing with tap water for 2 min. The slides were then immersed in Biebrich Scarlet/Acid Fuchsin for 15 min and rinsed in distilled water. Then a Phosphomolybdic/Phosphotungstic Acid Solution was used to differentiate the tissues for 15 min, followed by 10 min staining with Aniline Blue Solution. Slides then were rinsed in distilled water and immersed in 1% v/v acetic acid solution (Cat # 320099, Sigma-Aldrich) for 5 min. Finally, the samples were dehydrated, cleared, and mounted.

2.12. Caveolae isolation:

2.12.1. Tissue harvesting

Anaesthesia was induced, as explained in section 2.2 and hearts were harvested (See 2.3).

2.12.2. Sample Preparation:

2.12.2.1. NP 40 Lysis buffer preparation:

For preparing NP40 lysis buffer, sodium chloride (Cat # 793566, Sigma-Aldrich, Australia) was mixed with Tris (Cat # BIO3099T, Astral Scientific, Australia) at the final concentration of 150 mM and 50 mM, respectively. Then IGE PAL (Cat # CA-630, Sigma-Aldrich) was added to the final concentration of 1% v/v, and then one tablet of phosphatase inhibitor cocktail (Roche, PhosSTOP EASYpack REF: 04 906 837 001) and

one tablet of protease inhibitor cocktail (Roche, cOmplete ULTRA Tablets, Mini, EASYpack, REF: 05 892 970 001) per 10 mL of NP40 buffer were added.

2.12.2.2. Lysing Tissues:

The frozen hearts were weighed and transferred to a 5mL specimen container and immersed in the ice-cold NP40 lysis buffer in a ratio of 6 to 1. The tissue was mechanically homogenised using a tissueRuptor (Cat No./ID: 9002758, QIAGEN, Australia). The lysed tissue was transferred to 2 mL tubes and spun down at the highest speed for 15 min at 4°C. The supernatant was transferred to a fresh tube and stored at -80° C.

2.12.2.3. Quantification of the total proteins in the lysed samples

The total protein of each sample of lysed tissues was determined using the "Pierce™ BCA Protein Assay Kit"(Cat # 23225, ThermoFisher Scientific, Australia), which is a colourimetric assay. The manufacturer's working instructions were followed to prepare samples and standards. Briefly, samples were diluted 10 times in NP40 buffer; a calibration curve was prepared using the standard provided in the kit. Twenty-five microliters of standards (7.8µg/mL –2000 µg/mL) (Figure 2.5) in duplicate and samples in triplicate were transferred to the 96-well plate. The BCA working solution was prepared by mixing reagent B and reagent A, to the ratio of 1(reagent B) in 50 (reagent A) and then BCA working solution (200 µL) was dispensed into the wells. The plate was briefly shaken and incubated at 37°C for 30 min. The optical density of samples was determined using the Synergy 2 plate reader at 562 nm.

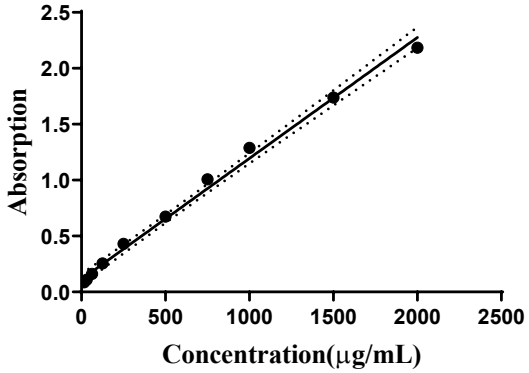


Figure 2.5: The standard curve obtained from the Pierce™ BCA protein assay

2.13. Discontinuous Sucrose gradient ultracentrifugation

Caveolae were isolated by applying the standard discontinuous sucrose gradient ultracentrifugation method (Lisanti et al. 1994). Briefly, 2 mL of prepared heart lysates were adjusted to 45% w/v sucrose by adding an equal volume of 90% w/v sucrose in MBS (25 mM MES and 150 mM NaCl, pH 6.5). The preparation was mixed thoroughly. Then the 4 mL mixture of heart lysate and sucrose was transferred to an ultracentrifuge tube (Cat # 344059, Beckman Coulter, Australia) and overlaid with 4 mL 35% w/v sucrose and then 4 mL 5% sucrose (both in MBS containing 250 mM sodium carbonate) (Song, Scherer, et al. 1996). The tubes were inserted in the tube holders, carefully weighed, sealed, and then swung into the centrifuge rotor (Beckman, SW 41Ti). The samples were subjected to 20 hours of centrifugation (Beckman Optima L-80XP Ultracentrifuge, Swing Bucket; SW41Ti) at 39,000 RPM (187813 RCF) at 4°C. Twelve sub-fractions (each fraction almost 1 mL) were collected carefully to avoid any mixing.

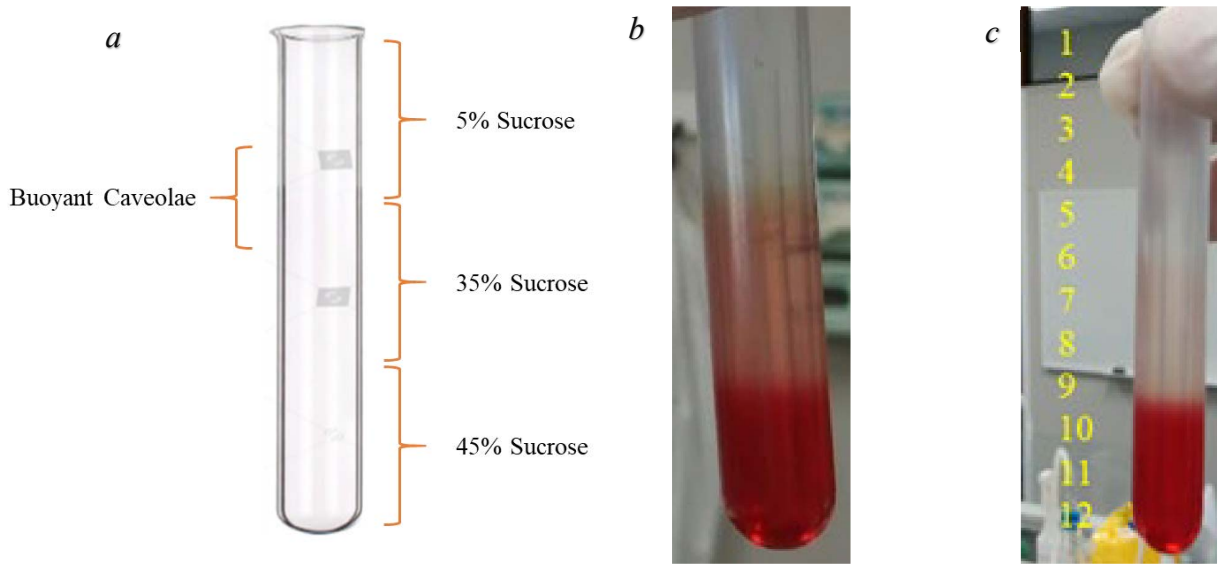


Figure 2.6 :a) Shows schematic caveolae subfractions, b) shows tube containing sample before centrifugation, c) shows fractioned sample after 20 hours centrifugation

2.14. Protein Electrophoresis & Western Blotting

Protein electrophoresis and Western blot were performed using the tissue lysate, the preparation of which was detailed in section 2.12.2. Briefly, after determining the concentration of total protein, this was used to calculate the required amount of lysate that contained 10-30 µg of protein. A master-mix of NuPAGE® LDS sample buffer (4

times diluted, Cat # NP0007, Thermofisher Scientific) and NuPAGE® sample reducing agent (10-fold diluted) (Cat # NP0009, Thermofisher Scientific) were prepared and added to the samples. The mixtures of samples and master-mix were heated at 85°C for 2 min, then kept on ice until loading into the wells of an electrophoresis Bis-Tris; 4-12% gel cassette (Cat # NW04122BOX, Invitrogen, Australia). The gel electrophoresis cassette was inserted into the tank (Mini Gel tank, Invitrogen, Australia) filled with 1 × Bolt™ MES SDS Running Buffer (Cat # B000202, Thermofisher Scientific, Australia). The apparatus was connected to the power supply (BioVolt 250, Select Bioproduct, Australia), and the samples were run for 60 min at 100V. A pre-stained protein ladder (Cat # ab116028, Abcam) was loaded in the gel to determine the molecular weight of proteins of interest in samples. After electrophoretic separation, the proteins from the gel were transferred to the Immobilon-FL Membrane (0.45µm) (Supplier Merck, Cat # 00010) after activating the membrane using 100% methanol. The gel was transferred to the membrane using the sponge pads (Cat # EI9052, Thermofisher Scientific) and filter papers (Cat # 88600, Thermofisher Scientific) (Figure 2.7) in an electrical field at 0.35A (constant) for 75 min.

Figure 2.7: The schematic show how proteins transferred to the membrane

A Ponceau S solution (Cat # 1770, Sigma-Aldrich, Australia) was used to check the transferred protein's quality to the membrane. The membrane was immersed in a five-fold diluted Ponceau S solution for 5 min, which stains the transferred proteins red. The Ponceau S solution was removed from the membrane by washing the membrane three times for 5 minutes with distilled water. The membrane was then blocked using Intercept®

(TBS) Blocking Buffer (Cat # 927-6000, LI-COR, Australia) for an hour on a tilting device at RT. The blocked membrane was incubated with the primary antibody in the cold room, overnight. The membrane was then washed with Tris-Buffered Saline with 0.1% v/v Tween 20 (TBST) (four times, each time for 5 min), then it was incubated with the secondary antibody for an hour on a tilting deceive at RT. The secondary antibody varied, because of the source of tissue and colour of the secondary antibody (Cat # 926-32211, 925-68023, 925-68024, 926-32210). The secondary antibody was diluted in Intercept® (TBS) blocking buffer with 0.2% Tween 20 v/v (Cas # 9005-64-5, Sigma-Aldrich) and 10% v/v sodium dodecyl sulphate (SDS) (Cas # L3771, Sigma-Aldrich). After four washes with TBST and one wash with TBS (Tris-Buffered Saline), the proteins on the membrane were visualised by the Odyssey CLx imaging system (LI-COR, Australia).

The collected images were analysed using Image Studio software (LI-COR Image Studio Software).

2.15. Electron Microscopy

Lipids in the biological membrane are randomly distributed (Di Paolo & De Camilli 2006) and the resolution of the imagining technique using light microscopy, which was originally developed for detecting the distribution of lipids (Stauffer, Ahn & Meyer 1998; Varnai & Balla 1998) was not high enough to detect the correlation of lipid distribution within the subcellular structure (Downes, Gray & Lucocq 2005). Using an electron beam instead of a beam of light, the high resolution of the electron microscope (EM), is a research tool that further expands the scope of structural research into unique areas of cell and molecular biology. Two major electron microscopes, which are mainly used in biological studies, are the transmission electron microscope (TEM) and the scanning electron microscope (SEM).

2.15.1. TEM

TEM consists of an electron gun, a series of apertures, and a set of magnetic lenses (Figure 2.8). In TEM a high-voltage electric, current-heated tungsten filament forms a fine electron beam that is controlled by the magnetic lenses. This fine electron beam is first adjusted by condenser, objective and intermediate apertures and then passed through the specimen, and the electron beam is focused onto a fluorescent screen or captured as an image by a light-sensitive sensor, such as a charge-coupled device camera. Hence, as

the name connotes, TEM forms images by capturing an electron beam transmitted through the samples (Graham & Orenstein 2007).

Despite all advantage of TEM as a research tool, the limitations of TEM need to be considered. The first and most important limitation is that TEM provides black and white two-dimensional images. Also, the limitation of the specimen's is another TEM issue (Graham & Orenstein 2007).

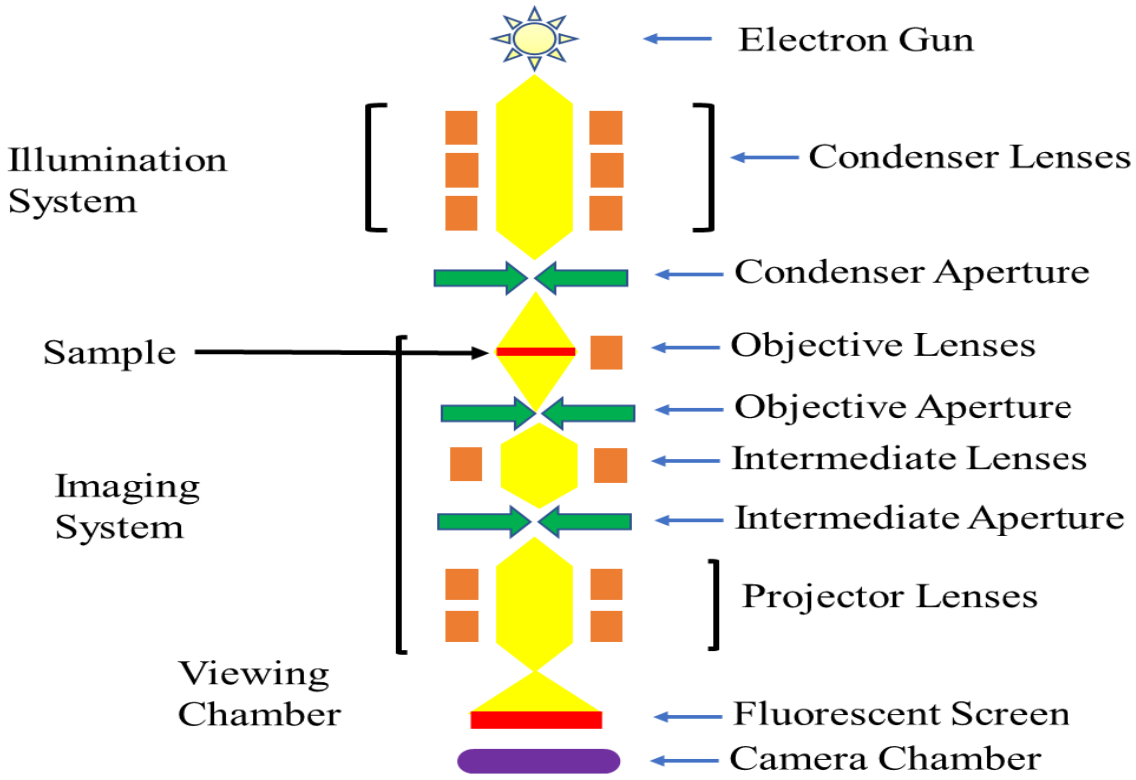


Figure 2.8: The Optics of a basic transmission electron microscope (TEM)

2.15.2. SEM

Like TEM, SEM consists of an electron gun, a series of apertures and a set of magnetic lenses, also in SEM some detectors and scan coils have been used (Figure 2.9).

Unlike TEM, which beams cross the thin sample, SEM is a type of electron microscope that creates images of a sample by scanning the surface using a focused beam of electrons. The electrons, which hit the samples, interact with atoms in the sample and producing different signals that contain information about the sample. The images produced by SEM are particularly appreciated for their high depth of field and excellent image resolution (Kourkoutis, Plitzko & Baumeister 2012; Mignot 2018).

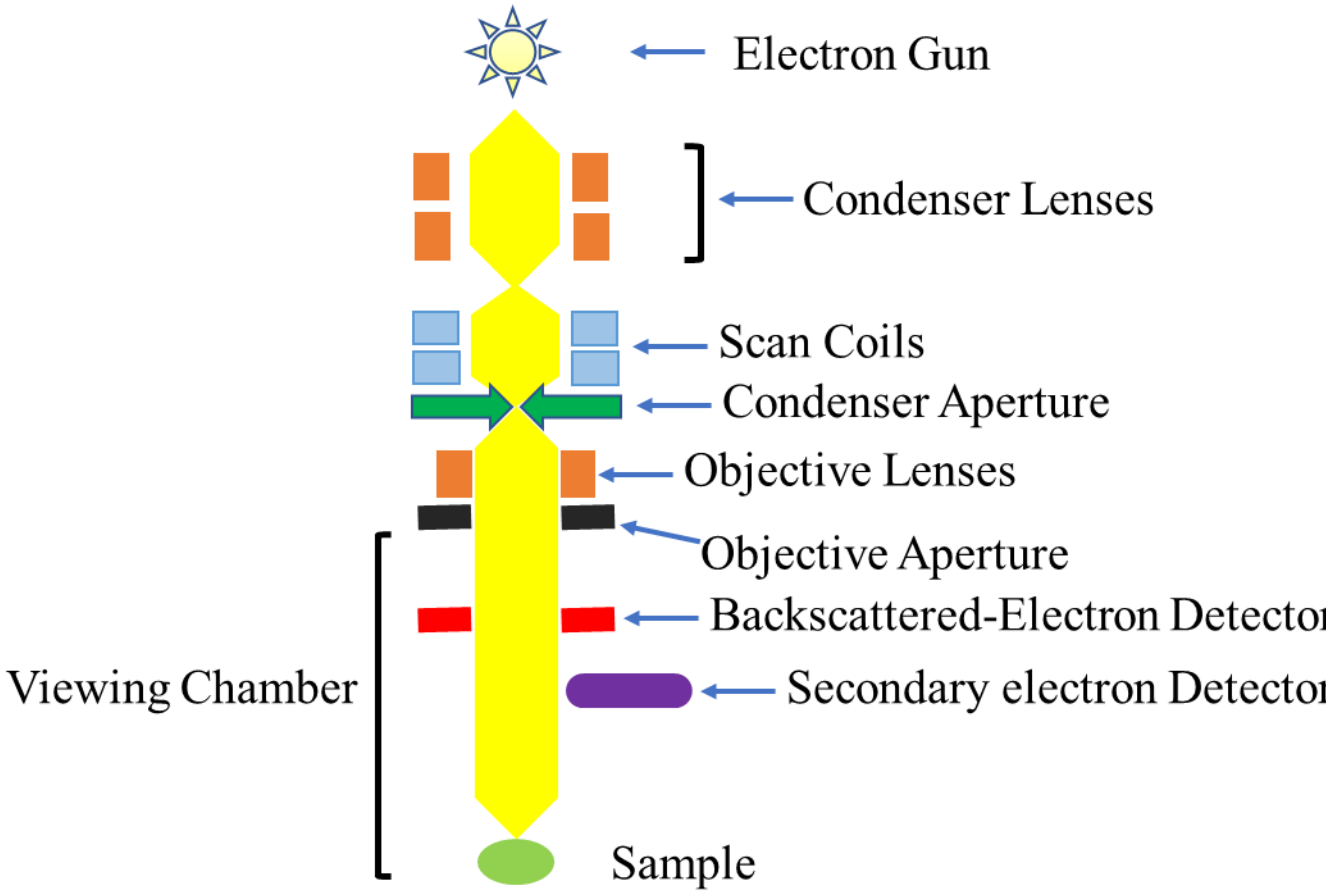


Figure 2.9: The optics of a basic scanning electron microscope (SEM)

The SEM was mainly used to determine the size and number of caveolae in this project.

2.15.3. Biological samples preparation

The mice were euthanised as detailed in section 2.2. Then the hearts of FXYD1WT and FXYD1KO mice were perfused using saline (Product code: SC3050, BRIEMARPAK, 0.9% v/v sodium chloride for irrigation) to remove most of the blood. Then, hearts were removed from mice and the left ventricular dissected, cut in approximately 1-2 mm^2 pieces and immediately immersed in the primary fixative (2.5% v/v glutaraldehyde prepared from stock solution, glutaraldehyde aqueous solution, EM Grade 25% v/v, Cat # C001, ProSciTech, Australia) and 4% v/v paraformaldehyde prepared form stock solution, (paraformaldehyde (formaldehyde) aqueous solution (CAS # C004, ProSciTech, Australia) in 0.1M phosphate buffer (pH=7)). The primary fixative was removed, and samples were washed three times with phosphate buffer (0.1% v/v, pH=7) to obliterate

primary fixative, for five minutes on the rotor. The secondary fixative (1% v/v Osmium in 0.1M phosphate buffer(pH=7) was added to the samples. Samples were incubated for one hour at RT in the secondary fixative while rotating. Subsequently, the secondary fixative was removed after an hour, and the samples were washed with 0.1% v/v phosphate buffer, pH 7. Samples were dehydrated by being taken through serial concentrations of ethanol (30% to absolute ethanol) to remove water from the samples. Figure 2.10 illustrates the process of immersing sample to the EPON resin. EPON resin is a very viscose resin, which was serially diluted with absolute ethanol (Procure-Araldite embedding kit, CAS# C039, ProSciTech).

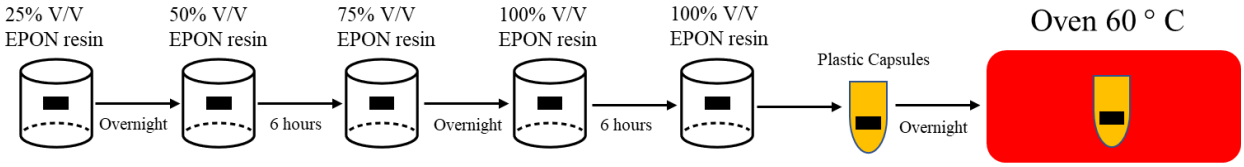


Figure 2.10: The samples were immersed in a serial dilution of EPON resin at RT on the rotor, the resin infiltrates into the sample and hardens it, the EPON resin polymerised at 60°C overnight

2.15.4. Samples Sectioning

The plastic capsules were removed from around the resin blocks. The blocks were trimmed and placed face-up by removing the resin around the tip of the sample and making a rectangle or trapezium shape on the tip. The samples were sectioned at 500 nm (Semi-thin section) using a microtome (Leica, WILD M3Z) and transferred on glass slides. The sections were stained with Toluidine Blue (Cat # 89640-5G, Sigma Aldrich, Australia) and observed with a light microscope to ensure the sample's orientation and position in the block were correct. The samples were then sectioned at 50-70 nm and transferred to a copper mesh grid (emgrid Australia, Cat#FCF200-Cu-SB-25).

2.15.5. Post-staining:

The samples on the mesh grid were stained with heavy metals. The grids were briefly immersed in 2% w/v aqueous uranyl acetate (Cat # C079, ProSciTech, Australia), which provided contrast by interacting with lipid and proteins (Tuijtel et al. 2017) and incubated in the dark for 10 min. Then the grids were rinsed in warm distilled water. The grids were then stained with 3% w/v lead citrate (Cat # C073, ProSciTech, Australia) in the presence of sodium hydroxide pellets (Cat # ST000-500G, Chem-Supply, Australia) to avoid CO_2

contamination, which may lead to lead carbonate precipitation on the sections, for 10 min. Then, samples were rinsed with warm distilled water left to be dry and visualised with the electron microscope (ZEISS Sigma SEM).

2.15.6. Analysis of electron microscopy images

The obtained electron microscopy images were analysed using the "ImageJ 1.53C" (Schneider, Rasband & Eliceiri 2012) software.

2.16. Proteomics

2.16.1. Samples preparation

Two type of samples were used in this part of study. The whole heart from WT and FXYD1 KO mice and the caveolae subfractions that extracted from the WT and FXYD1 KO mouse whole hearts.

The extraction buffer (6 M urea, 2 M thiourea, 2% v/v sodium dodecyl sulfate (SDS), 2 mM diethylenetriamine pentaacetic acid (DTPA), 10 mM N-ethylmalemide (NEM), 1 µM aprotinin, 1 µM pepstatin A, 1 mM sodium orthovanadate, 20 µM leupeptin and 1x Halt phosphatase inhibitor cocktail (Thermo Scientific)) was added to the heart tissues which was collected from mice and (See section 2.2 and 2.3) homogenised (Omni International, Kennesaw GA) on ice. The homogenized tissues were centrifuged at 14,000 × *rcf* for 15 mins at 4°C, and the supernatant was collected. The pellet was resuspended in extraction buffer with acid-washed glass beads (≤ 106 µm), and proteins re-extracted by bead-beating (FastPrep-24, MP Biomedicals, Shandong, China) using two rounds at 40 m/s for 45 sec with 1 min incubation on ice between each round. Samples were centrifuged as above, and the supernatant pooled with that collected previously.

2.16.2. Protein Precipitation (Chloroform/Methanol)

The collected supernatant from the previous step contained proteins that needed to be precipitated. For separating proteins from the extraction buffer, four volumes of ice-cold methanol were added to the supernatant followed by 1 volume of ice-cold chloroform, then three volumes of ice-cold water with vortexing in between each addition. Subsequently, the samples were centrifuged at 14,000 x *rcf* for 2 mins at 4°C and the upper aqueous phase discarded. The pellet was washed with three volumes of ice-cold methanol with vortexing, then proteins collected by centrifugation at 14,000 x *rcf* for 2 mins at 4°C and the supernatant discarded. The previous step was repeated without washing the collected pellet.

2.16.3. Protein reduction and alkylation

The previous step's collected protein pellets were resuspended in 6 M urea, 2 M thiourea and reduced with 25 mM dithiothreitol (DTT) and then incubated at RT in the dark for 1 hr. Subsequently, methylmethanethiosulfonate 60 mM (MMTS; diluted 1:10 in acetonitrile (MeCN)) was added to the solution to alkylate the new free thiols and incubated for 1.5 hr at 35°C.

2.16.4. Trypsin digest of proteins

For digesting the alkylated proteins, the porcine sequencing grade modified trypsin (Promega, Madison, WI USA) was added to the proteins and incubated at RT for 18 hrs, the ratio of trypsin to protein was 1:25. The concentration of the proteins was determined with Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, CA USA) according to the manufacturer's instructions.

2.16.5. Peptide's concentration and desalting (Solid Phase Extraction)

Trifluoroacetic acid (0.1% v/v TFA) was used to acidify the peptides, and hydrophilic-lipophilic balance (HLB) solid-phase extraction (SPE) columns (Waters Corp., Sydney, Australia) were used to desalt the peptides. Briefly, columns were activated with 100% methanol (MeOH) followed by 100% acetonitrile (MeCN). Columns were equilibrated with 0.1% v/v TFA and peptide samples allowed to bind to the column by passing the sample through twice. The column was washed with 0.1% v/v TFA and peptides eluted with 65% MeCN, 0.1% v/v TFA. Peptides were subsequently lyophilised by vacuum evaporation until required.

2.16.6. Isobaric labelling of peptides (total proteome)

From each sample, 15 µg of the peptide was used and labelled with TMT6plex (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Peptides were mixed (1:1) for each sample type. Then the HLB columns were used to remove excess labelled materials. Biological replicates were run individually.

2.16.7. Isobaric labelling of peptides (reversibly redox modified Cys)

The TMT 6-plex application kit (Thermo Fisher Scientific, Waltham, MA) was used for isobaric tandem mass tag (TMT) labelling of the peptides. Fifteen micrograms of digested peptide incubated with TMT reagents were resuspended in an equal volume of MeCN (HPLC grade) for two hours at RT. Samples from across the biological groups were pooled, and MeCN was removed by vacuum centrifugation before incubation with 10 mM tributylphosphine (TBP) for 30 mins at 25°C to remove the MMTS protecting

group. The samples were acidified and diluted with 0.1% v/v TFA, followed by HLB-SPE clean-up as described in 2.16.5, prior to enrichment. Biological replicates were run individually for LC-MS/MS.

2.16.8. Analysis of MS/MS data for protein identification and quantitation

The Proteome Discoverer vers. 2.4 (Thermo Scientific) was used to identify and quantify the proteins, and an in-house MASCOT server was used to search against *Mus muscularis* SwissPROT databases consecutively (Mouse: UP000000589; organism ID 10090, 17,042 protein entries; last updated May 27, 2020). The fixed parameters applied for the searches were: precursor mass tolerance of 20 ppm, product ion mass tolerance of 0.1 Da, and two missed cleavages; and variable parameters were: Cys alkylation (N-ethylmalemide; NEM and carbamidomethylation; IAA), oxidation of Met, TMT6plex labelling of Lys and N-termini, acetylation of protein N-termini. Peptide level false discovery rate (FDR) was decided using Percolator (v. 2.08.01) and all searches were strained for <1% FDR, peptide length between 6-40 residues and rank one peptide identifications. For further analysis, a minimum of two unique peptides was required for confident identification and quantitation of proteins.

The median signal intensity from across all WT was used to normalise the results. Median log2 ratios were calculated at the protein level within each biological replicate. The weighted average log2 ratio was calculated across replicate experiments with the summed reporter ion intensities used to rank the proteins for generation of the zScore. Proteins were deemed to be significantly regulated based on a zScore of ±1.96 and students t-test $p<0.05$. Redox modified peptides were deemed to be significantly regulated based on a zScore of ±1.00 and students t-test $p<0.05$.

2.16.9. Analysis of MS/MS data for redox modified Cys peptide identification and quantitation

As explained in section 2.16.8, Cys peptide identification and quantitation were performed using ProteomeDiscoverer (vers. 2.4, Thermo Scientific) and searched using an in-house MASCOT server against *Mus muscularis* SwissPROT databases sequentially (Mouse: UP000000589; organism ID 10090, 17,042 protein entries; last updated May 27, 2020). Searches were performed using fixed parameters: precursor mass tolerance of 20 ppm, product ion mass tolerance of 0.1 Da, and two missed cleavages; and variable parameters: Cys alkylation (N-ethylmalemide; NEM and carbamidomethylation; IAA), oxidation of Met, TMT6plex labelling of Lys and N-termini, acetylation of protein N-

termini. Peptide FDR was determined using Percolator (v. 2.08.01) and all searches were filtered for <5% FDR, peptide length between 6-40 residues and rank one peptide identifications. Modified Cys peptides were normalised using median signal intensity from non-captured species across all WT samples. Median log2 ratios were calculated at the redox modified site level within each biological replicate. The weighted average log2 ratio was calculated across replicate experiments with the summed reporter ion intensities used to rank the proteins for generation of the zScore. Redox modified peptides were deemed to be significantly regulated based on a zScore of ±1.00 and students t-test $p<0.05$.

2.17. Statistical Analysis

The data are expressed as mean ± SEM. The direct comparisons between two treatments were performed using unpaired t-test, when the variance between two group were significant the nonparametric Mann-Whitney test.

To determine differences between multiple treatments ordinary one-way and two-way ANOVAs were applied to the data. GraphPad Prism 9.12.1 was used for analysis. A $P<0.05$ was considered statistically significant. To show the effect size, p values lower than 0.05 were specified.

CHAPTER THREE

Investigating FXYD1 dependent redox signalling in pre-clinical models of CVD

3.1. Introduction:

The role and function of oxidative stress and ROS on CVD development have been well studied (Kattoor et al. 2017; Tsutsui, Kinugawa & Matsushima 2011) and the results of different studies confirm that ROS was one of the main causes of development and progression of atherosclerosis, hypertension, arrhythmia, cardiomyopathy, congestive heart failure, ischemic heart disease and diabetes (Sugamura & Keaney 2011; Zalba et al. 2001). Figure 3.1 shows the pathways for the production of ROS in mammalian cells and enzymes that are important in hypertension (Harrison & Gongora 2009)

Hypertension is a main and modifiable risk factor for CVD and stroke (Roger et al. 2012). Hypertension is a multifactorial and highly complex disorder. However, despite advance therapeutic management, its contribution to mortality and disability remains one of the major public health challenges (Yoon et al. 2015). One of the most well-established and well-studied mechanisms that leads to the development of hypertension is hyperactivity of the renin-angiotensin system (RAS). Dysregulation of the RAS mechanism has a pivotal role in generating and maintaining hypertension (Brasier & Li 1996). Circulating of angiotensin II (AngII) is the main effector peptide of RAS that causes hypertension in kidney and blood vessels (Biancardi et al. 2017). AngII contributes to the development of hypertension by stimulating sodium reabsorption in the kidney's proximal tubule (Middleton 1996; Triggle 1995). In addition, AngII has potent effects on regulation of NO bioavailability through modified redox signalling. The uncoupling of eNOS is evident in different types of hypertension, including animal models of genetic hypertension, angiotensin II-induced hypertension and deoxycorticosterone acetate salt hypertension (Li & Forstermann 2014).

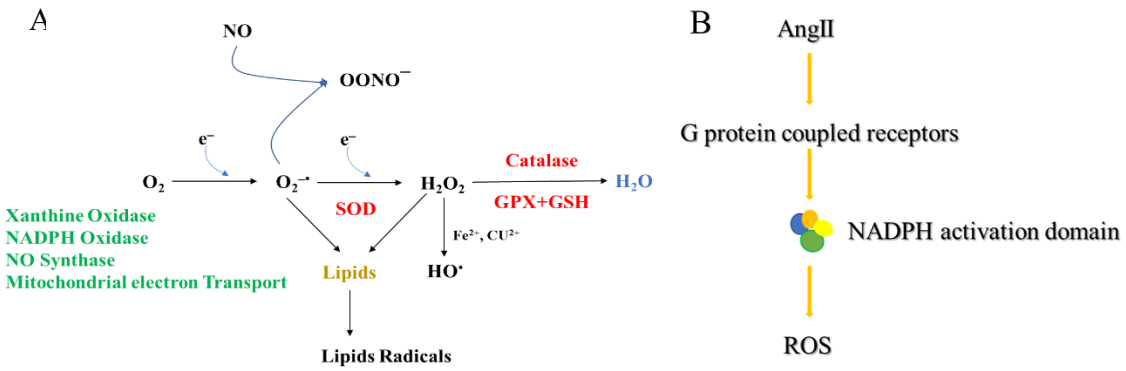


Figure 3.1: Figure A shows the pathways and enzymes that contribute to ROS production in mammalian cells and are important in hypertension (Harrison & Gongora 2009). AngII activates the NADPH oxidase (B)(Sirker et al. 2007).

As a powerful pro-inflammatory mediator, the AngII role in circulatory homeostasis has been well studied (Marchesi, Paradis & Schiffrin 2008). AngII also induces NADH oxidase (NOX enzymes) and generate ROS (Griendling & Ushio-Fukai 2000).

The impact of Ang II on stimulation of Na-K-ATPase activity has been studied and it has been shown that AngII gradually and directly stimulates the Na-K-ATPase pump function (Bharatula, Hussain & Lokhandwala 1998). Also our laboratory previously showed the FXYD1, which has a crucial role in regulating Na-K-ATPase activity (Geering 2006) in the heart and skeletal muscles (Pavlovic, Fuller & Shattock 2013), protects against AngII induced hypertension (Bubb et al. 2016). In addition, the protective role of FXYD1 in vascular health and association of FXYD1 knockout with dysregulation of oxidative and nitrosative stress and increasing of blood pressure following AngII infusion has been demonstrated (Bubb et al. 2021). Based on these results in this part of the project, the impact of FXYD1 protein in the hypertension mouse model was investigated.

Another disease model investigated in this study was T2DM. T2DM is characterised by relative insulin insufficiency caused by pancreatic β-cell dysfunction and insulin resistance in target organs. T2DM was listed as the sixth leading cause of disability in 2015 with the economic burden of US$ 825 billion to global health (Chatterjee, Khunti & Davies 2017). T2DM is a multifactorial disease and factors such as the environment, genetics, lifestyle, increased concentrations of LDL cholesterol, decreased concentrations of HDL cholesterol, hypertension and smoking play critical role in pathogenesis and

progression of this disease (Bergman 2013; Turner et al. 1998). In addition to all the factors, the rate of CVD in adults with diabetes is two to three times greater than adults without diabetes (Henning 2018). Moreover, CVD is the leading cause of premature death in adults with diabetes (Sarwar et al. 2010).

The growing evidence supports a crucial role for oxidative stress in the etiopathogenesis of insulin resistance and diabetes (Styskal et al. 2012). It has been shown that superoxide and other ROS production in the vascular wall plays a crucial role in vascular disease pathogenesis in general and has characteristic features in diabetes (Lassegue, San Martin & Griendling 2012). Inoguchi et al. (2000) showed that the elevation of circulating glucose and fatty acid concentrations might increase NADPH oxidase activity (Inoguchi et al. 2000). It has also been demonstrated that NADPH oxidase's expression level increases in a T2DM rat model (Kim et al. 2002).

Atherosclerosis is another disease model investigated in this part of the study. Atherosclerosis, which is a leading cause of death and disability globally (Murray & Lopez 2013), is characterised by the deposition of lipids and fibrous elements in the large and medium-sized arteries (Galkina & Ley 2009). The accumulation of LDL in the arterial intima, where the LDL could be modified by oxidation or aggregation, has a crucial role in the development of atherosclerosis (Steinberg & Witztum 2010). It has been shown that individuals with extremely low LDL typically do not develop clinically relevant atherosclerosis regardless of the presence of other risk factors (Steinberg, Glass & Witztum 2008). Additionally, ROS's role and function in the development of CVD has been demonstrated (Sugamura & Keaney 2011) and the impact of the lipoprotein oxidation in the maturation of atherosclerosis had been shown (Steinberg & Witztum 2010). It is widely held that the starting step in developing an atherosclerotic lesion is damage to the endothelium (Hadi, Carr & Al Suwaidi 2005). This monolayer of cells is indispensable for regulating vascular tone and the maintenance of vascular homeostasis and function (Tschudi et al. 1996).

Previously it has been demonstrated that FXYD1 features in oxidative modification of the Na^+/ K^+ pump (Bibert et al. 2011) while the recent work where we have identified a role for FXYD1 in eNOS regulation (Bubb et al 2021, submitted). Therefore, I predict that FXYD1 has an important role in the control of oxidative signalling. I hypothesise that an absence of FXYD1 will exacerbate the disease conditions of diabetes,

hypertension, and atherosclerosis due to greater redox activity. I will investigate this predominantly through protein expression analysis.

3.2. Method

3.2.1. Development of mouse models

All animal studies performed were approved by the Northern Sydney Local Health District Animal Ethics Committee (approval numbers RESP/17/96, RESP/17/56, RESP/17/88) in confirmation with the National Health and Medical Research Council of Australia's Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.1.1. AngII Induced hypertension mice model

In this study, both FXYD1 KO and FXYD1 WT mice were used (See chapter 2, section 2.1.4). To investigate the impact of FXYD1 gene on hypertension, at 3-4 months of age the FXYD1 KO and FXYD1 WT mice received either vehicle (saline) or 2.1 mg/kg per day for 28 days of AngII via Alzet mini-osmotic pumps (Alzet Model 2002, ALZET® Osmotic Pumps, USA, CA). These were implanted beneath the skin behind the scapula while the mouse was under isoflurane (1.5/0.5%isoflurane/air) anaesthesia, to mimic the neurohormonal dysregulation.

3.2.1.2. STZ induced diabetes mice model

Both FXYD1 KO and FXYD1 WT mice at age of six to eight weeks old were rendered diabetic beside littermate controls. The mice were weighed and administered 55 mg/kg of streptozotocin in 0.1 mol/L citrate buffer, pH 4.5 (CAS#18883-66-4, Sigma Aldrich, Sydney, Australia) injected intraperitoneally, daily for the first three days. The control mice were injected with buffer alone. After the last injection, the food was changed to the high fat (Cat#SF04-001, Specialty Feeds, WA, Australia) for thirty weeks (See 2.1.3 for more details).

3.2.1.3. Development of an atherosclerosis novel mouse model

To study atherosclerosis, standard laboratory mice are not suitable due to the resistance to plaque development (von Scheidt et al. 2017). We utilised a strain of mice that are susceptible to atherosclerosis, apolipoprotein E knockout mice. In chapter 2 section 2.1.2 the full description and characterisation of the mouse model were explained.

3.2.2. Collecting Tissue

The mice were anaesthetised then the heart, mesentery and aorta were collected. The heart and mesentery snap frozen in liquid nitrogen at the end of study. Subsequently, the collected tissues were stored at -80°C for later experiments. The aortas were immersed in formalin solution, neutral buffered, 10% v/v (Cat # HT501640, Sigma-Aldrich) and stored at 4°C

3.2.3. Redox Enzymes Detection

Heart and mesentery tissue, which were collected and stored at -80º C, were processed and prepared for immunoblotting. The details of sample preparation and immune blotting were explained in chapter 2, section 14.

3.2.4. Detection of plaques using oil red O

To confirm the successful development of atherosclerosis mouse model (ApoE KO), the aorta of C57BL/6 wild type and FXYD1 KO mice were collected and stained with oil red O (See section 5.2.1.8.2 for more details) to detect the plaque formation in C57BL/6 wild type and FXYD-1 KO samples were compared with ApoE KO/FXYD1 KO and ApoE KO/ FXYD1 WT samples.

3.3. Results

3.3.1. Ang II-induced hypertension

3.3.1.1. FXYD1 and blood pressure

To investigate the impact of FXYD1 on blood pressure in this hypertension mice model, the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and heart rate (HR) in WT control, FXYD1 KO control, WT Ang II induced and FXYD1 KO Ang II induced mouse were measured. Figure 3.2, demonstrates and compares the SBP (3.2, A), DBP (3.2, B) and MABP (3.2, C) in FXYD1 KO and WT mice. Figure 3.3 shows the heart rate (HR) in Ang II-induced WT and FXYD1 KO mice.

The results showed that while treatment with Ang II and significantly increase blood pressure and heart rate the absence of FXYD1 KO does not have an impact upon either blood pressure or heart rate.

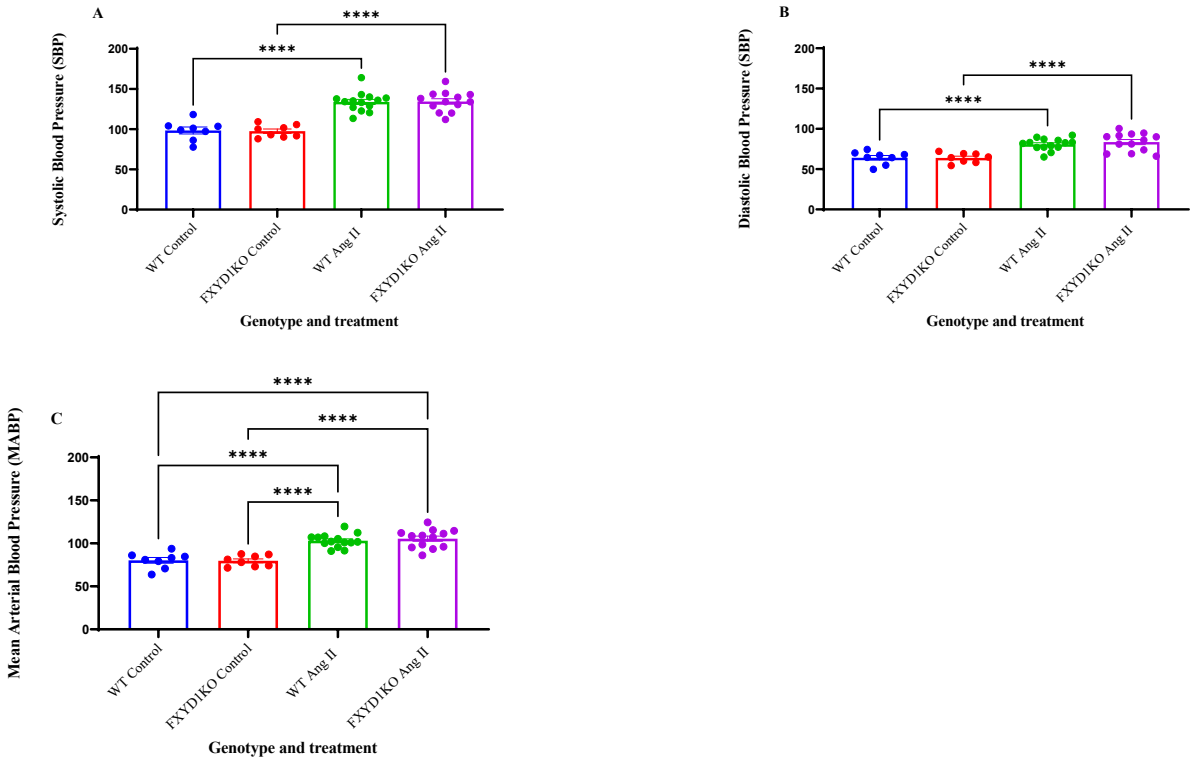


*Figure 3.2: SBP(A), DBP (B), MABP (C) in WT and FXYD1 AngII induced hypertension mice (n=8-14). Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO, (**P<0.001, ****P<0.0001).*

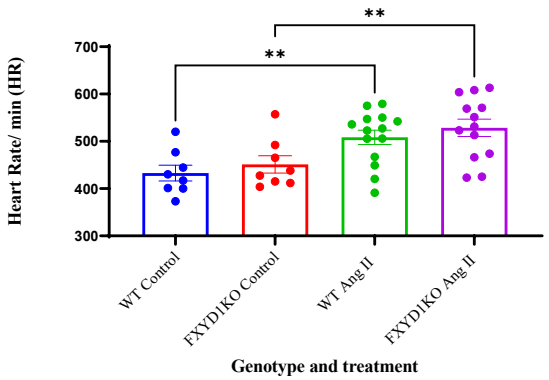


*Figure 3.3: HR in WT and FXYD1 AngII induced hypertension mice (n=8-14). Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO, (*P<0.05).*

3.3.1.2. Protein expression of Redox Enzymes in **Mesentery** tissues

Angiotensin II is an octapeptide that is a powerful vasopressor and stimulus for producing aldosterone from the adrenal cortex (Gigante et al. 1997). Elevated AngII concentrations lead to a number of diseases, including hypertension, atherosclerosis, kidney disease and probably cancer (Deshayes & Nahmias 2005; Sadoshima 2000). Because the mesenteric vascular bed is representative of a large portion of the resistance vasculature in the body where the endothelium function is critical for maintaining vascular tone and it is also an easily accessible source of a large amount of blood vessels. Previous work has shown this vascular bed to undergo redox signalling modification during diabetes and hypertension (Bubb, Ritchie & Figtree 2019) to investigate the impact of FXYD1 on the expression of NOX2, NOX4, PRDX6, GLRX-1 and eNOS western blots were performed on mesentery tissues (See chapter 2, section 2.14).

It has been showen that in the setting of Ang II-induced hypertension the level of NOX2 increases (Murdoch et al. 2011), which is one of the main sources of superoxide in biological system that cause damages to the organs. Our results (Figures 3.4) showed neither treatment with Ang II nor absence of FXYD1 had impact upon the expression level of NOX2 enzymes in our mesentery samples.

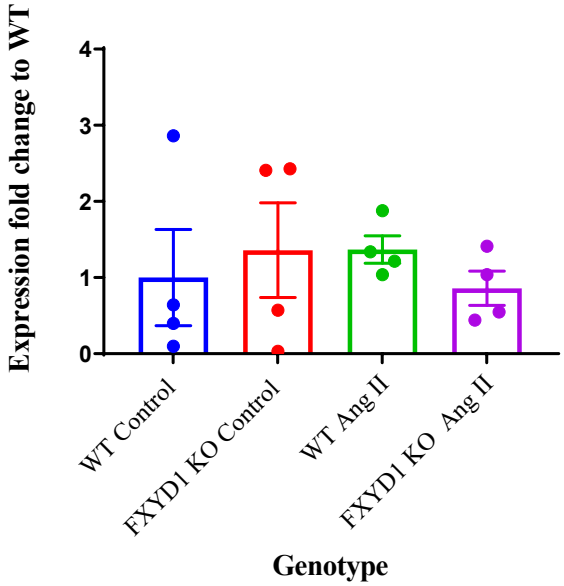


Figure 3.4: Protein expression level of NOX2 in FXYD1 WT and FXYD1 KO male (A, N=4) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO.

NOX4 is a key source of ROS involved in hypertension and it increases in Ang II treated mice (Zhang et al. 2014). Our NOX4 detection results (Figure 3.5) did not show significant differences between the expression level of NOX4 in Ang II treated mesentery and control samples. In addition, the expression level of NOX4 in FXYD1 KO did not increase when compared to WT.

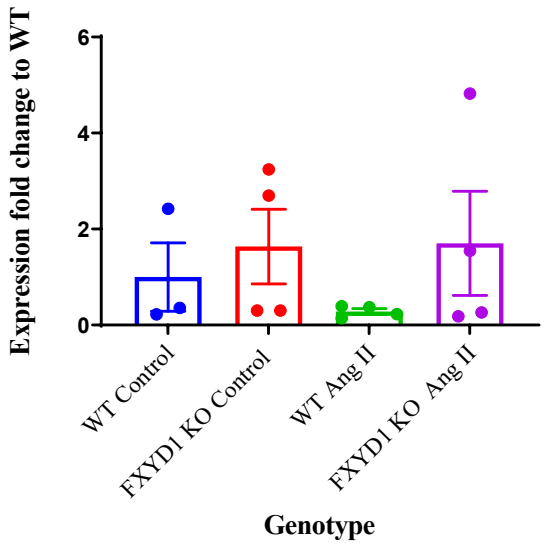


Figure 3.5: Protein expression level of NOX4 in FXYD1 WT and FXYD1 KO male (n=4) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO

Peroxiredoxins (PRDXs) are antioxidant enzymes that mainly act as scavengers of peroxide in biological system (Patel & Chatterjee 2019). PRDX6, a member of PRDX family, is a bifunctional enzyme. While PRDX6 is able to function as an antioxidant by reducing short-chain hydroperoxides through its peroxidase activity, it is able to generate oxidants through its phospholipase (PL)A2 activity (Chatterjee et al. 2011). The results of detection of PRDX6 (Figure 3.6) showed that Ang II caused significant increase in expression level of PRDX6 and, FXYD1 knock out significantly blocked that increase.

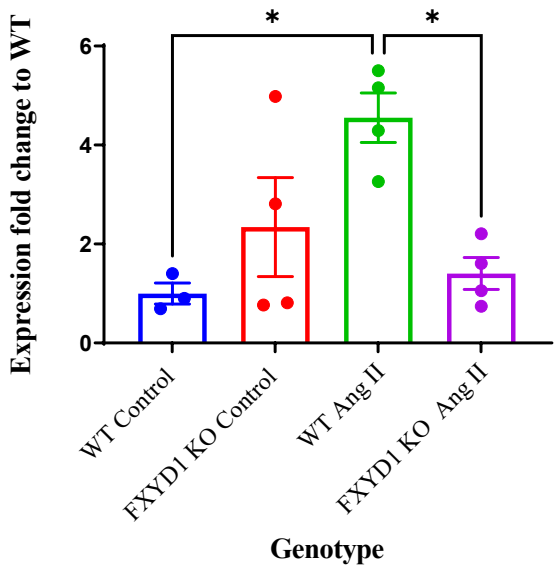


*Figure 3.6: Protein expression level of PRDX6 in FXYD1 WT and FXYD1 KO male (n=3-4) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO, (*P<0.05).*

Glrx1 is a thioltransferase that modulates protein S-glutathiolation (Matsui et al. 2020). In Ang II treated Glrx KO mice activation of NADPH was significantly decreased leading to a reduction of oxidant production (Bachschmid et al. 2010). Our Glrx1 detection results (Figure 3.7) demonstrated that neither Ang II nor FXYD1 had any impact upon Glrx1 expression.

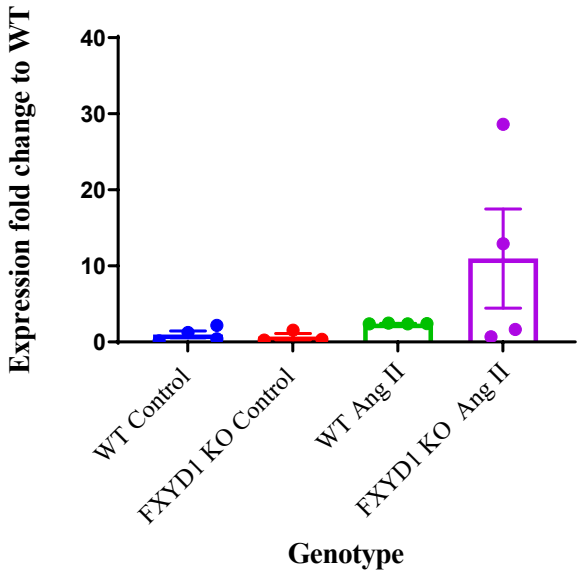


Figure 3.7: Protein expression level of GLRX-1 in FXYD1 WT and FXYD1 KO male (n=3-4) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO

eNOS uncoupling, which leads to endothelial dysfunction (Yang et al. 2009), was observed in Ang II experimental model (Mollnau et al. 2002). Hence, it is crucial to investigate the expression level of eNOS in our hypertension experimental model. The expression of eNOS in our mesentery samples was not detected, which may be due to the very low level of eNOS in our samples or our method's sensitivity.

Figure 3.8 shows the representative images of western blot membranes.

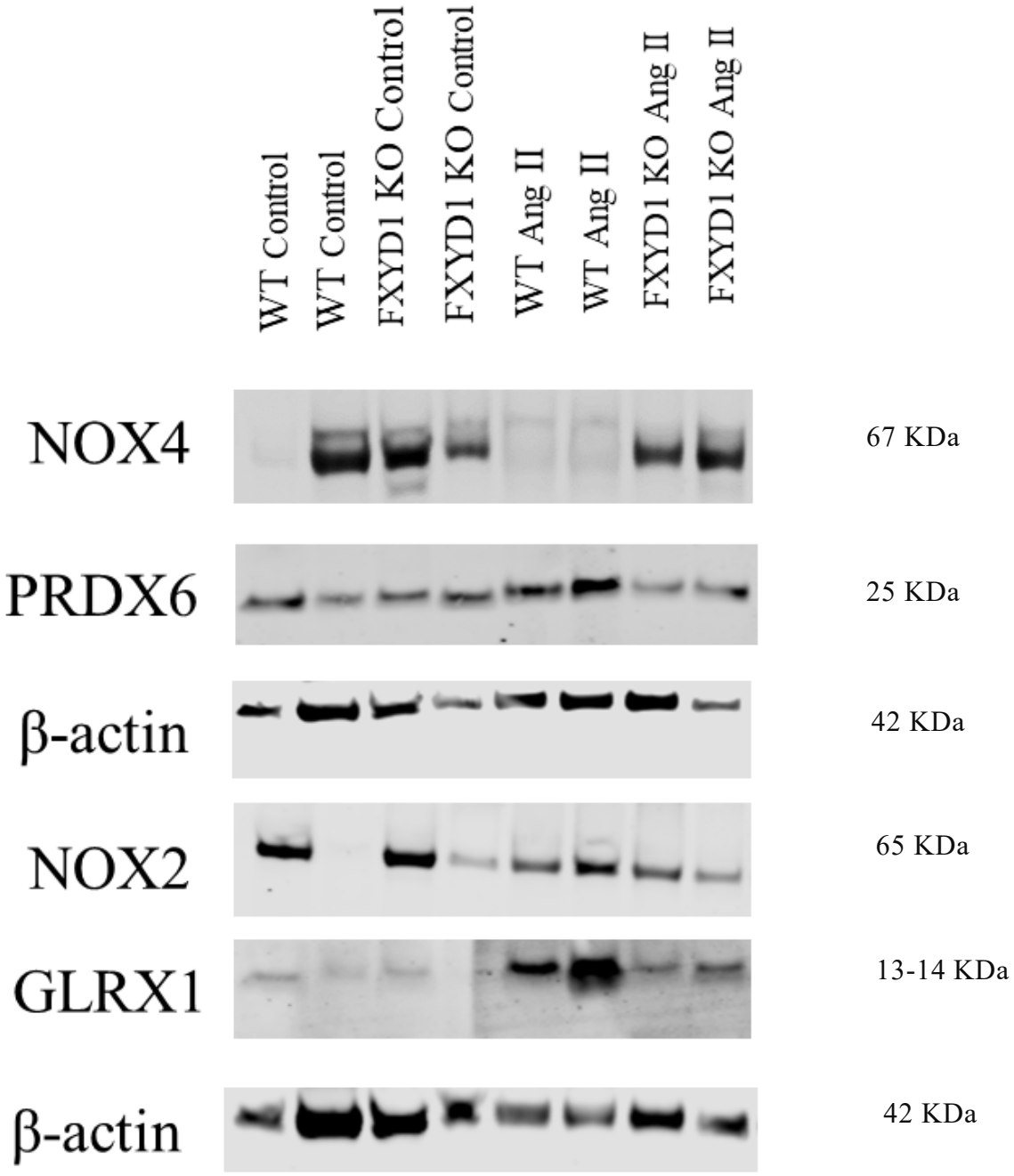


Figure 3.8: Western blot analysis of the expression levels of NOX2, NOX4, PRDX6 and GLRX1 in mesentery of Ang II-induced hypertension WT control, FXYD1 KO control, AngII WT and Ang II FXYD1 KO

3.3.2. STZ induced diabetes

3.3.2.1. Body Mass

To investigate the impact of STZ and FXYD1 knock out upon weight, the mice were weighed biweekly and monitored daily during this study. The results of weight changes showed that the weight of WT induced diabetes mice (pooled samples, male and female) was significantly increased. However, the weight of FXYD1 KO STZ induced diabetes samples did not increase significantly (Figure 3.9 A), which shows that FXYD1kock out does not lead to significant weight gain in diabetic mice. When the data is separated based on the sex, the results showed that induction of diabetes or FXYD1 knock out do not lead to significant weight gain in male mice (Figure 3.9, B). However, the result of body weight changes for female samples showed that in STZ induced WT mice body weight was significantly increased. The comparison between female FXYD1 KO control with female FXYD1 KO, STZ induced diabetes littermates showed no significant weight differences between the groups (Figure 3.9, C).

This body weight experiment in males and females showed that FXYD1 protein does not impact upon weight gain or loss in STZ-induced diabetic mice.

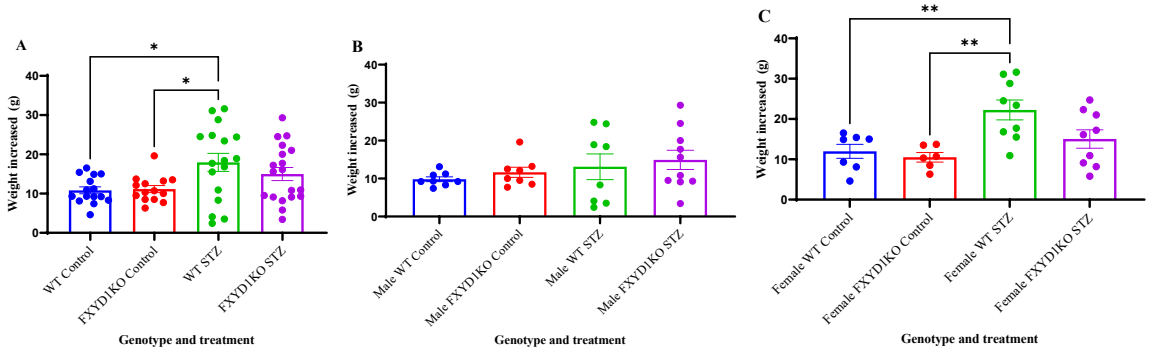


*Figure 3.8: Weight changes in WT and FXYD1 KO male and female (A, n=14-19) male (B, n=8-10), female (C, n=6-9). Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO, (*P<0.05, **P<0.01).*

3.3.2.2. Blood glucose changes in FXYD1 WT and FXYD1 KO STZ induced mice

The results of the endpoint glucose blood test showed that in male STZ-induced WT and mice the level of glucose in circulation blood was significantly increased, which means the hyperglycaemia achieved. However, the results collected by Dr Bubb showed 54% of FXYD1 KO the male responded STZ 55 mg/kg/day and none of the female responded to this treatment (These results collected by Dr Bubb and have not been shown here). However, in the comparison between STZ induced WT and FXYD1KO mice the blood glucose was significantly lower in FXYD1 KO samples (Figure 3.10 (A)). This implies in the absence of FXYD1 the induction of diabetes is lessened. In contrast, in the STZ induced female mice hyperglycaemia was not achieved (Figure 3.10 B), which shows sex differences in the response to STZ that might happen due to hormonal differences between males and females (Kim et al. 2020; Manfredini et al. 2019).

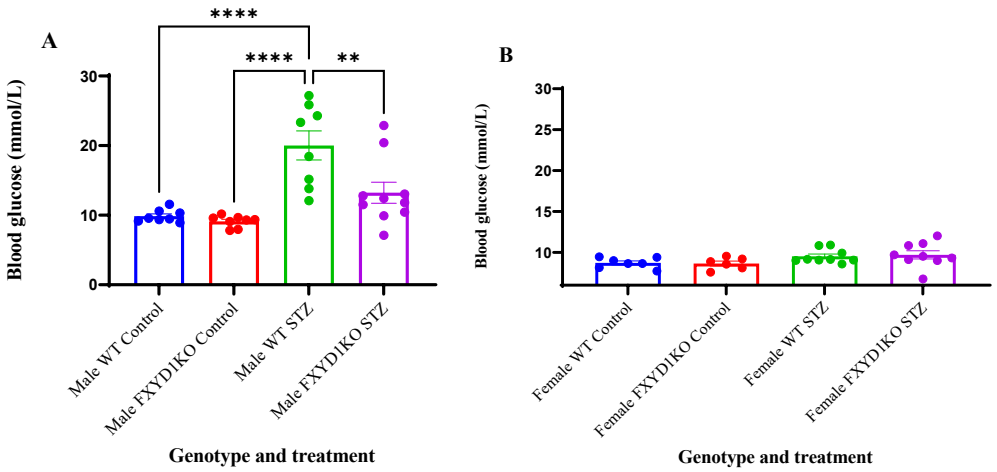


*Figure 3.9: The endpoint blood glucose data are shown as mean ± SEM in all samples (A, n=14-19), male (B, n = 8-10) and female mice (C, n=6-9), statistical analysis was performed by two-way ANOVA, (** P<0.01, ****P<0.0001)*

3.3.2.3. Redox Enzymes

3.3.2.3.1. Protein expression of Redox Enzymes in **Heart** tissues

The expression level of NOX2, NOX4, PRDX6, GLRX-1 and eNOS in heart tissue from FXYD1 knock out and WT STZ induced mice was studied.

Figure 3.11 shows the results of expression level of NOX2 enzyme in heart tissues of WT and FXYD1 KO control samples, as well as STZ induced WT and FXYD1 KO samples. The results showed in the pooled samples (Male and female) that there were no differences in different treatment or genotypes. In addition, in the male samples the results did not show any differences. However, in female samples the expression level of NOX2 in STZ-induced WT and FXYD1 KO mice was significantly decreased when compared with WT control.

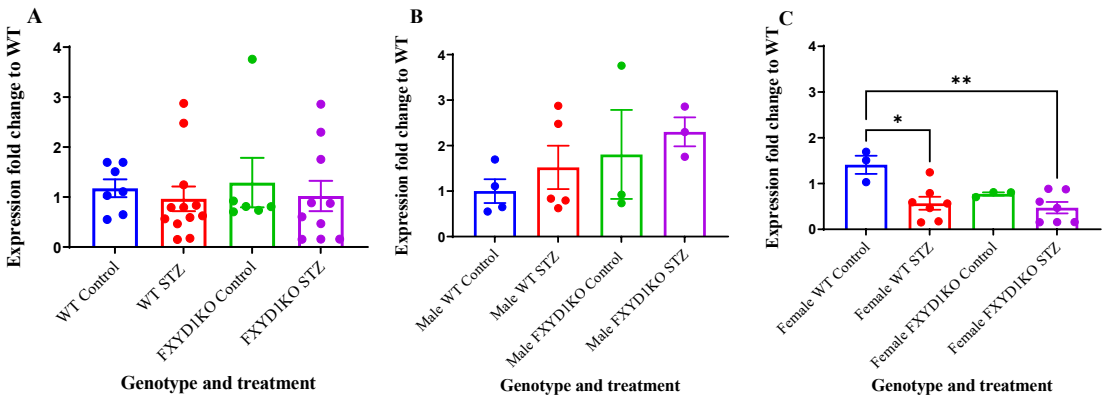


*Figure 3.10: Protein expression level of NOX2 in FXYD1 WT and FXYD1 KO male and female (A, n=6-12, male (B, N=3-5) and female (C, n=3-7) in mouse heart. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO, (*p<0.05** p<0.01).*

Figure 3.12 shows the results of expression level of NOX4 in the pooled samples. The results show while STZ significantly increased the expression level of NOX4 there were no differences between the FXYD1 KO and WT controls (Figure 3.12, A). The results in male samples show that the STZ had no impact upon expression level of NOX4 in WT mice. However, in the absence of FXYD1the expression of NOX4 in male diabetes samples was significantly decreased, which may happen due to ability of FXYD1 to protect against ROS. In addition, the combination of STZ and absence of FXYD1 significantly reduced the expression level of NOX4.

The results of NOX4 in female samples were different from the results of the male samples. The results show that STZ increased the expression level of NOX4 in heart tissues, in the presence or absence of FXYD1.

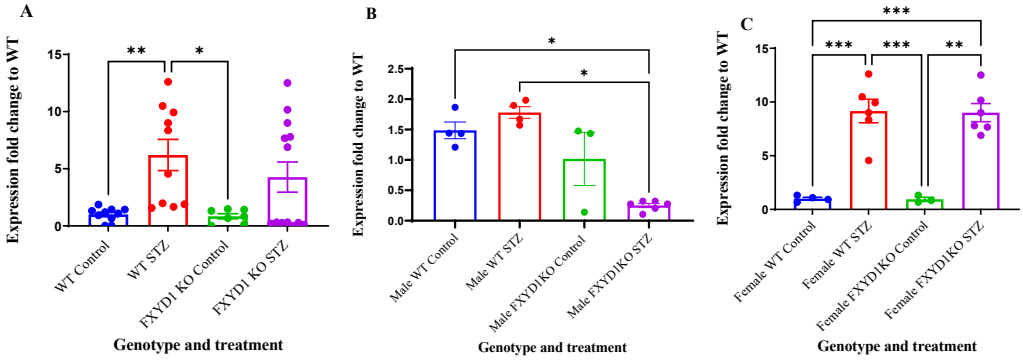


Figure 3.11: Protein expression of NOX4 in FXYD1 WT and FXYD1 KO male and female (A, n=7-13), male (B, N=3-6) and female (C, n=3-6) in mouse heart. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO. (p<0.05, **p<0.01, ***p<0.001).*

Figure 3.13 shows the results of PRDX6 expression level in heart tissues. These results show that the induction of STZ and absence of FXYD1 did not have an impact upon the level of PRDX6 in these samples with the same trend in male and female mice.

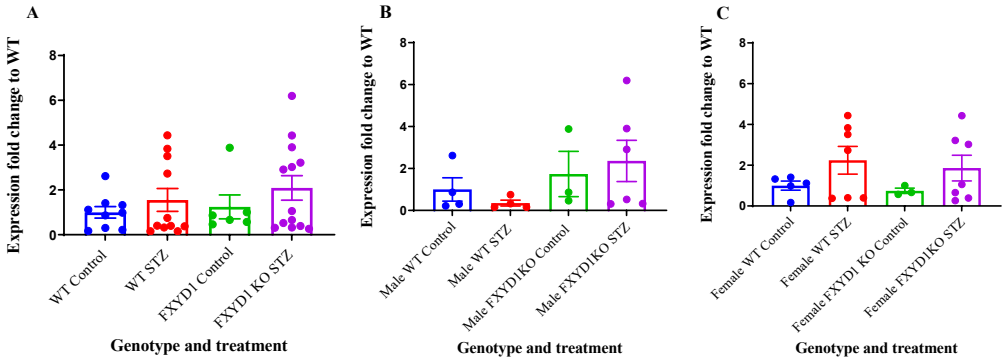


Figure 3.12: Protein expression of PRDX6 in FXYD1 WT and FXYD1 KO male and female (A, n=6-13), male (B, n=3-6) and female (C, n=3-7) in mouse heart. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO.

The protective role of GLRX1 in cardiomyocytes and endothelia cell against oxidative stress has been demonstrated (Burns et al. 2020). Our results showed (Figure 3.14, A) that in pooled samples in the absence of FXYD1, in STZ induced samples the expression level of GLRX-1 was significantly increased. The results of male (Figure 3.14, B) samples did not show any differences in STZ induced or FXYD1KO samples. However, in the female samples (Figure 3.14, C) the expression level of GLRX-1 in STZ induced FXYD1 KO samples was significantly increased.

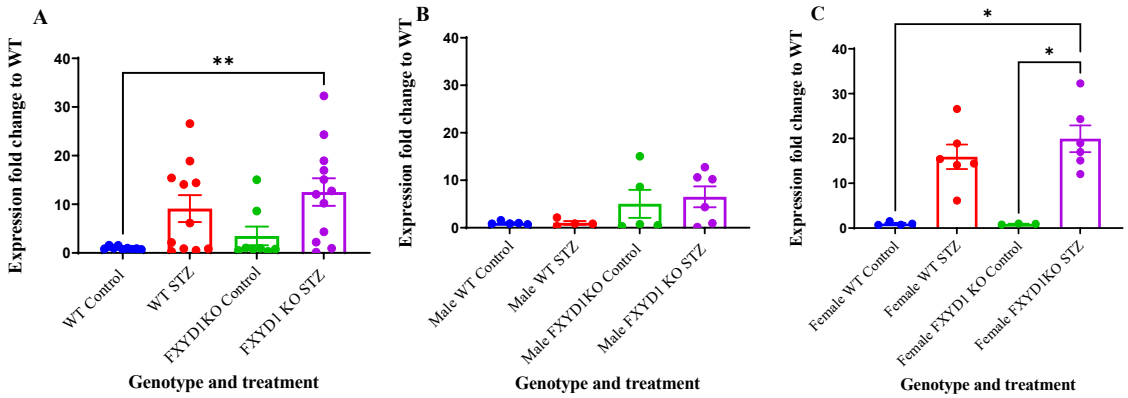


Figure 3.13: Protein expression of GLRX-1 in FXYD1 WT and FXYD1 KO male and female (A, n=8-12), male (B, N=4-6) and female (C, n=3-7) in mouse heart. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between WT and FXYD1 KO.

The results of eNOS expression level in pooled (Figure 3.15) samples showed that in the absence of FXYD1, STZ increased the expression level of eNOS. However, separating results based on the sex did not show differences in our samples.

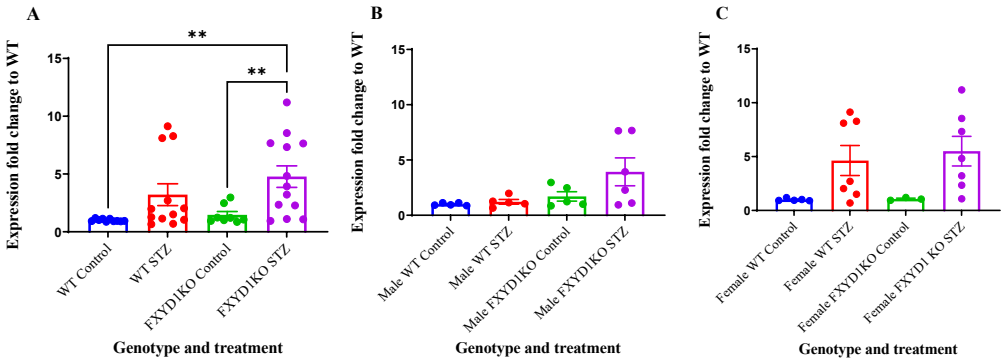


Figure 3.14: Protein expression of eNOS in FXYD1 WT and FXYD1 KO male and female (A, n=8-13), male (B, N=5-6) and female (C, n=3-7) in mouse heart. Data are presented as mean ± SEM. Statistical analysis was performed by ordinary one-way ANOVA test to determine differences between WT and FXYD1 KO.

Figure 3.16 shows the representative gel images for redox enzymes in heart tissues of STZ-indued diabetic samples.

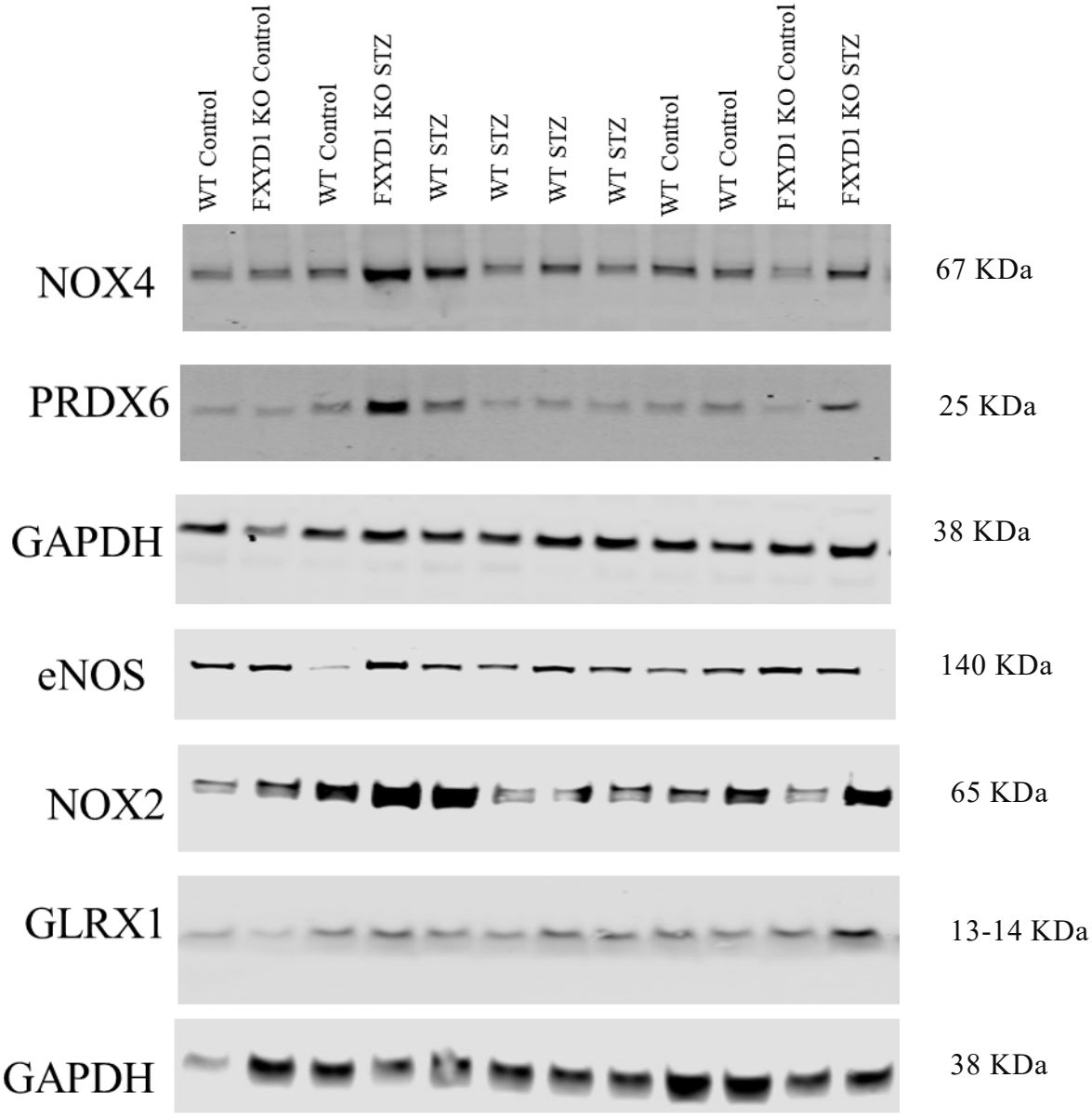


Figure 3.15: Western blot analysis of the expression levels of NOX2, NOX4, PRDX6, GLRX1 and eNOS in HEART of STZ-induced Diabetes WT control, FXYD1 KO control, STZ WT and STZ FXYD1 KO

3.3.2.3.1. Protein expression of Redox Enzymes in **Mesentery** tissues

The western blot was performed to decide whether FXYD1 had an impact upon the expression of redox enzymes in the mice's vasculature in STZ induced diabetes samples. The expression of NOX2, NOX4, PRDX6, GLRX-1 and eNOS in male STZ induced mice mesentery were tested.

The results showed that the induction of STZ or absence of FXYD1 did not have an impact on expression level of NOX2 (Figure 3.17) in our samples.

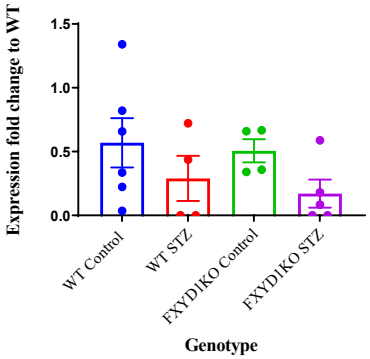


Figure 3.16: Protein expression level of NOX2 in FXYD1 WT and FXYD1 KO male (n=4-6) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between FXYD1 WT and FXYD1 KO

As with NOX2, the expression level of NOX4 did not change with STZ induction (Figure 3.18). In addition, the absence of FXYD1 did not affect the NOX4 expression in the samples.

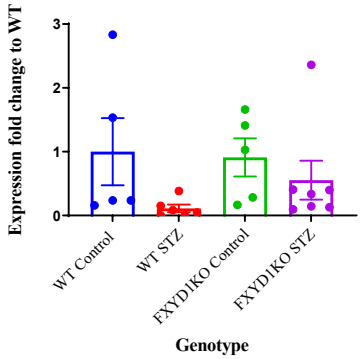


Figure 3.17: Protein expression level of NOX4 in FXYD1 WT and FXYD1 KO male (n=5-7) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA test to determine differences between FXYD1 WT and FXYD1 KO

Figure 3.19 shows the results of GLRX-1 detection in the mesentery samples. The results showed that STZ or FXYD1 did not have an impact upon expression of GLRX-1.

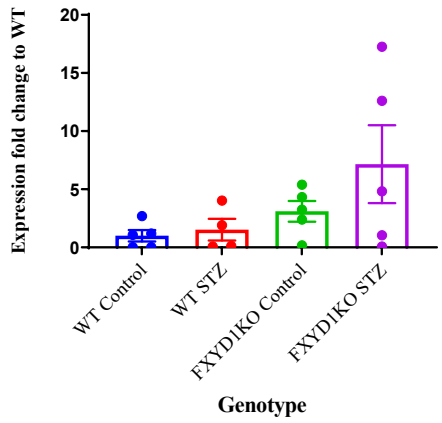


Figure 3.18: Protein expression level of GLRX-1 in FXYD1 WT and FXYD1 KO male (n=4-6) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO.

Further analysis showed that expression level of the eNOS was not significantly altered in our samples (Figure 3.20).

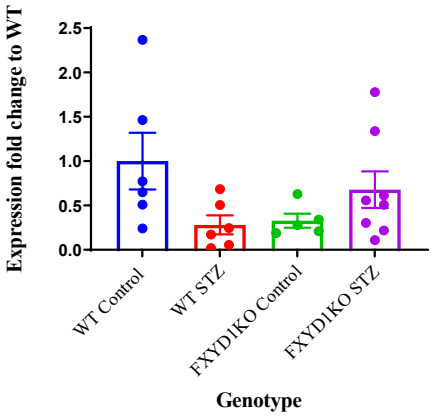


Figure 3.19: Protein expression level of eNOS in FXYD1 WT and FXYD1 KO male (n=4-6) in mouse mesentery. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA to determine differences between FXYD1 WT and FXYD1 KO.

Despite three attempts, the PRDX6 enzyme could not be detected in our mesentery samples, which may happen due to sensitivity of our detection method or low level of PRDX6 in our samples.

Figure 3.21 illustrates the representative images of western blot membrane of the redox enzymes in mesentery of STZ-induced diabetic mice.

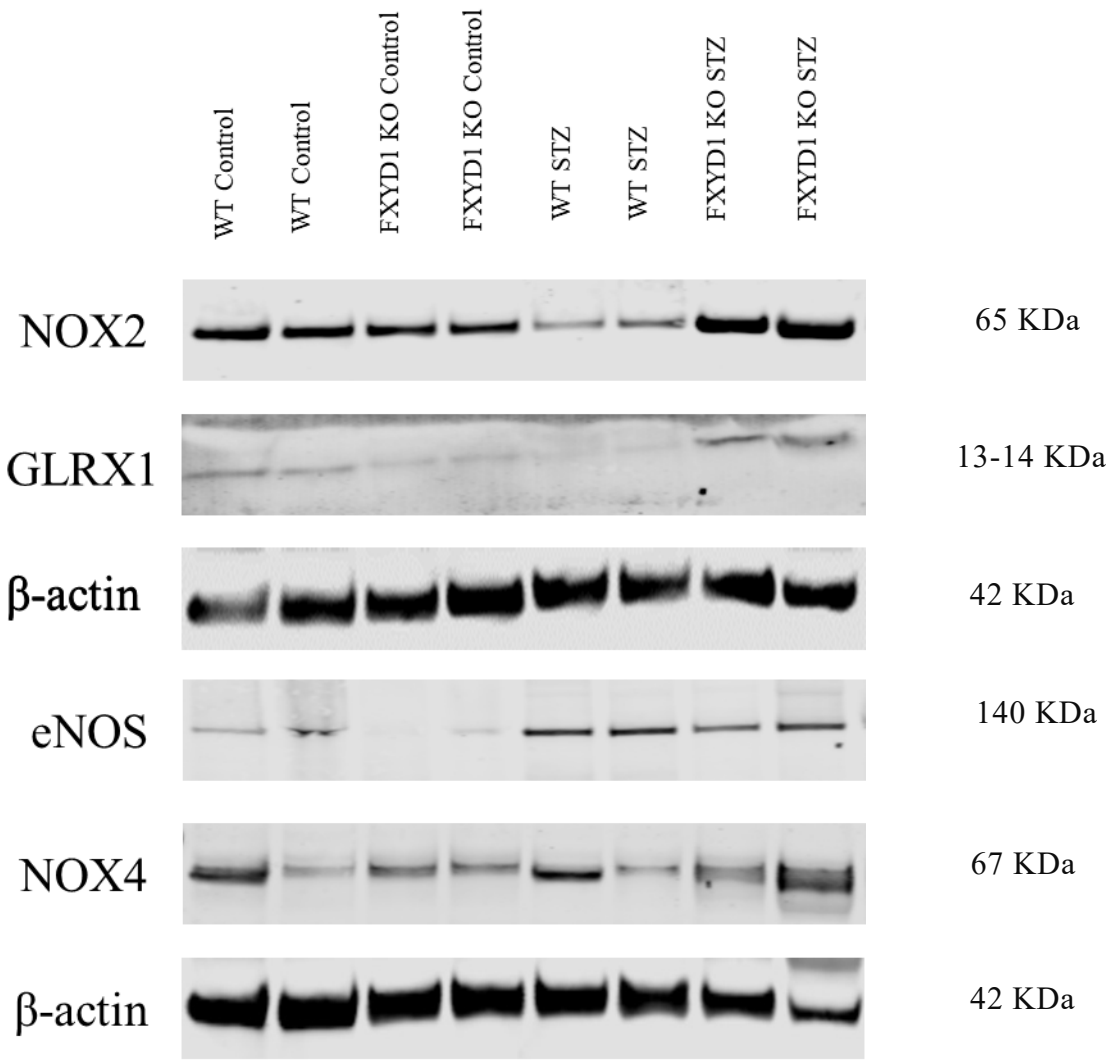


Figure 3.20: Western blot analysis of the expression levels of NOX2, NOX4, GLRX1 and eNOS in Mesentery of STZ-induced Diabetes WT control, FXYD1 KO control, STZ WT and STZ FXYD1 KO

3.3.3. Atherosclerosis

3.3.3.1. Detection of plaques using oil red O

Figure 3.22 shows the results of plaque detection in stained aorta of C57BL/6 wild type and FXYD-1 KO mice, and ApoE KO/FXYD1 KO and ApoE KO/FXYD1 WT mice, that were on high fat/high cholesterol diet for 16 weeks. The results showed that in the stained aorta of C57BL/6 wild type and FXYD-1 KO mice a few very small plaques have been detected, however in ApoE KO/FXYD1 KO and ApoE KO/FXYD1 WT plaques were detected in all part of the aorta with highest deposition in aortic arches confirming successful development of atherosclerosis in this mice model.

Figure 3.21: Plaque detection in aorta of C57BL/6 WT and FXYD1 KO by Oil Red O and compare with the Oil red O stained aorta from ApoE KO/FXYD1 KO and ApoE KO/ FXYD1 WT mice that were on high fat/high cholesterol diet for 16 weeks. The samples are representative of a total number of 91 samples

3.3.3.1. Redox Enzymes

3.3.3.1.1. Protein expression of Redox Enzymes in **Heart** tissues

Figures 3.23 shows the results of NOX2 expression level in atherosclerosis samples. The results show that in male ApoE KO/FXYD1 KO samples (Figure 3.23 B) the expression level of NOX2 was significantly decreased, which implies in atherosclerosis mouse model the absence of FXYD1 leads to less NOX2 which is the source of superoxide in biological system. However, absence of FXYD1 in female samples did not affect the expression level of NOX2 enzyme (Figure 3.23, C). Also, the results demonstrate that when we mixed the results of male and female samples together, there is not differences between WT and FXYD1 KO samples (Figure 3.23, A).

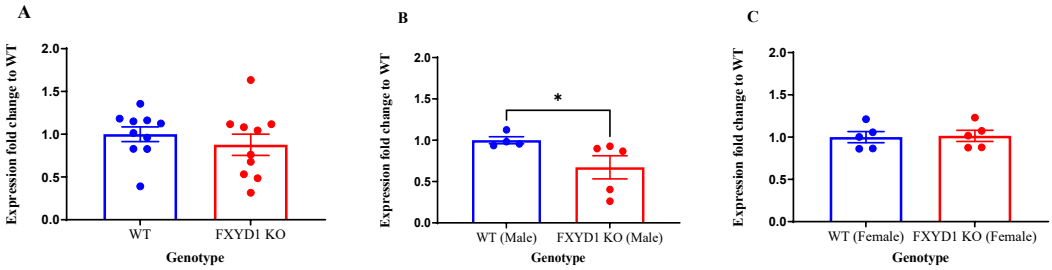


*Figure 3.22: Protein expression of NOX2 in FXYD1 WT and FXYD1 KO male and female (A, n=10, male (B, N=4-5) and female (C, n=5) in mouse heart. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO (*P<0.05).*

The expression levels of NOX4, a family member of NADPH oxidase, were determined in heart tissues and results (Figure 3.24) showed that FXYD1 did not have an impact upon the expression level of NOX4 in heart tissue samples.

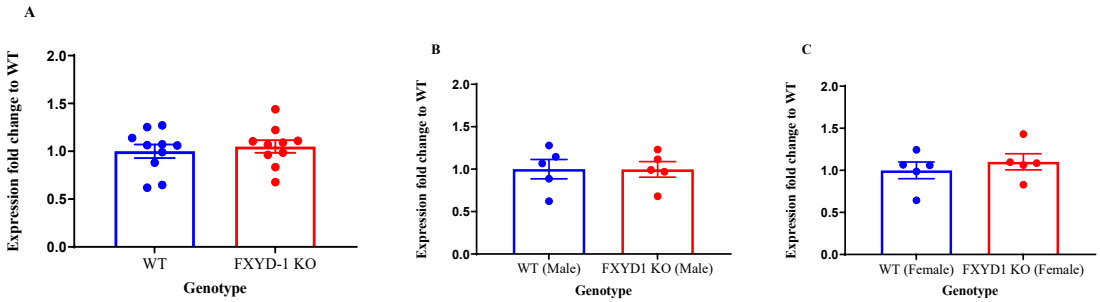


Figure 3.23: Protein expression of NOX4 in FXYD1 WT and FXYD1 KO male and female, A, n=10), male (B, N=5) and female (C, n=5) in mouse heart. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO.

Figure 3.25 shows the results of expression level of PRDX6 in heart tissue samples. The results show that FXYD1 did not have an impact upon expression level of PRDX6 in the samples.

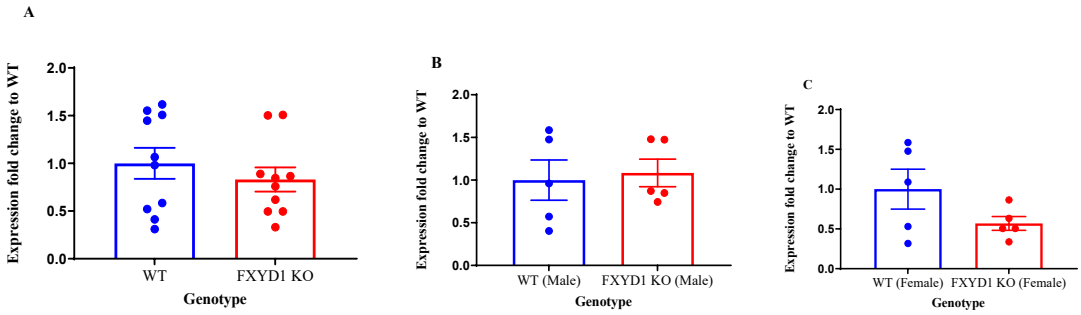


Figure 3.24: Protein expression of PRDX6 in FXYD1 WT and FXYD1 KO male and female A, n=10), male (B, N=5) and female (C, n=5) in mouse heart. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO

It has been shown that the expression of GLRX-1 is enhanced in human atherosclerotic lesions to protect against coronary artery disease (Okuda et al. 2001). The expression levels of GLRX-1 in ApoE KO/ FXYD1 KO and ApoE KO/ FXYD1 WT were determined and results depicted in Figure 3.26. As it can be seen, the absence of FXYD1 did not have any impact on expression level of GLRX-1 in the heart tissue samples.

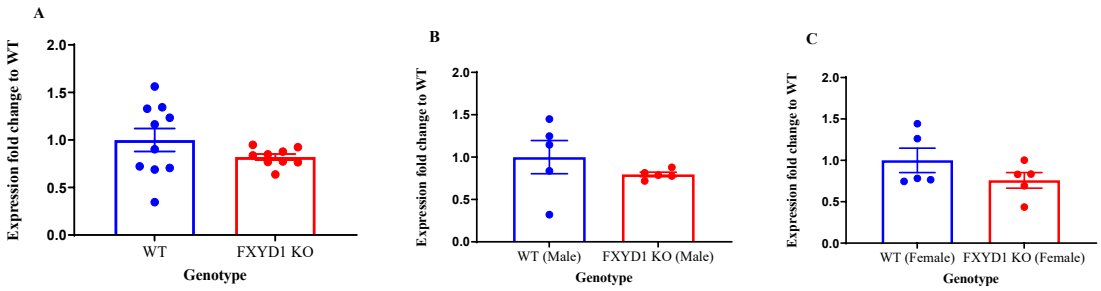


Figure 3.25: Protein expression of GLRX-1 in FXYD1 WT and FXYD1 KO male and female (A, n=9-10, male (B, N=5) and female (C, n=5) in mouse heart. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO.

The pivotal role of eNOS in CVD has been well studied and it has been shown that optimal expression level of eNOS is crucial for improvement of cardiovascular system (Daiber et al. 2019; Galougahi et al. 2014; Heiss & Dirsch 2014; Karbach et al. 2014). The expression levels of eNOS in heart samples were determined and the impact of FXYD1 on the expression level of eNOS was investigated. Figure 3.28 shows the results, it seems that the absence of FXYD1 did not have any impact in male mice (Figure 3.28 B). However, the expression level of eNOS in female samples was significantly decreased in the knockout animals (Figure 3.27, C).

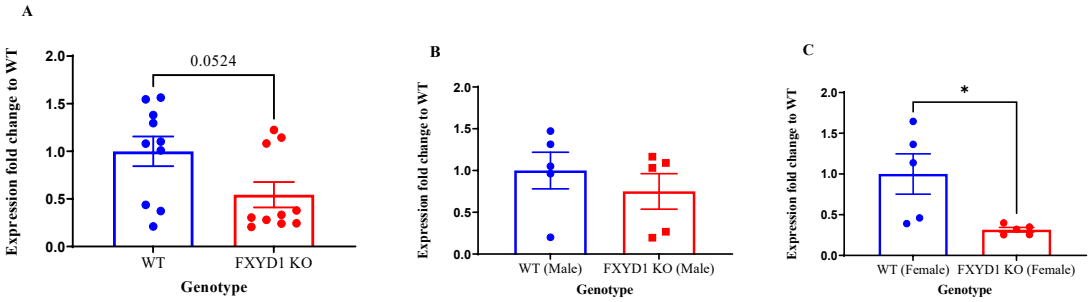


*Figure 3.26: Protein expression of eNOS in FXYD1 WT and FXYD1 KO male and female, (A, n=9-10, male (B, n=5) and female (C, n=5) in mouse heart. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO (*p<0.05)*

Figure 3.28 shows the representative western blot membrane images of redox enzymes in the heart tissues samples.

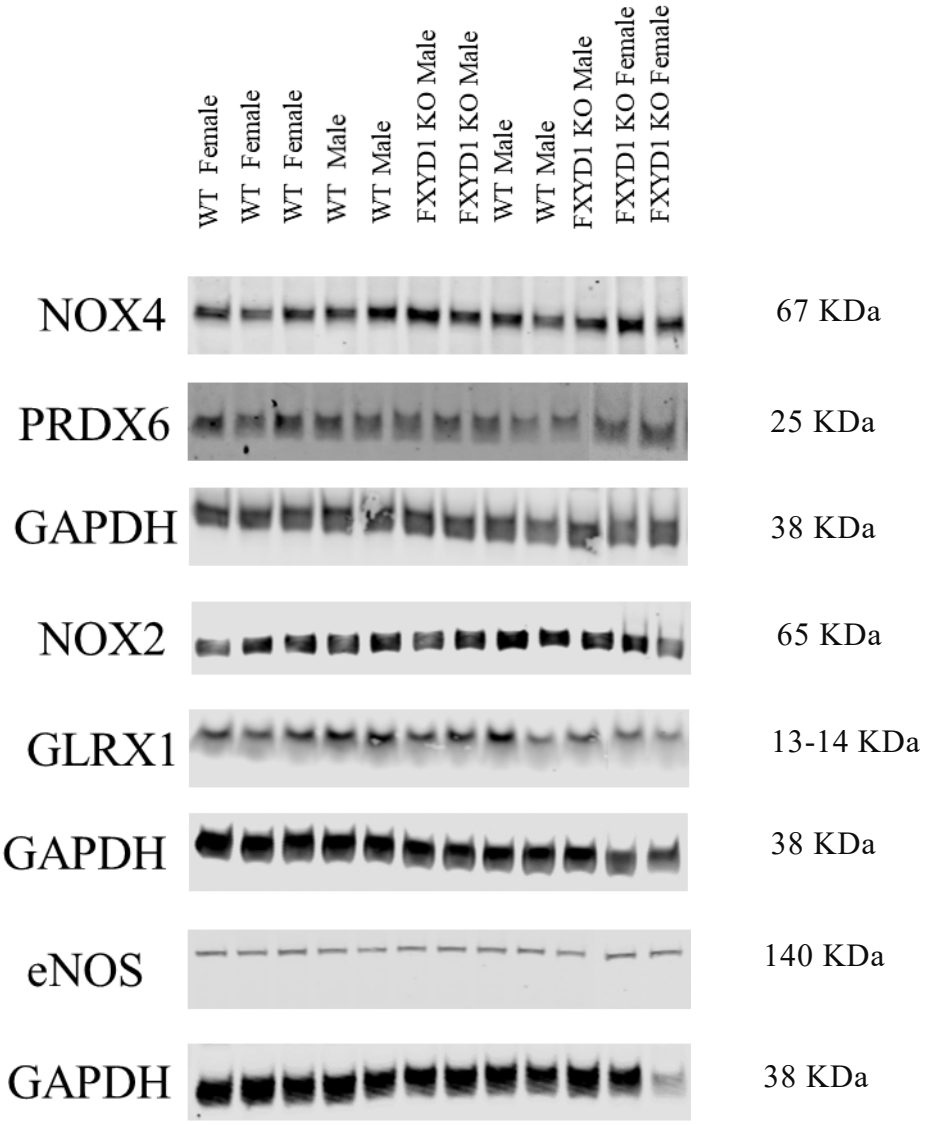


Figure 3.27: Western blot analysis of the expression levels of NOX2, NOX4, PRDX6, GLRX1 and eNOS in Heart of ApoE KO/ FXYD1 KO and ApoE KO/ FXYD1 WT

3.3.3.1.2. Protein expression of Redox Enzymes in the **Mesentery** tissues

The expression level of 5 redox enzymes NOX2, NOX4, PRD-6, GLRX-1 and eNOS in mice mesentery tissues were studied.

The results for detection of NOX2 in mesentery samples (Figure 3.29) showed no differences between FXYD1 KO and WT ApoE KO samples. In addition, when the results separated based on sex of samples no differences were seen in either males or females.

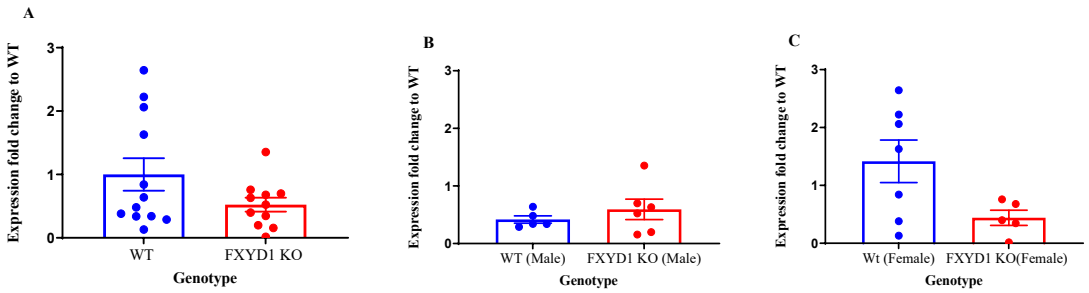


Figure 3.28: Protein expression of NOX2 in FXYD1 WT and FXYD1 KO male and female, A, n=11-12, male (B, N=5-6) and female (C, n=5-7) in mouse mesentery. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO.

As with NOX2, the absence of FXYD1 did not alter the expression level of NOX4 in mesentery samples (Figure 3.30 A). This was the case for both males (Figure 3.30 B) and females (3.30 C) samples.

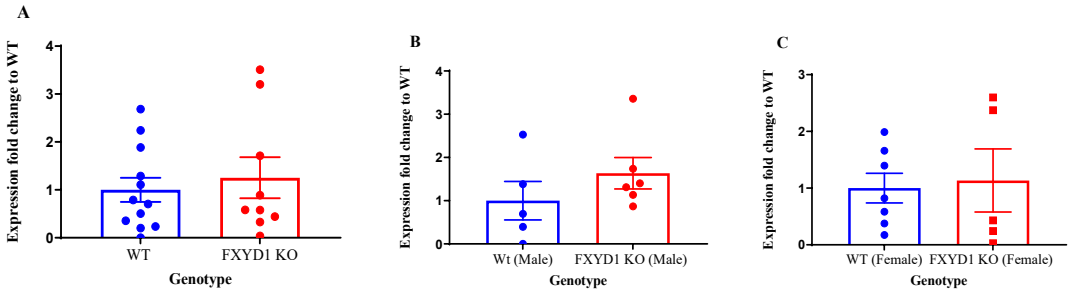


Figure 3.29: Protein expression of NOX4 in FXYD1 WT and FXYD1 KO male and female, (A, n=9-12), male (B, N=5-6) and female (C, n=5-7) in mouse mesentery. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO

Figure 3.31 shows the results of PRDX6 expression level in WT and FXYD1 atherosclerosis mouse model. The results show that the absence of FXYD1 did not alter the expression level of PRDX6 in mesentery tissues, regardless of if the mice were male (Figure 3.31 B) or female (Figure 3.31 C).

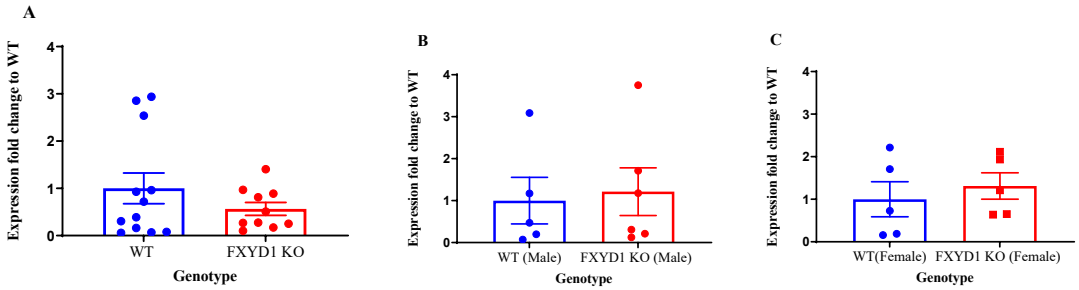


Figure 3.30: Protein expression of PRDX6 in FXYD1 WT and FXYD1 KO male and female (A, n=10-12, male (B, N=5-6) and female (C, n=5) in mouse mesentery. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO

Figure 3.32 shows that the absence of FXYD1 had no effect on expression level of GLRX-1 in mesentery tissues.

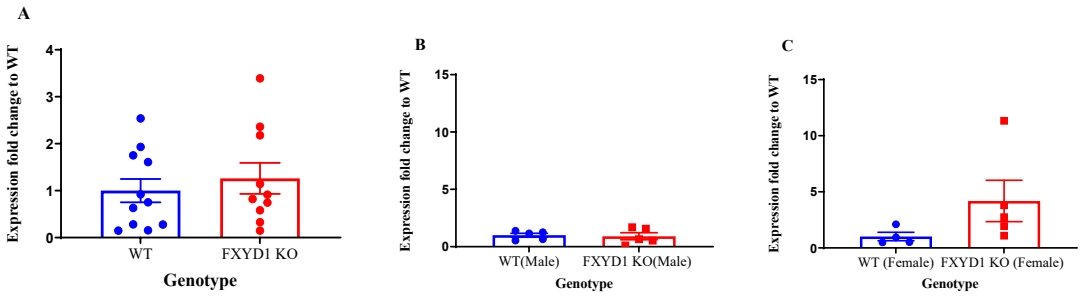


Figure 3.31: Protein expression of GLRX-1 in FXYD1 WT and FXYD1 KO male and female (A, n=10-11), male (B, N=5) and female (C, n=4-5) in mouse mesentery. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by the Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO

As with the other enzymes that investigated in this part of study, the results of eNOS detection in mesentery tissue showed that the absence of FXYD1 did not have any impact on expression level of eNOS (Figure 3.33).

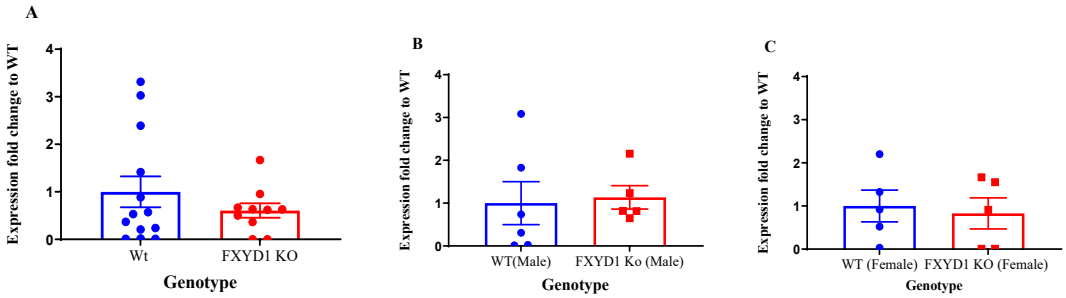


Figure 3.32: Protein expression of eNOS in FXYD1 WT and FXYD1 KO male and female, (A, n=10-13), male (B, N=5-6) and female (C, n=5) in mouse mesentery. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney non-parametric test to determine differences between FXYD1 WT and FXYD1 KO

Figure 3.34 shows the representative western blot membrane images of redox enzymes in the mesentery tissues samples.

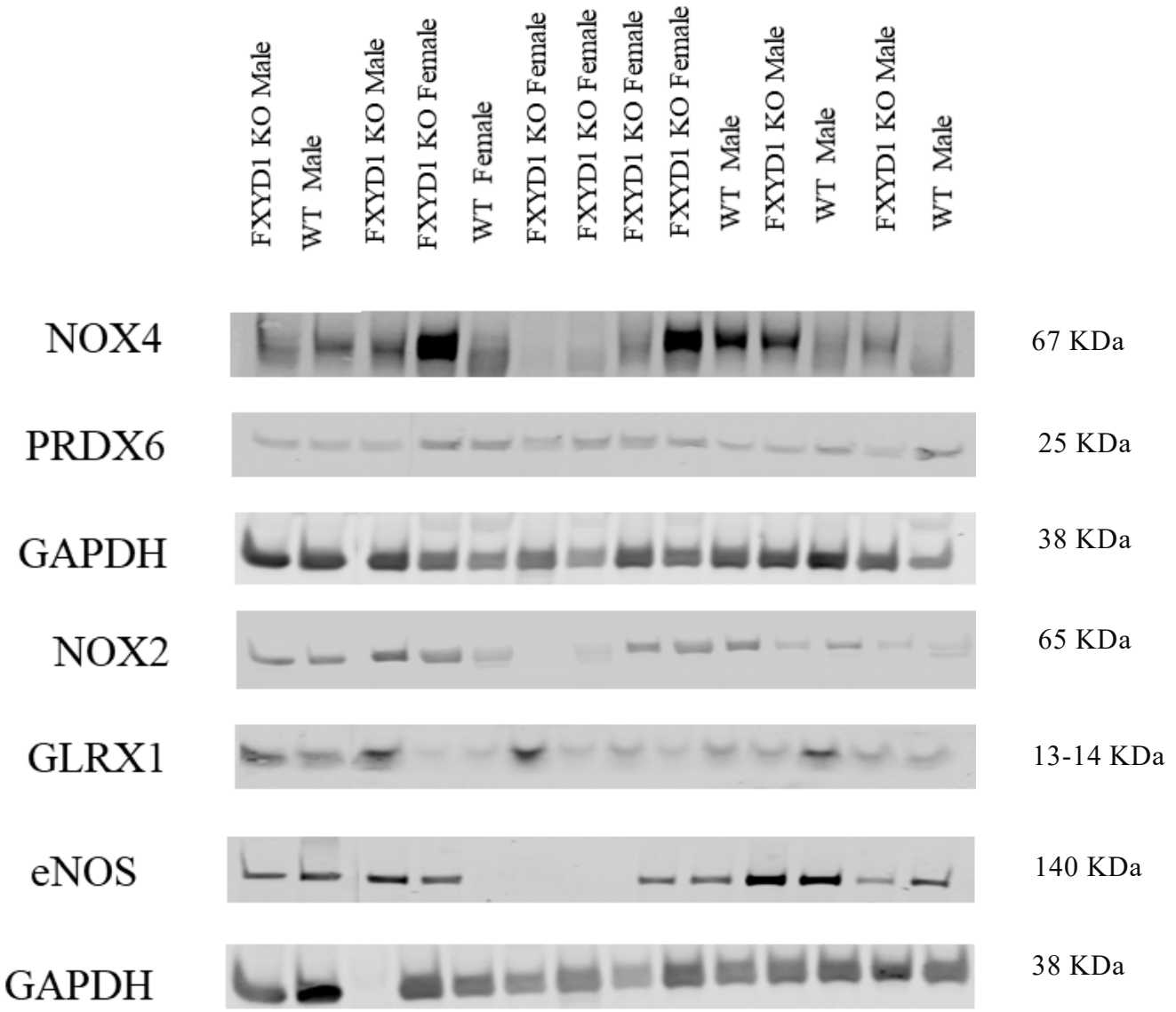


Figure 3.33: Western blot analysis of the expression levels of NOX2, NOX4, PRDX6, GLRX1 and eNOS in Mesentery of ApoE KO/ FXYD1 KO and ApoE KO/ FXYD1 WT

3.4. Summary protein expression of Redox Enzyme in different disease models

Tables 3.1 and 3.2 are summary of the results of expression of redox enzymes experiments in male and female heart tissues. Tables 3.3 and 3.4 summarise the results of expression level of redox enzymes experiments in female and male mesentery tissues.

Table 3.1: Summary of protein expression change inFXYD1 KO male mice's heart tissues of disease models (NA=Not available)

Redox Enzyme	Atherosclerosis	Hypertension	Diabetes
GLRX-1		NA	
NOX2		NA	
NOX4		NA	
PRDX-6		NA	
eNOS		NA	

Table 3.2: Summary of protein expression change in FXYD1 KO female mice's heart tissues of disease models (NA=Not available)

Redox Enzyme	Atherosclerosis	Hypertension	Diabetes
GLRX-1		NA	
NOX2		NA	
NOX4		NA	
PRDX-6		NA	
eNOS		NA	

Table 3.3: Summary of protein expression changes inFXYD1 KO male mice's mesentery tissues of disease models (NA=Not available)

Redox Enzyme	Atherosclerosis	Hypertension	Diabetes
GLRX-1	⟷	⟷	⟷
NOX2	⟷	⟷	⟷
NOX4	⟷	⟷	⟷
PRDX-6	⟷	↓	NA
eNOS	⟷	NA	⟷

Table 3.4: Summary of protein expression changes in FXYD1 KO female mice's mesentery tissues of disease models (NA=Not available)

Redox Enzyme	Atherosclerosis	Hypertension	Diabetes
GLRX-1	⟷	NA	NA
NOX2	⟷	NA	NA
NOX4	⟷	NA	NA
PRDX-6	⟷	NA	NA
eNOS	⟷	NA	NA

3.5. Discussion

We have shown that in the hypertension mouse model used, the systolic, diastolic, and mean arterial blood pressure in Ang II treated mice were significantly increased. This is an indication of success of this mouse model. (Busse et al. 2017; Iulita et al. 2018). In the STZ induced samples the blood glucose significantly increased in WT male samples which is the sign of successful of induction of (Zhang et al. 2008). However, in female mice this procedure was not successful which might happen due to resistance of the female mice to this procedure or the hormonal differences between males and females. Also, the detection of a large number of plaques in the aorta and aortic arch of ApoE KO mice confirm the successful generation of the atherosclerosis in this model.

The successful development of different mice models provides us the opportunity to study the impact of FXYD1 on redox enzymes in these models.

As shown in Table 3.1 to 3.4 in the atherosclerosis mice model, the results of redox enzymes' protein expression showed that NOX2 was significantly decreased in FXYD1 KO male mice heart tissues. Lowering of NOX2 protein, which is an enzyme that involves in many pathways including, detoxification of ROS, cellular response to stress, innate immune system, and immune system (Burtenshaw et al. 2017; Chen, Craige & Keaney 2009; Drummond & Sobey 2014; Lambeth 2007; Santillo et al. 2015), may reduce the ROS production in the biological system and subsequently reduces the risk of CVD (Diebold et al. 2015). In addition, these results showed that the expression level of NOX2 in hypertension and diabetic mouse model in FXYD1 KO samples did not change. The unchanged expression level of NOX2 in diabetic and hypertension models in FXYD1 KO mouse might be due to nature of the disease and the level of contribution and importance of NOX2 in these disease models. In addition, the function and role of FXYD1 gene may vary in different disease models and target different pathways in different diseases.

Our results showed the expression level of eNOS, a membrane protein that catalyses the production of NO, was significantly decreased in female FXYD1KO mice. The benefits of endothelial-derived NO are well studied, and the mechanisms have been explained (Cockcroft 2005; Moyes et al. 2014; Villanueva & Giulivi 2010). The reduction of NO may be due to dysfunction of endothelium and endothelial dysfunction considered an early phase of the atherosclerotic process (Forstermann, Xia & Li 2017). Lowering the

NOX2 and eNOS in FXYD1 KO atherosclerotic mice inferred a contribution of FXYD1 in atherosclerosis and ROS promotion. However, the reduced expression of NOX2 in male FXYD1 KO atherosclerotic mice and the lowered expression level of eNOS in female ApoE KO/FXYD KO mice, raises the point that the function and effect of FXYD1 in male and female may be different.

The significant contribution of the oxidative stress to the development of hypertension has been well studied (Harrison & Gongora 2009; Harrison et al. 2011; Shuvaev et al. 2009). Previously it has been shown that FXYD proteins have important role in the vasculature under oxidative stress by reducing the inhibition of Na^+/K^+ pump (Liu et al. 2013). Hence, it is important for this study to investigate whether the absence of FXYD1 has any impact on hypertension in a mouse model in general and specifically on redox signalling pathway.

The blood pressure test results showed that blood pressure and heart rate significantly increased in Ang II treated mice. However, the absence of FXYD1 KO did not have an impact upon the blood pressure or heart rate. The results of redox enzymes detections showed that in the absence of FXYD1 protein the expression of PRDX6 in mesentery tissues was significantly decreased. PRXD6 is a bifunctional enzyme as it can be an antioxidant or a stimulation factor for oxidants production (Chatterjee et al. 2011). How PRDX6 played in Ang II induced hypertension mesentery vasculature remains unresolved. In this study, the mesentery samples from female mice and heart tissues were not available so the data could not be collected and compared.

Despite a success in generating the male WT diabetic mouse model, that procedure was not successful in female mice. Notably, the circulation glucose level in male FXYD1 KO reduced, and the reduction of blood glucose reduces the risk of diabetes (Kharroubi & Darwish 2015) and subsequently reduces the oxidative stress, inflammatory response and risk of CVD (Domingueti et al. 2016). This was a finding that we encountered with STZ induced mice model that needs to be investigated further.

The results of body weight for the STZ induced diabetics mice compared with the control mice showed the absence of FXYD1 did not have an impact on STZ induced diabetes and also sex did not show any effect upon weight changes.

The results of redox enzymes detection for STZ induced diabetic mouse showed that the expression level of NOX4, which is one of the major sources of oxidative stress in

the failing heart (Kuroda et al. 2010), in FXYD1 KO male mice significantly decreased. Reduction of NOX4 attenuates the oxidative stress and subsequently reduces the risk of heart failure. The redox enzymes detection results imply that the absence of FXYD1 in STZ induced male mice reduced the oxidative stress caused by NOX4, which contrasts with previous studies that demonstrated the protective role of FXYD proteins (Bibert et al. 2011; Figtree et al. 2009). However, in the studies mentioned above, rabbits and Xenopus oocytes were tested, and species differences may cause the differences in the results. Moreover, the limited number of samples used in this study could be considered another source of discrepancy.

The discrepancy of our experiments' results leads us to proteomic and electron microscopic approaches to investigate the impact of FXYD1 on the structure and protein contents of FXYD1 KO samples. Because FXYD1 is the resident of caveolae, it has become the centre of this study.

CHAPTER FOUR

Investigating the role of FXYD1 in caveolae morphology and proteome

4.1. Introduction

FXYD1, a small plasma membrane protein, is expressed in a wide variety of tissues, but is enriched particularly in the heart, liver, and skeletal muscle. The primary role of FXYD1 in skeletal muscle is in potassium homeostasis (Bogaev et al. 2001; Nielsen & Clausen 2000; Rembold et al. 2005). However, in the heart the role of FXYD1is more extensive, regulating not only the Na^+-K^+-ATPase but also regulating the Na^+-Ca^+ exchanger and L-type calcium channels (Cheung et al. 2013a; Pavlovic, Fuller & Shattock 2013; Zhang et al. 2015).

Within the plasma membrane, FXYD1 is predominantly located in the sub-cellular regions known as the caveolae, which are omega or flask-shaped, protein-coated, invaginations of the plasma membrane (Parton, Tillu & Collins 2018). These structures are 50-100 nanometre (nm) in diameter (Rothberg et al. 1992) and enriched in sphingolipids (Schnitzer et al. 1995), cholesterol (Pike et al. 2002) and a specific class of proteins, the caveolins (Kurzchalia et al. 1992; Way & Parton 1995). The caveolae are the home to a high number of signalling proteins, and dysfunction of caveolae is linked to cardiac disease (Parton, Tillu & Collins 2018; Rajab et al. 2010).

Na^+-K^+-ATPase is predominantly located within caveolae (Liu & Askari 2006; Liu et al. 2011; Liu et al. 2003; Nie et al. 2020). Previous work in our laboratory has highlighted a role for FXYD1 in protecting the cardiac Na^+-K^+-ATPase from oxidative dysregulation (Bibert et al. 2011). Therefore, we postulated that FXYD1 might interact with other proteins within the caveolae to modify redox signalling. We also hypothesised that FXYD1 might be integral in the structure and morphology of caveolae. We aimed to determine the effect of FXYD1 on caveolae protein content, size, and the number of caveolae in heart tissue samples. To achieve this aim, I isolated cardiac tissue from FXYD1 knock out mice and wildtype littermates. I conducted an unbiased proteomic analysis on the whole heart and caveolae sub-fractions, as omics approaches provide a great understanding of cellular signalling networks (Shin et al. 2018). I also utilised electron microscopy to visualise the caveolae and determine the size and the number of caveolae in the heart sections.

4.2. Methods:

4.2.1. Extraction of hearts from mice

All animal studies performed were approved by the Northern Sydney Local Health District Animal Ethics Committee (approval number RESP/17/56; 17/96) and conform with the National Health and Medical Research Council of Australia's *Code of Practice for the Care and Use of Animals for Scientific Purposes*. Hearts were extracted from a total of 108 mice. Ten FXYD1 WT and KO mice were used for electron microscopy and 18 for whole heart proteomic analysis (9 per each group), of which 6 (three per group) were used for optimisation of the method. An additional 80 mice were used for caveolae sub-fractionation, with five hearts pooled together per sample to ensure adequate protein concentration in sub-fractions, with total of 40 mice per group.

4.2.2. Quantification of the total proteins in the lysed and caveolae subfraction samples

The total protein of each sample of lysed tissues and caveolae subfraction were determined using the Pierce™ BCA Protein Assay Kit (Cat # 23225, ThermoFisher Scientific, Australia), which is a colourimetric assay. (See 2.12.2.3. for more dials).

4.2.3. Electron Microscopy

4.2.3.1. Biological sample preparation

The sample preparation for electron microscopy was comprehensively explained in section 2.15. Briefly, the mice were euthanised, and the hearts of FXYD1WT and FXYD1KO mice were perfused using saline to remove most of the blood. The left ventricle and septum were dissected free of the right ventricle, cut in approximately 1-2mm^2 pieces and immediately immersed in the primary fixative (2.5% v/v glutaraldehyde plus 4% v/v paraformaldehyde in 0.1M phosphate buffer (pH=7)). Subsequently, the primary fixative was replaced with the secondary fixative (1% v/v osmium in 0.1M phosphate buffer(pH=7)). Samples were dehydrated through graduations of ethanol and then immersed in EPON resin, which was polymerised at 60°C overnight.

4.2.3.2. Sample sectioning and placing on mesh grids

Sectioning the samples and placement on the mesh grid have been explained in chapter 2, section 15.4. Briefly, samples were removed from around the resin blocks, sectioned at 500 nm and transferred onto glass slides. The sections were stained with Toluidine Blue and observed with a light microscope to ensure the sample's orientation and position

in the block were correct, before being further sectioned to 50-70 nm sections and transferred to copper mesh grids.

4.2.3.3. Sample post-staining and visualising

Samples on the mesh grid were stained with heavy metals (See 2.15.5). The grids were stained with 2% w/v aqueous uranyl acetate and 3% w/v lead citrate. Then samples were rinsed with warm distilled water left to dry and visualised with the electron microscope (See 2.16.6).

4.2.3.4. Analysis of electron microscopy images

The ImageJ software (Schneider, Rasband & Eliceiri 2012) was used to analyse the captured images. The length of the plasma membrane and the number of caveolae were determined, in order to calculate the number of caveolae per µm. Also, the diameter (nm) and circumference of caveolae were calculated, and results were compared between FXYD1 KO and WT samples.

4.2.4. Proteomics

4.2.4.1. Protein sample preparation

The extraction buffer (6 M urea, 2 M thiourea, 2% v/v sodium dodecyl sulfate (SDS), 2 mM diethylenetriamine pentaacetic acid (DTPA), 10mM N-ethylmalemide (NEM), 1 µM aprotinin, 1 µM pepstatin A, 1 mM sodium orthovanadate, 20 µM leupeptin and 1x Halt phosphatase inhibitor cocktail (Thermo Scientific)) was added to the heart tissues (See sections 2.16.1). The samples were subsequently homogenised (Omni International, Kennesaw GA) on ice. The tissue homogenates were centrifuged at 14,000 x rcf for 15 mins at 4°C and the supernatant collected. The pellet was resuspended in extraction buffer with acid-washed glass beads (≤106µm), and proteins re-extracted by bead-beating (FastPrep-24, MP Biomedicals, Shandong, China) using two rounds at 40 m/s for 45 sec with 1 min incubation on ice between each round. Samples were centrifuged as above, and each supernatant was pooled with that collected previously.

4.2.4.2. Protein Precipitation (Chloroform/Methanol)

The collected supernatant from the previous step contained proteins that needed to be precipitated. For separating protein from the extraction buffer four volumes of ice-cold methanol were added to the supernatant followed by 1 volume of ice-cold chloroform, then three volumes of ice-cold water with vortexing in between each addition. Subsequently, the samples were centrifuged at 14,000 x rcf for 2 mins at 4°C and the

upper aqueous phase discarded. The pellet was washed with three volumes of ice-cold methanol with vortexing; then the proteins were collected by centrifugation at 14,000 x rcf for 2 mins at 4°C with the supernatant discarded. Finally, for the last time, the collected pellet was resuspended in the ice-cold methanol and proteins collected by centrifugation at 14,000 x rcf for 2 mins at 4°C with the supernatant discarded.

4.2.4.3. Protein reduction and alkylation

The protein pellets collected from previous steps were resuspended in 6 M urea, 2 M thiourea and reduced with 25 mM dithiothreitol (DTT) and then incubated at RT in the dark for 1 hr. Subsequently, 60mM methylmethanethiosulfonate (MMTS; diluted 1:10 in acetonitrile (MeCN)) was added to the solution to modify the new free thiols, and the solution was incubated for 1.5 hr at 35°C.

4.2.4.4. Protein digestion (Trypsin)

For digesting the alkylated proteins, porcine sequencing grade modified trypsin (Promega, Madison, WI USA) was added to the proteins and incubated at RT for 18 hrs, the ratio of trypsin to protein was 1:25. The concentration of the proteins was determined with Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, CA USA) according to the manufacturer's instructions.

4.2.4.5. Peptide's concentration and desalting (Solid Phase extraction)

From each sample, 15 µg of the peptide was used and labelled with TMT6plex (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Peptides were mixed 1:1 for each of the sample types. Then the HLB columns were used to remove excess labels. Biological replicates were run individually.

4.2.4.6. Isobaric labelling of peptides (reversibly redox modified Cys)

The TMT 6-plex application kit (Thermo Fisher Scientific, Waltham, MA) was used for isobaric tandem mass tag (TMT) labelling the peptides. Fifteen micrograms of digested peptide incubated with TMT reagents was resuspended in an equal volume of MeCN (HPLC grade) for two hours at RT. Samples from across the biological groups were pooled, and MeCN was removed by vacuum centrifugation before incubation with 10mM tributylphosphine (TBP) for 30 mins at 25°C to remove the MMTS protecting group. The samples were acidified and diluted with 0.1% v/v TFA, followed by HLB-SPE clean-up as described in section 17.5 of Chapter 2, prior to enrichment. Biological replicates were run individually for LC-MS/MS.

4.2.4.7. Analysis of MS/MS data for protein identification and quantitation

The Proteome Discoverer v. 2.4 (Thermo Scientific) was used to identify and quantify the proteins, and an in-house MASCOT server was used to search against *Mus muscularis* SwissPROT databases consecutively (Mouse: UP000000589; organism ID 10090, 17,042 protein entries; last updated May 27, 2020). The fixed parameters applied for the searches were: precursor mass tolerance of 20 ppm, product ion mass tolerance of 0.1 Da, and two missed cleavages; and variable parameters were: Cys alkylation (N-ethylmalemide; NEM and carbamidomethylation; IAA), oxidation of Met, TMT6plex labelling of Lys and N-termini, acetylation of protein N-termini. Peptide level false discovery rate (FDR) was decided using Percolator (v. 2.08.01) and all searches were strained for <1% FDR, peptide length between 6-40 residues and rank one peptide identifications. For further analysis, a minimum of 2 unique peptides was required for confident identification and quantitation of proteins.

The median signal intensity from across all WT was used to normalise the results. Median log2 ratios were calculated at the protein level within each biological replicate. The weighted average log2 ratio was calculated across replicate experiments with the summed reporter ion intensities used to rank the proteins for generation of the zScore. Proteins were deemed to be significantly regulated based on a Z-Score of ±1.96 and students t-test $p<0.05$. Redox modified peptides were deemed to be significantly regulated based on a Z-Score of ±1.00 and students t-test $p<0.05$.

4.2.4.8. Analysis of MS/MS data for redox modified Cys peptide identification and quantitation

As explained in section 16.8 of chapter 2, Cys peptide identification and quantitation were performed using ProteomeDiscoverer v. 2.4 (Thermo Scientific) and searched using an in-house MASCOT server against *Mus muscularis* SwissPROT databases sequentially (Mouse: UP000000589; organism ID 10090, 17,042 protein entries; last updated May 27, 2020). Searches were performed using fixed parameters: precursor mass tolerance of 20 ppm, product ion mass tolerance of 0.1 Da, and two missed cleavages; and variable parameters: Cys alkylation (N-ethylmalemide; NEM and carbamidomethylation; IAA), oxidation of Met, TMT6plex labelling of Lys and N-termini, acetylation of protein N-termini. Peptide level FDR was determined using Percolator (v. 2.08.01) and all searches were filtered for <5% FDR, peptide length between 6-40 residues and rank one peptide identifications. Modified Cys peptides were normalised using median signal intensity

from non-captured species across all WT samples. Median log2 ratios were calculated at the redox modified site level within each biological replicate. The weighted average log2 ratio was calculated across replicate experiments with the summed reporter ion intensities used to rank the proteins for generation of the zScore. Redox modified peptides were deemed to be significantly regulated based on a Z-Score of ±1.00 and students t-test outcome with a $p<0.05$.

4.3. Results

4.3.1. Quantification of the total proteins in the lysed tissue and caveolae subfraction samples

The quantity of total protein in lysed heart and caveolae subfraction (Fraction 4-5-6) were determined. The results showed no significant differences in the total protein quantity in FXYD1 KO samples to compared with WT samples in either whole heart (Figure 4.1, A) or in caveolae subfractions (Figure 4.1, B).

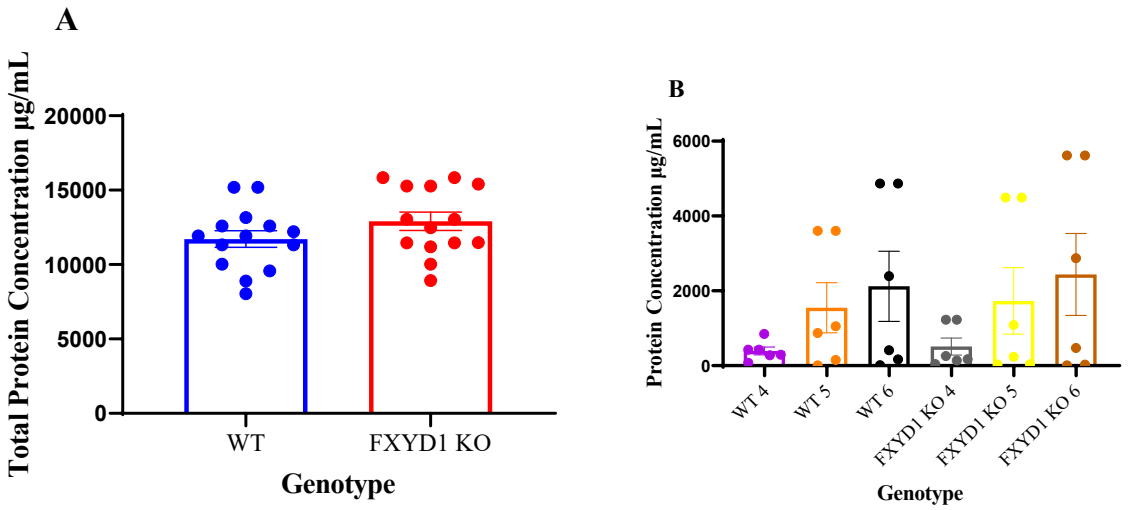


Figure 4.1: Total protein concentration in FXYD1 WT and FXYD1 KO lysed heart tissues male mice (A, n=14), and total protein concentration in caveolae subfractions (4-5-6) FXYD1 WT and FXYD1 KO male mice (B, n=6). Data [are] presented as mean ± SEM. Mann-Whitney was used to determine differences between FXYD1 WT and FXYD1 KO for heart tissues and nonparametric one-way ANOVA for caveolae subfractions.

4.3.2. Electron microscopy

The size, morphology, and distribution of caveolae were determined by electron microscopic techniques (McGuire & Twietmeyer 1983; Rothberg et al. 1992; Yamada 1955). The caveolae were morphologically defined as flask-shaped invaginations of the plasma membrane size of ≈ 70 nm average diameter (Stan 2005). It has also been shown that the distribution of caveolae varies in different tissues (Noguchi, Shibata & Yamamoto 1987; von Ruhland et al. 2004). This study determined the morphology, size, and distribution of caveolae in mouse heart tissues by applying electron microscopy

techniques (See 2.15). Representative images for each genotype are provided in Figure 4.2.

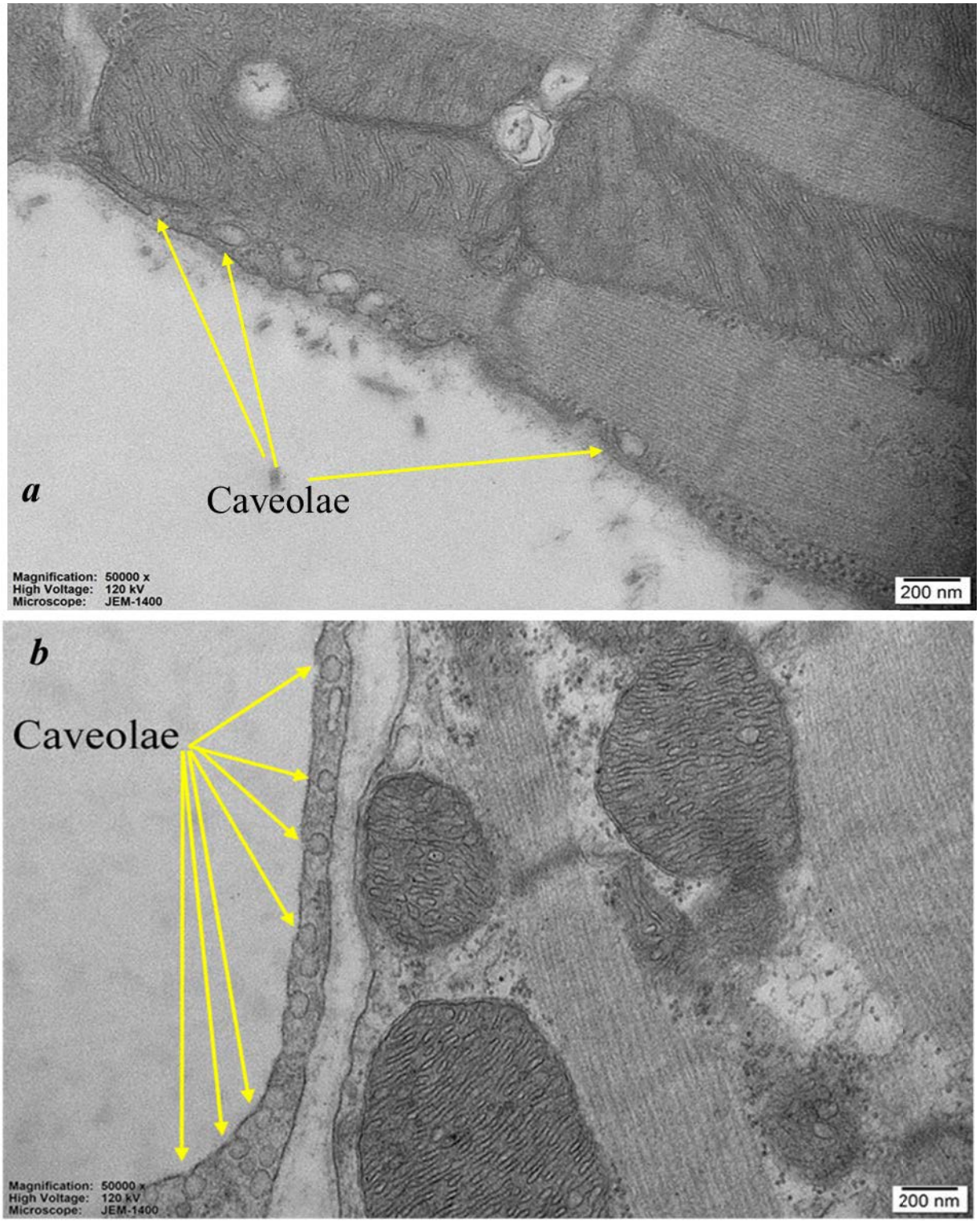


Figure 4.2: Representative image of caveolae imaging in the hearts of A) FXYD1 WT and B) FXYD1 KO MICE

The density of caveolae in the plasma membrane of heart tissue in FXYD1 KO mice was significantly increased ($p<0.0001$, Figure 4.3). However, diameter and circumference of caveolae in FXYD1 KO mice were significantly decreased (Figure 4.4 and 4.5; $p< 0.05$) when compared with WT mice.

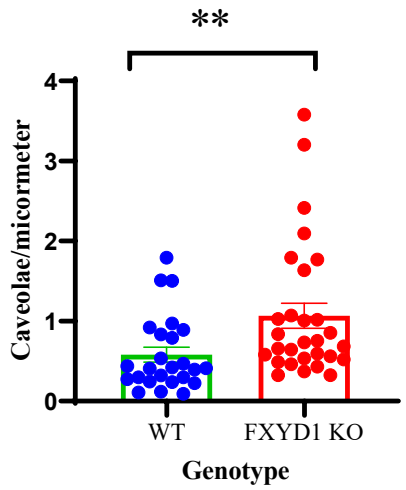


*Figure 4.3: The number of caveolae per µm of the plasma membrane for FXYD1 knockout and wild type littermates. Data are shown as mean ± SEM, and statistical analysis was performed by nonparametric Mann-Whitney test, the biological number N=5, with 2-10 images analysed per sample. **p<0.01*

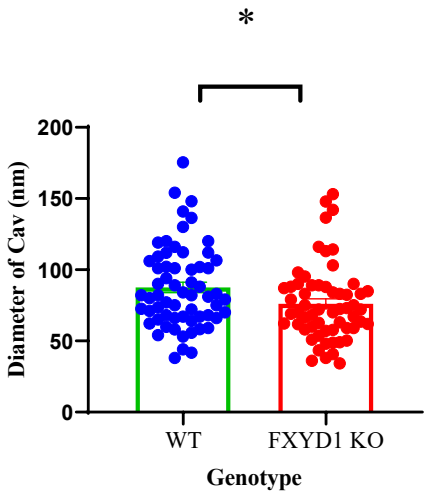


Figure 4.4: The diameter of caveolae (nm) of the plasma membrane for FXYD1knock out and wild type littermates. Data are shown as mean ± SEM, and statistical analysis

*was performed by nonparametric Mann-Whitney test, the biological number N=5, with 2-10 images analysed per sample. *p<0.05*

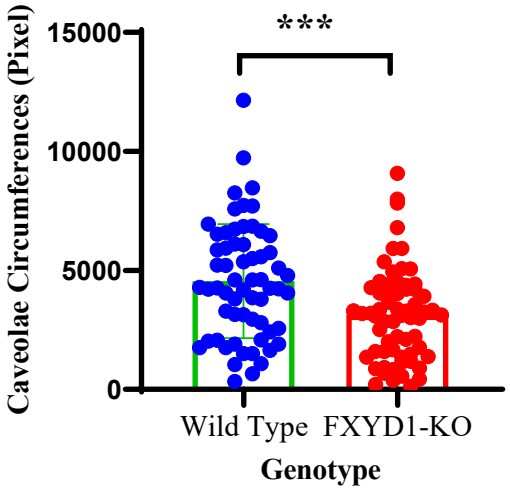


*Figure 4.5: The circumference (pixel ^{2}) of the plasma membrane's caveolae for FXYD1 knockout and wild type littermates. Data are shown as mean ± SEM, and statistical analysis was performed by nonparametric Mann-Whitney test, the biological number N=5, with 2-10 images analysed per sample, ***p<0.001*

4.3.3. Proteomics

4.3.3.1. Whole Hearts

The analysed proteomics data of the WT whole heart when compared with the proteomics results from FXYD1 KO whole heart showed that 11 proteins were significantly up-regulated in FXYD1 KO mice's heart (Table 4.1) and 61 proteins were significantly downregulated (Table 4.2).

Table 4.1: The list of proteins that are significantly up-regulated in FXYD1 KO hearts

Gene	Protein Name	n-fold changes	z-Score	Log2 KO vs WT
A1bg	Alpha-1B-Glycoprotein	+3.12	5.28	1.64
Znf638	Zinc finger protein 638 (Nuclear protein 220)	+1.58	1.85	0.66
Myh7	Myosin 7 (Myosin Heavy Chain, Cardiac Muscle Beta Isoform) (MyHC-beta)	+1.45	1.77	0.53
Ankzf1	Ankyrin Repeat and Zinc Finger Domain-Containing Protein 1	+1.38	1.50	0.46
Ngp	Neutrophilic Granule Protein (NGP) (Cystatin-Like Protein)	+1.32	1.30	0.40
Ntan1	Protein N-Terminal Asparagine Amidohydrolase	+1.28	1.16	0.36
Gfod1	Glucose-Fructose Oxidoreductase Domain-Containing Protein 1	+1.26	1.01	0.33
Tiprl	TIP41-Like Protein	+1.22	0.94	0.29
Atp5mf	ATP Synthase Subunit F, Mitochondrial	+1.22	0.85	0.28
Aldob	Fructose-Bisphosphate Aldolase B	+1.21	0.89	0.28

Pltp	Phospholipid Transfer Protein	+1.21	0.96	0.28

Of the up-regulated proteins, none were recognised as having a role in redox signalling. Also, the network map, which was prepared using STRING online software (Szklarczyk et al. 2019), does not show any connection between the up-regulated proteins (Figure 4.6).

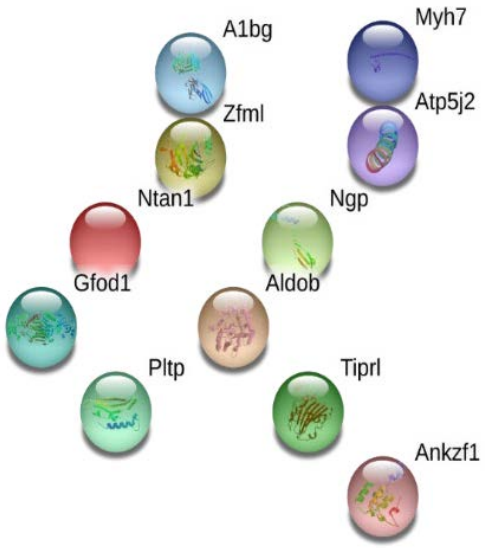


Figure 4.6: Network nods represent proteins, which were significantly increased in FXYD1 KO whole hearts mice

After analysing the up-regulated proteins in FXYD1 KO samples using online proteomic software "Reactome" (Fabregat, Jupe, et al. 2018; Fabregat, Korninger, et al. 2018; Fabregat, Sidiropoulos, et al. 2018; Jassal et al. 2020) the 25 most relevant pathways were identified, sorted by *p*-value, and listed in Table 4.2.

One of the most relevant pathways to the up-regulated proteins in FXYD1 KO samples is High-Density Lipoprotein (HDL) remodelling pathway. It has been demonstrated that HDL particles play a central role in the reverse transport of the cholesterol, by which the cholesterol in different tissues are transferred to the liver and converted to bile salts and supplied to tissues including adrenals and gonads for steroid hormone synthesis (Tall et al. 2008). Apoa-I activates the enzyme lecithin-cholesterol acetyltransferase (LCAT) at

the LDL surface, catalysing the reaction of cholesterol and phosphatidylcholine to yield cholesterol esterified with a long-chain fatty acid and the 2-lysophosphatidylcholine. While the hydrophobic cholesterol ester reaction product is strongly associated with HDL, 2- lysophosphatidylcholine product is released (Cooke et al. 2018; Rousset et al. 2009).

Another important pathway is NR1H3 & NR1H2. NR1H3 and NR1H2 modulate the activation of some genes which are closely associated with reverse cholesterol transport (RCT) pathway, including ATP-binding cassette subfamily A type 1 (ABCA1), ATP-binding cassette subfamily G type 1 (ABCG1), type 5 (ABCG5) and type 8 (ABCG8) and a cluster of apolipoprotein genes such as APOE, APOC1, APOC2 and APOC4 (Back et al. 2013; Jakobsson et al. 2009).

Moreover, plasma lipoprotein remodelling pathway is involved in clearing LDL (Gibbons et al. 2004), converting VLDL to LDL (Redgrave 2004) and remodelling HDL (Rye, Clay & Barter 1999).

Table 4.2: The 25 most relevant pathways related to the proteins which significantly up-regulated in FXYD1 KO heart tissue sorted by p-value

	Pathway name	P-Value
1	HDL remodelling	0.003
2	Hereditary fructose intolerance	0.003
3	NR1H3 & NR1H2 regulate gene expression linked to cholesterol transport and efflux	0.008
4	Plasma lipoprotein remodelling	0.015
5	NR1H2 and NR1H3-mediated signalling	0.018
6	Formation of ATP by chemiosmotic coupling	0.031
7	Cristae formation	0.042
8	Fructose catabolism	0.046
9	Plasma lipoprotein assembly, remodelling, and clearance	0.060
10	IRAK1 recruits IKK complex	0.060
11	IRAK1 recruits IKK complex upon TLR7/8 or 9 stimulation	0.060
12	Fructose metabolism	0.062
13	BBSome-mediated cargo-targeting to cilium	0.125
14	Diseases of carbohydrate metabolism	0.133

15	Gluconeogenesis	0.144
16	Glycolysis	0.158
17	RUNX3 regulates WNT signalling	0.184
18	Binding of TCF/LEF: CTNNB1 to target gene promoters	0.206
19	TFAP2 (AP-2) family regulates transcription of cell cycle factors	0.215
20	Platelet degranulation	0.216
21	Glucose metabolism	0.241
22	Response to elevated platelet cytosolic Ca2+	0.281
23	Signalling by NOTCH1 PEST Domain Mutants in Cancer	0.312
24	Constitutive Signalling by NOTCH1 HD+PEST Domain Mutants	0.312
25	Signalling by NOTCH1 HD+PEST Domain Mutants in Cancer	0.312

$P<0.05$ is significant

Of interest in my research, Phospholipid Transfer Protein (Pltp) was increased 1.21-fold in FXYD1 KO mice, and this protein has a close connection to the proteins that impact on atherosclerosis development (Figure 4.7). Notably, Pltp facilitates the transfer of phospholipids among plasma lipoproteins (Nishida, Arai & Nishida 1993). In addition, Pltp mediates transformation of a range of different lipid molecules, including diacylglycerol, phosphatidic acid, sphingomyelin, phosphatidylcholine, phosphatidylglycerol, cerebroside and phosphatidylethanolamine into vesicular structures (Nishida et al. 1997; Rao et al. 1997). Moreover, it has been shown that Pltp transfers the antioxidant α-tocopherol, which evokes the hypothesis that Pltp plays a role in protecting endothelium against oxidation of lipoproteins and tissues (Desrumaux et al. 1999; Kostner et al. 1995).

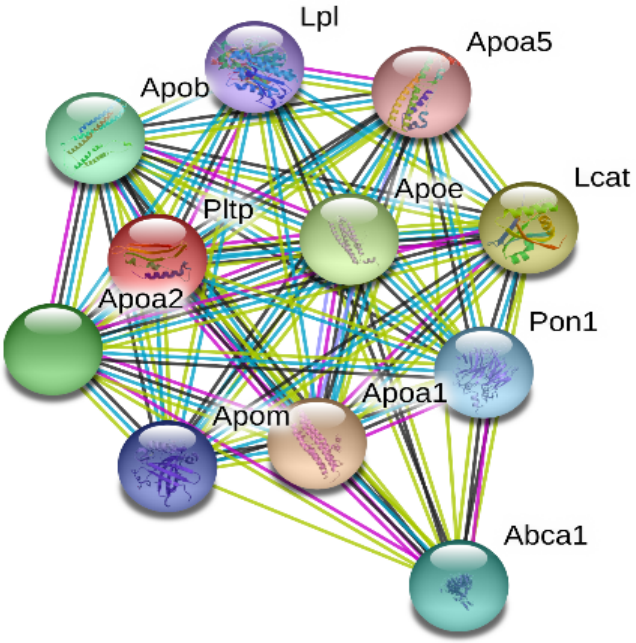


Figure 4.7: Network nodes represent proteins, which has a close connection with Pltp

As shown in Figure 4.7, Apoe, Apoa1, Apoa2, Apoa5, Apob, Apom and Lpl, which have a close connection to Pltp, are involved in lipoprotein transportation. Thus, while these proteins themselves were not modified, that signalling pathway may have been altered.

From the proteins listed in Table 4.3, which shows the proteins that were downregulated in FXYD1KO mice heart tissues, only Prdx5 has a redox regulatory role acting as a cytoprotective antioxidant enzyme in inflammation (Knoops et al. 2016). Another protein that was downregulated in the hearts of FXYD1 knock out mice was Phospholamban (Pln). Pln has an essential role in calcium homeostasis in the heart muscle as it reversibly inhibits the activity of ATP_2A_2 in cardiac sarcoplasmic reticulum by decreasing the apparent affinity of the ATPase for Ca^{2+} and in so doing, it regulates the contractility of the heart muscle in response to physiological stimuli via its effects on ATP_2A_2 (Koss & Kranias 1996).

Moreover, Cytochrome *c* Oxidase Subunit 6A1 (Cox6a1), which was downregulated 1.33-fold, is an enzyme of the mitochondrial electron transport chain that catalyses the transfer of electrons from cytochrome *c* to oxygen (Grossman & Lomax 1997). xanthine dehydrogenase (XDH) is another protein that significantly downregulated in FXYD1 KO

heart tissue. It has been shown that XDH is involved in oxidative metabolism of purine and can be converted to xanthine oxidase, which is a source of ROS (Berry & Hare 2004).

Figure 4.8 illustrates the network map for the proteins that were significantly downregulated in FXYD1 KO mice. The map does not show many close connections between the proteins, and there is no connection between Prdx5 and Pln with other proteins.

After analysing the downregulated proteins in FXYD1 KO samples using online proteomic software "Reactome" (Fabregat, Jupe, et al. 2018; Fabregat, Korninger, et al. 2018; Fabregat, Sidiropoulos, et al. 2018; Jassal et al. 2020) the most relevant pathways were identified, sorted by *p*-value, and listed in Table 4.4.

All of the pathways listed in Table 4.4 have impacts on some of the biological functions, which are out of the scope of this study; however, some of these pathways and their functions are important and will be briefly explained.

Interestingly the NR1H3 & NR1H2, which is one of the important pathways in up-regulated proteins, is listed as one of the relevant pathways for downregulated proteins. However, the function is different, and the NR1H2 & NR1H3 regulate gene expression linked to triglyceride lipolysis in adipose. It has been shown that NR1H2 & NR1H3 are expressed in adipocytes (Juvet et al. 2003) and the expression of NR1H3 is up-regulated during adipocyte differentiation (Darimont et al. 2006; Juvet et al. 2003). Moreover, Dib et al. (2004) showed that the absence of NR1H3 resulted in elevated adiposity in white tissues (Dib, Bugge & Collins 2014).

Mitochondrial uncoupling pathway is another relevant pathway to the downregulated proteins in FXYD1 KO mice. Mitochondria consist of transport carrier family called uncoupling proteins (UCPs). These proteins are involved in a wide range of physiological and pathological conditions. Physiological conditions include thermogenesis, fatty acid metabolism and protection against free radicals and ageing. The pathological conditions include involvement in obesity, diabetes, and degenerative, neurological, and immunological diseases (Esteves & Brand 2005; Kunji 2004). It has been shown that regulated uncoupling caused by UCPs attenuates mitochondrial ROS production and protects against cellular damage (Brand & Esteves 2005; Krauss, Zhang & Lowell 2005).

Table 4.3: List of proteins significantly downregulated in FXYD1 KO mice

Gene	Protein Name	n-fold changes	z-Score	Log2 KO vs WT
Mbp	Myelin Basic Protein (Myelin A1 Protein)	-2.43	-4.12	-1.28
Hif1an	Hypoxia-Inducible Factor 1-Alpha Inhibitor (Hypoxia-Inducible Factor Asparagine Hydroxylase)	-2.00	-3.47	-1.00
Svs3a	Seminal Vesicle Secretory Protein 3A	-1.84	-3.01	-0.88
Svs2	Seminal Vesicle Protein Secretion 2	-1.75	-2.86	-0.81
Ucp1	Mitochondrial Brown Fat Uncoupling Protein 1 (UCP1)	-1.71	-2.47	-0.77
Pln	Cardiac Phospholamban PLB	-1.70	-2.47	-0.76
Uba5	Ubiquitin-Like Modifier Activating Enzyme 5 (Ubiquitin-Activating Enzyme E1 Domain-Containing Protein 1)	-1.68	-2.40	-0.75
Mpz	Myelin Protein P0 (Myelin Peripheral Protein)	-1.62	-2.07	-0.69
Nefl	Neurofilament Light Polypeptide (68kDa Neurofilament Protein)	-1.56	-2.09	-0.64
Fasn	Fatty Acid Synthase	-1.55	-2.01	-0.64
Bri3	Brain Protein I3	-1.54	-2.01	-0.63
Acly	ATP-Citrate Synthase	-1.49	-1.79	-0.58
Rrm1	Ribonucleoside-Diphosphate Reductase Large Subunit	-1.46	-1.57	-0.55
Banf1	Barrier-To-Autointegration Factor (Breakpoint Cluster Region Protein 1) (LAP2-Binding Protein 1)	-1.44	-1.54	-0.53
Plin1	Perilipin-1 (Lipid Droplet-Associated Protein) (Perilipin A)	-1.41	-1.59	-0.49

Dpp4	Dipeptidyl Peptidase 4 (T-Cell Activation Antigen CD26) (Thymocyte-Activating Molecule) (THAM)	-1.39	-1.50	-0.47
Tkt	Transketolase (TK)	-1.38	-1.40	-0.46
Nr3c1	Glucocorticoid Receptor (GR) (Nuclear Receptor Subfamily 3 Group C Member 1)	-1.36	-1.43	-0.45
Cnst	Consortin	-1.36	-1.45	-0.44
Hist1h1c	Histone H1.2 (H1c)	-1.36	-1.39	-0.44
Itga8	Integrin Alpha 8	-1.34		
Cox6a1	Cytochrome c Oxidase Subunit 6A1 Mitochondrial	-1.33	-1.36	-0.42
Galns	N-Acetylgalactosamine-6-Sulfatase	-1.33	-1.38	-0.41
Snca	Alpha-Synuclein	-1.33	-1.25	-0.41
Lsm12	Protein LSM12 Homolog	-1.32	-1.26	-0.40
Rel	Proto-Oncogene c-Rel	-1.31	-1.20	-0.39
Slc25a1	Tricarboxylate Transport Protein (Citrate Transport Protein) (Solute Carrier Family 25 Member 1)	-1.29	-1.13	-0.36
Tmsb4x	Thymosin Beta-4	-1.28	-1.13	-0.36
Golph3l	Golgi Phosphoprotein 3-Like	-1.28	-1.16	-0.35
Ddx1	ATP-Dependent RNA Helicase DDX1	-1.28	-1.10	-0.35
Ca3	Carbonic Anhydrase 3	-1.28	-1.11	-0.35
S100a4	Protein S100-A4 (Metastatin) (S100 Calcium-Binding Protein A4)	-1.28	-1.07	-0.35
Fth1	Ferritin Heavy Chain	-1.27	-1.11	-0.35
Uba2	SUMO-Activating Enzyme Subunit 2 (Ubiquitin-Like Modifier-Activating Enzyme 2)	-1.27	-1.10	-0.35
Snrnp40	U5 Small Nuclear Ribonucleoprotein 40 kDa Protein	-1.27	-1.15	-0.35

Ptgs1	Prostaglandin G/H Synthase 1 (EC 1.14.99.1) (Cyclooxygenase-1) (COX-1)	-1.27	-1.10	-0.34
Prdx5	Peroxiredoxin-5 Mitochondrial	-1.27	-0.98	-0.34
Tmem35a	Transmembrane Protein 35A (Peroxisomal Membrane Protein 52) (PMP52)	-1.26	-1.12	-0.34
Erlin2	Erlin-2 (Endoplasmic Reticulum Lipid Raft-Associated Protein 2)	-1.26	-1.11	-0.33
S100a10	Protein S100-A10 (Calpactin I Light Chain) (Calpactin-1 Light Chain) (S100 Calcium-Binding Protein A10)	-1.26	-0.93	-0.33
Myl1	Myosin Light Chain 1/3, Skeletal Muscle	-1.26	-1.02	-0.33
Snx9	Sorting Nexin-9	-1.25	-0.99	-0.32
Xdh	Xanthine Dehydrogenase / Oxidase	-1.24	-0.81	-0.31
Fyn	Tyrosine Protein Kinase Fyn	-1.23	-1.02	-0.30
Igf2bp2	Insulin-Like Growth Factor 2 mRNA-Binding Protein	-1.23	-0.91	-0.30
Rgs10	Regulator of G-Protein Signalling 10	-1.23	-0.83	-0.30
Rhot1	Mitochondrial Rho GTPase 1 (MIRO-1)	-1.23	-0.81	-0.30
Tpm4	Tropomyosin Alpha 4 Chain	-1.23	-0.88	-0.29
Vps33b	Vascular Protein Sorting-Associated Protein 33B	-1.22	-0.90	-0.29
Ptgis	Prostacyclin Synthase	-1.22	-0.95	-0.29
Ndrg4	Protein NDRG4	-1.22	-0.96	-0.29
Fhl2	Four and A Half LIM Domains Protein 2	-1.22	-0.88	-0.28
Tagln2	Transgelin 2	-1.22	-0.86	-0.28
Copa	Coatomer Subunit Alpha (Alpha-COP)	-1.22	-0.93	-0.28
Mybphl	Myosin Binding Protein H-Like	-1.22	-0.88	-0.28

Pgp	Glycerol-3-Phosphate Phosphatase	-1.21	-0.87	-0.28
Nme2	Nucleoside Diphosphate Kinase B	-1.21	-0.85	-0.28
Cpa3	Mast Cell Carboxypeptidase 3	-1.21	-0.88	-0.27
Fnta	Protein Farnesyltransferase / Geranylgeranyl transferase	-1.21	-0.96	-0.27
Lclat1	Lysocardiolipin Acyltransferase 1	-1.20	-0.79	-0.27
Csnk1e	Casein Kinase I Isoform Epsilon	-1.20	-068	-0.26

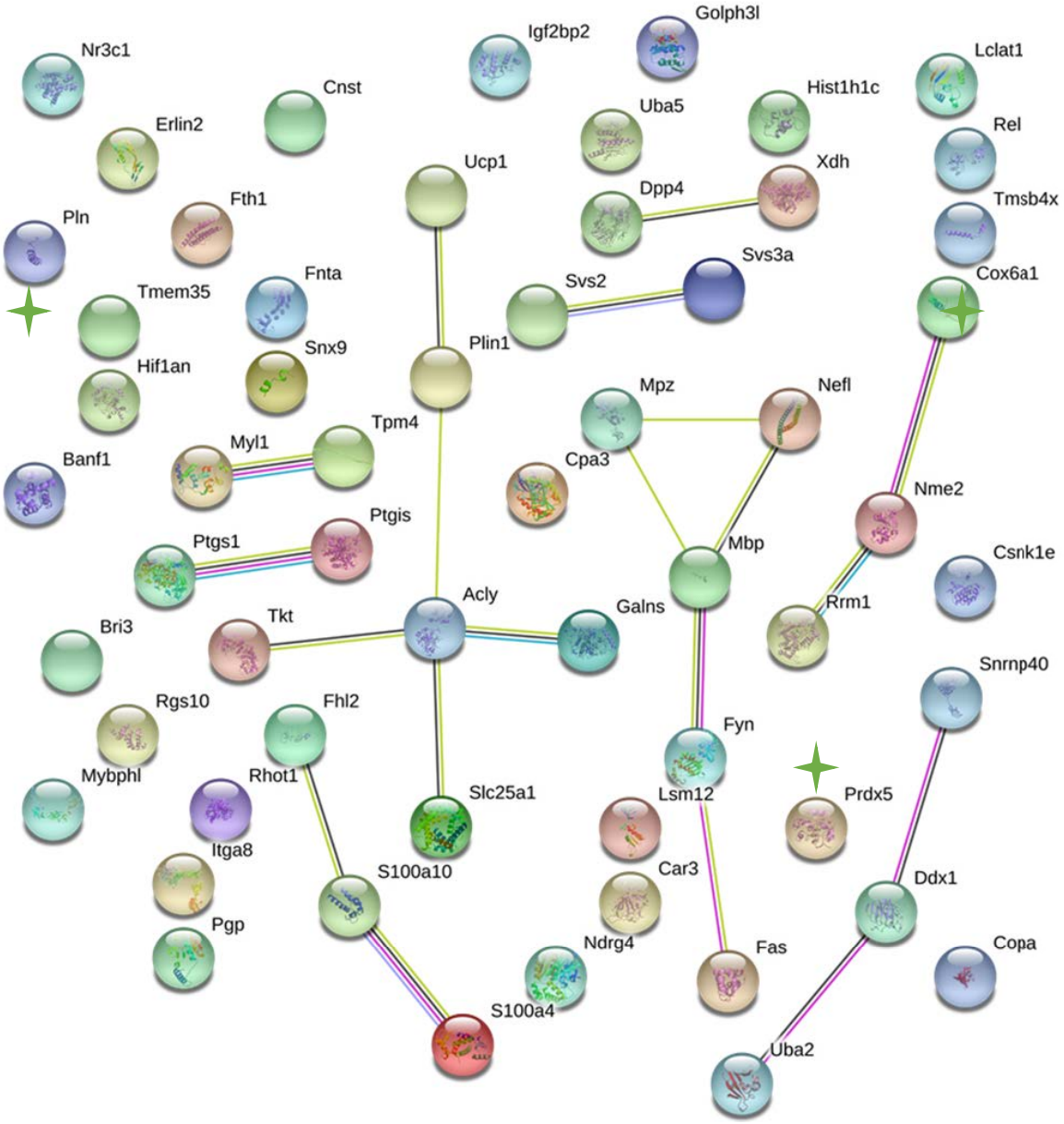


Figure 4.8: Network nodes represent proteins, which significantly downregulated in FXYD1 KO mice's heart

Table 4.4: The 25 most relevant pathways related to the proteins which significantly decreased in FXYD1 KO mice sorted by p-value

Pathway name	P-Value
Nuclear Receptor transcription pathway	0.0001
ChREBP activates metabolic gene expression	0.0044
NR1H2 & NR1H3 regulate gene expression linked to triglyceride lipolysis in adipose	0.0106
MPS IV - Morquio syndrome A	0.0450
Signalling by plasma membrane FGFR1 fusions	0.0739
The fatty acid cycling model	0.1155
COX reactions	0.1290
Autointegration results in viral DNA circles	0.1423
Insulin effects increased synthesis of Xylulose-5-Phosphate	0.1423
Integration of viral DNA into host genomic DNA	0.1423
The proton buffering model	0.1554
Striated Muscle Contraction	0.1577
Sterols are 12-hydroxylated by CYP8B1	0.181
Reuptake of GABA	0.181
Synthesis of dolichyl-phosphate mannose	0.181
Regulation of gene expression in endocrine committed (NEUROG3+) progenitor cells	0.193
2-LTR circle formation	0.193
APOBEC3G mediated resistance to HIV-1 infection	0.206
AKT-mediated inactivation of FOXO1A	0.218
Pre-NOTCH Processing in the Endoplasmic Reticulum	0.218
Scavenging by Class H Receptors	0.218
Synthesis of dolichyl-phosphate-glucose	0.23
Reversible hydration of carbon dioxide	0.23
Apoptosis induced DNA fragmentation	0.23
Mitochondrial Uncoupling	0.241

$P<0.05$ is significant

4.3.3.2. Caveolae Subfractions Four and Five:

Caveolae and its proteins components can be isolated from the cells by discontinuous sucrose gradient ultracentrifugation technique (Foster, De Hoog & Mann 2003), which was described in Chapter 2 Section 13. Caveolae are mostly concentrated in fraction 4-5 and 6 of sucrose gradient (Foster, De Hoog & Mann 2003; Wypijewski et al. 2015) and FXYD1, which is the centre of this study, is preponderantly located in the caveolae (Wypijewski et al. 2015). We therefore isolated caveolae by this approach and applied proteomics techniques to investigate the impact of FXYD1 KO caveolar protein abundance and post-transitional modifications.

FXYD1 KO resulted in substantial alteration in protein abundance in the caveolae. Table 4.5 lists the proteins that were significantly upregulated in caveolae subfractions 4 and 5 isolated from the FXYD1 KO heart mice compared to WT counterparts. One hundred thirty-nine (139) proteins were significantly increased in the FXYD1 KO mouse.

From these upregulated proteins in FXYD1 KO mice's heart caveolae, glutathione peroxidase 1 (Gpx1) acts as a protector of haemoglobin from an oxidative breakdown (Ho et al. 1997); while apolipoprotein A-I (Apoa-I) take part in the reverse transport of cholesterol from tissues to the liver for excretion by boosting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase (LCAT). Bothe Gpx1and ApoA-I are candidates for mediating altered sensitivity to cardiovascular pathology (Meng et al. 1995). Also, apolipoprotein C-I (Apoc-I) is another upregulated protein, which is the inhibitor of lipoprotein binding to the low-density lipoprotein (LDL) receptor and overexpression of that leads to an increase in the circulating triglycerides level (Berbee et al. 2005; Gautier et al. 2007; Westerterp et al. 2006). Moreover, ApoC-I binds to free fatty acids and reduces their intercellular esterification (Westerterp et al. 2007). In addition, Cavin 2, which is caveolae related protein and plays a role in shaping caveolae (Hansen et al. 2009) was upregulated in FXYD1 KO mice.

The listed proteins in Table 4.5 are involved in the number of different pathways, with the 25 most relevant pathways illustrated in Table 4.6.

Figure 4.9 shows the network nodes in caveolae subfractions 4 and 5 in FXYD1 KO mice' heart for the significantly increased proteins.

Table 4.5: List of proteins significantly up-regulated in caveolae subfraction 4 and 5 FXYD1 KO mice

Gene	Protein Name	n-Fold cha	z-Score	Log2 KO vs
Fgg	Fibrinogen gamma chain	3.52	6.20	1.81
Gpi	Glucose-6-phosphate isomerase	3.38	5.95	1.76
Fh	Fumarate hydratase, mitochondrial	2.98	4.87	1.54
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	2.74	4.77	1.45
Ech1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondria	2.56	4.49	1.36
Nipsnap2	Protein NipSnap homolog 2	2.45	4.44	1.3
Aldh5a1	Succinate-semialdehyde dehydrogenase, mitochondrial	2.41	5.68	1.27
Aldoa	Fructose-bisphosphate aldolase A	2.41	3.74	1.27
Mug1	Murinoglobulin-1	2.27	3.97	1.18
Aldob	Fructose-bisphosphate aldolase B (EC 4.1.2.13) (Aldolase 2 (Liver-type aldolase)	2.21	4.34	1.15
Dync1h1	Cytoplasmic dynein 1 heavy chain 1 (Cytoplasmic dynein h chain 1) (Dynein heavy chain, cytosolic)	2.11	3.15	1.08
Acadl	Long-chain specific acyl-CoA dehydrogenase, mitochondria	2.07	3.22	1.05
Rpl15	60S ribosomal protein L15	1.95	3.2	0.96
Phb	Prohibitin	1.94	3.08	0.96
Psmc3	26S proteasome regulatory subunit 6A	1.92	3	0.94
Tf	Serotransferrin	1.9	2.7	0.93
Pkm	Pyruvate kinase PKM	1.89	2.66	0.92
Atad3	ATPase family AAA domain-containing protein 3	1.87	2.9	0.9
Ogdh	2-oxoglutarate dehydrogenase, mitochondrial	1.86	2.62	0.9
Phb2	Prohibitin-2 (B-cell receptor-associated protein BAP37) (Re of estrogen receptor activity)	1.85	2.85	0.89
Slc4a1	Band 3 anion transport protein	1.85	4.42	0.89
Phb2	Prohibitin-2	1.85	2.85	0.89

Slc4a1	Band 3 anion transport protein (Anion exchange protein 1) ((Anion exchanger 1) (MEB3) (Solute carrier family 4 memb (CD antigen CD233)	1.85	4.42	0.89
Ucp1	Mitochondrial brown fat uncoupling protein 1	1.84	2.5	0.88
Dnaja2	DnaJ homolog subfamily A member 2	1.81	3.27	0.85
Ndufa5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su (Complex I subunit B13) (Complex I-13kD-B) (CI-13kD-B (NADH-ubiquinone oxidoreductase 13 kDa-B subunit)	1.81	4.24	0.85
Rpl18	60S ribosomal protein L18	1.8	3.25	0.85
Mief2	Mitochondrial dynamics protein MID49 (Mitochondrial dyn protein of 49 kDa homolog) (Mitochondrial elongation fact (Smith-Magenis syndrome chromosomal region candidate g protein homolog)	1.72	2.20	0.78
Cbr2	Carbonyl reductase [NADPH] 2	1.71	3.35	0.78
Aldh2	Aldehyde dehydrogenase, mitochondrial (EC 1.2.1.3) (AHD (ALDH class 2) (ALDH-E2) (ALDHI)	1.70	2.42	0.77
Cltc	Clathrin heavy chain 1	1.7	3.8	0.76
Ndufb8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub mitochondrial	1.7	2.9	0.77
Ndufs3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	1.7	2.83	0.77
Aldh2	Aldehyde dehydrogenase, mitochondrial	1.7	2.42	0.77
Mtnd5	NADH-ubiquinone oxidoreductase chain 5 (EC 7.1.1.2) (NA dehydrogenase subunit 5)	1.69	2.79	0.76
Fga	Fibrinogen alpha chain	1.66	3.74	0.74
Ces1d	Carboxylesterase 1D	1.65	3.1	0.72
Acad10	Acyl-CoA dehydrogenase family member 10	1.64	3.3	0.71
Chchd3	MICOS complex subunit Mic19	1.63	2.3	0.7
Ndufa2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su	1.63	2.6	0.71
Ndufa8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su	1.63	2.21	0.7
Vcp	Transitional endoplasmic reticulum ATPase	1.63	2.1	0.71
Chchd3	MICOS complex subunit Mic19 (Coiled-coil-helix-coiled-c helix domain-containing protein 3)	1.63	2.29	0.70

Dhrs4	Dehydrogenase/reductase SDR family member 4 (EC 1.1.1. (NADPH-dependent carbonyl reductase/NADP-retinol dehydrogenase) (CR) (PHCR) (NADPH-dependent retinol dehydrogenase/reductase) (NDRD) (mouNRDR) (Peroxisor short-chain alcohol dehydrogenase) (PSCD)	1.62	2.70	0.70
Atp5mc1	ATP synthase F(0) complex subunit C1, mitochondrial (AT synthase lipid-binding protein) (ATP synthase membrane su locus 1) (ATP synthase proteolipid P1) (ATPase protein 9) (ATPase subunit c)	1.62	2.94	0.70
Ndufb10	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub	1.62	3.4	0.7
Atp5mc1	ATP synthase F	1.62	2.94	0.7
Dhrs4	Dehydrogenase/reductase SDR family member 4	1.62	2.7	0.7
Hibadh	3-hydroxyisobutyrate dehydrogenase, mitochondrial	1.62	3.52	0.7
Oxct1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitocl (EC 2.8.3.5) (3-oxoacid CoA-transferase 1) (Somatic-type su CoA:3-oxoacid CoA-transferase) (SCOT-s)	1.61	1.99	0.69
Psmd6	26S proteasome non-ATPase regulatory subunit 6	1.61	1.98	0.68
Oxct1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitoch	1.61	1.99	0.69
Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitocho	1.6	1.86	0.64
Got2	Aspartate aminotransferase, mitochondrial	1.6	1.95	0.67
Psmc6	26S proteasome regulatory subunit 10B	1.59	2.1	0.67
Ndufa6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su	1.56	3.2	0.64
Got1	Aspartate aminotransferase, cytoplasmic	1.56	1.83	0.65
Fgb	Fibrinogen beta chain	1.55	2.7	0.63
Ndufb5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub mitochondrial	1.55	2.44	0.63
Uba1	Ubiquitin-like modifier-activating enzyme 1	1.54	1.96	0.63
Mtnd4	NADH-ubiquinone oxidoreductase chain 4	1.54	1.91	0.63
Immt	MICOS complex subunit Mic60	1.53	1.75	0.62
Klhl41	Kelch-like protein 41 (Kelch repeat and BTB domain-conta protein 10)	1.52	2.14	0.61
Mmut	Methylmalonyl-CoA mutase, mitochondrial	1.52	2.91	0.6
Rpl5	60S ribosomal protein L5	1.52	2.12	0.6

Klhl41	Kelch-like protein 41	1.52	2.13	0.61
Tln1	Talin-1	1.52	2.93	0.6
Nme2	Nucleoside diphosphate kinase B	1.5	2.02	0.59
Isoc1	Isochorismatase domain-containing protein 1	1.5	1.6	0.6
Rpl19	60S ribosomal protein L19	1.47	1.58	0.52
Apip	Methylthioribulose-1-phosphate dehydratase	1.47	1.76	0.56
Mrps36	28S ribosomal protein S36, mitochondrial	1.47	1.68	0.55
Sptan1	Spectrin alpha chain, non-erythrocytic 1	1.46	1.71	0.55
Me3	NADP-dependent malic enzyme, mitochondrial	1.46	1.96	0.55
Cavin2	Caveolae-associated protein 2	1.46	2.66	0.54
Ndufb3	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub	1.45	2	0.54
Ndufv1	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitoch	1.45	1.47	0.53
Sdha	Succinate dehydrogenase [ubiquinone] flavoprotein subunit mitochondrial	1.45	1.52	0.54
Gorasp2	Golgi reassembly-stacking protein 2	1.44	1.5	0.53
Fmo2	Dimethylaniline monooxygenase [N-oxide-forming] 2	1.43	1.56	0.52
Mccc2	Methylcrotonoyl-CoA carboxylase beta chain, mitochondria	1.43	2.25	0.52
Hsd17b10	3-hydroxyacyl-CoA dehydrogenase type-2	1.43	1.93	0.52
Ndufb11	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub mitochondrial	1.42	2.55	0.51
Hk2	Hexokinase-2	1.42	1.52	0.5
Myh3	Myosin-3	1.41	2.06	0.49
Ndufs2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	1.41	1.49	0.5
Myh6	Myosin-6	1.41	1.54	0.5
Vcl	Vinculin	1.41	1.81	0.5
Myh3 Myhs	Myosin-3 (Myosin heavy chain 3)	1.41	2.07	0.49
Slc25a12	Calcium-binding mitochondrial carrier protein Aralar1	1.4	1.35	0.48

Ndufs4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	1.4	1.88	0.47
Rpl8	60S ribosomal protein L8	1.4	1.95	0.45
Ca2	Carbonic anhydrase 2	1.39	1.5	0.48
Epb41	Protein 4.1	1.39	1.4	0.47
Psmc5	26S proteasome regulatory subunit 8	1.37	1.31	0.46
Slc25a13	Calcium-binding mitochondrial carrier protein Aralar2	1.36	1.2	0.44
Ndufv2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitoch	1.36	1.54	0.44
Copg2	Coatomer subunit gamma-2	1.36	1.27	0.44
Rpl34	60S ribosomal protein L34	1.36	1.86	0.44
Ndufb4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub	1.35	1.98	0.44
Apoc1	Apolipoprotein C-I	1.35	1.83	0.43
Tln2	Talin-2	1.35	1.27	0.42
Psmd12	26S proteasome non-ATPase regulatory subunit 12	1.33	1.2	0.41
Prkaa2	5'-AMP-activated protein kinase catalytic subunit alpha-2	1.33	1.05	0.41
Cryab	Alpha-crystallin B chain	1.32	1.45	0.4
Samm50	Sorting and assembly machinery component 50 homolog	1.32	1.1	0.42
Pygl	Glycogen phosphorylase, liver form	1.32	1.2	0.4
Eprs	Bifunctional glutamate/proline--tRNA ligase	1.32	1.15	0.4
Kyat3	Kynurenine--oxoglutarate transaminase 3	1.32	1.34	0.4
Cul2	Cullin-2	1.32	1.1	0.4
Aldh4a1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochond	1.31	1.03	0.38
Sptbn1	Spectrin beta chain, non-erythrocytic 1	1.3	1.53	0.38
Snca	Alpha-synuclein	1.3	1.76	0.36
Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial	1.3	1.27	0.38
Ckap4	Cytoskeleton-associated protein 4	1.3	1.2	0.38
Pygm	Glycogen phosphorylase, muscle form	1.3	0.1	0.37
Serpina1e	Alpha-1-antitrypsin 1-5	1.3	1.52	0.34

Ndufa3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su	1.3	1.22	0.36
Ca1	Carbonic anhydrase 1	1.28	1.24	0.35
Rpl13a	60S ribosomal protein L13a	1.27	1.43	0.34
Gdpd1	Lysophospholipase D GDPD1	1.27	0.93	0.34
Ndufa12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex su 12	1.27	1.21	0.34
Plin1	Perilipin-1	1.27	1.54	0.34
Ldhb	L-lactate dehydrogenase B chain	1.27	0.87	0.34
Mccc1	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochon	1.26	1	0.33
Cct2	T-complex protein 1 subunit beta	1.26	1.04	0.33
Rps2	40S ribosomal protein S2	1.25	1.14	0.32
Vwa8	von Willebrand factor A domain-containing protein 8	1.25	0.81	0.32
Psmc4	26S proteasome regulatory subunit 6B	1.23	0.83	0.31
Adhfe1	Hydroxy acid-oxoacid transhydrogenase, mitochondrial	1.23	0.1	0.3
Gpx1	Glutathione peroxidase 1	1.23	0.95	0.3
Cat	Catalase	1.22	0.94	0.3
Apoa1	Apolipoprotein A-I	1.22	0.67	0.3
Ndufb7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex sub	1.22	1.12	0.3
Rpl14	60S ribosomal protein L14	1.2	1	0.25

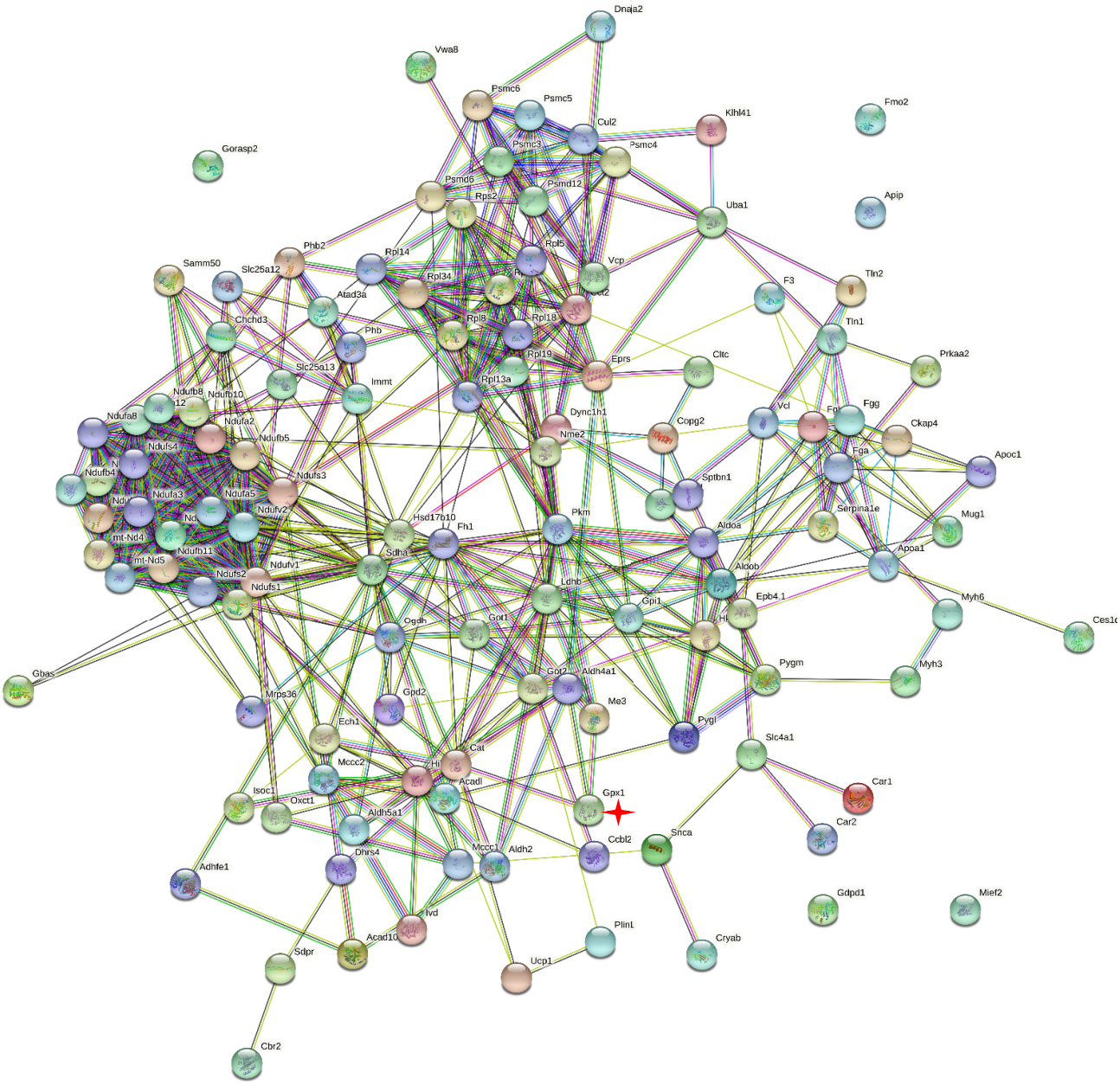


Figure 4.9: Network nodes represent proteins, which significantly up-regulated in Caveolae subfractions 4 and 5 in FXYD1 KO mice's heart

Table 4.6: The 25 most relevant pathways sorted by p-value caveolae (upregulate subfractions 4 and 5)

	Pathway name	P-Value
1	Complex I biogenesis	5.83E-09
2	Respiratory electron transport	1.28E-04
3	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	7.27E-04
4	The citric acid (TCA) cycle and respiratory electron transport	0.00935475
5	Selenocysteine synthesis	0.012105254
6	Hh mutants that do not undergo autocatalytic processing are degraded by ERAD	0.021121828
7	Formation of a pool of free 40S subunits	0.023294436
8	Eukaryotic Translation Termination	0.025901359
9	Hh mutants abrogate ligand secretion	0.028165751
10	Cross-presentation of soluble exogenous antigens (endosomes)	0.029169917
11	SCF-beta-TrCP mediated degradation of Emi1	0.033966827
12	SRP-dependent co-translational protein targeting to membrane	0.036667465
13	Regulation of ornithine decarboxylase (ODC)	0.039246016
14	Vif-mediated degradation of APOBEC3G	0.039246016
15	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	0.04024027
16	L13a-mediated translational silencing of Ceruloplasmin expression	0.042113258
17	Regulation of expression of SLITs and ROBOs	0.056754064
18	Mitochondrial protein import	0.061441769
19	Auto degradation of Cdh1 by Cdh1:APC/C	0.061662012
20	Erythrocytes take up oxygen and release carbon dioxide	0.063872251
21	Peptide chain elongation	0.064161343
22	CDT1 association with the CDC6:ORC:origin complex	0.06921338
23	NR1H2 & NR1H3 regulate gene expression linked to triglyceride lipolysis in adipose	0.070226194
24	Defective HLCS causes multiple carboxylase deficiency	0.070226194
25	Viral mRNA Translation	0.07199421

$P<0.05$ is significant

Some of the pathways listed in Table 4.6 directly or indirectly implicate in electron transportation including; complex I (NADH-ubiquinone oxidoreductase), an enzyme involved in ATP generation via oxidative phosphorylation which utilises NADH to pump the protons out of the mitochondrial matrix (McKenzie & Ryan 2010). Other pathways such as “respiratory electron transport”, “Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins” and “The citric acid (TCA) cycle and respiratory electron transport” are involved in reduction or oxidation of NADH (Martinez-Reyes & Chandel 2020; Schertl & Braun 2014).

Knock out of FXYD1 was associated with significant downregulation of 59 proteins in caveolae subfractions 4 and 5 (Table 4.7). Glutaredoxin-1 (GLRX1) is among the downregulated proteins (1.29-fold) which plays a key role in cellular protection against oxidative stress via its glutathione-disulphide oxidoreductase activity (requiring the presence of NADPH and glutathione reductase) reducing lower molecular weight disulphides and proteins (Reynaert, Ckless, et al. 2006; Reynaert, van der Vliet, et al. 2006). The cardioprotective function of GLRX1 has been shown (Malik et al. 2008), so this enzyme's significant downregulation may increase CVD risk. Also, Caveolin 3 (Cav3), which is one of the core structural components required for caveolae formation (Parton 2018), was significantly downregulated (1.27 fold). Annexin A1, which is a well-known anti-inflammatory protein, is another protein that significantly downregulated (1.32). The crucial role of this protein in inflammation (D'Acquisto et al. 2007; Hannon et al. 2003) and wound healing has been demonstrated (Leoni et al. 2015).

Table 4.8 reports the 25 most relevant pathways sorted by p-value for proteins which were significantly downregulated in caveolae subfractions 4 and 5.

Figure 4.10 shows the network nodes in caveolae subfractions 4 and 5 in FXYD1 KO mice' heart for the significantly decreased proteins.

Table 4.7: List of proteins significantly downregulated in caveolae subfraction 4 and 5 FXYD1 KO mice

Gene	Protein Name	n-Fold changes	z-Score	Log2 KO vs WT
Clic4	Chloride intracellular channel protein 4	-2.11	-1.32	-0.40
Hist2	Histone H2B type 2-B (H2b 616)	-2.08	-1.32	-0.40
Ank1	Ankyrin-1 (ANK-1) (Erythrocyte ankyrin)	-1.89	-1.37	-0.45
Sdhc	Succinate dehydrogenase cytochrome	-1.81	-1.26	-0.34
Glrx-1	Glutaredoxin-1 (Thioltransferase-1) (TTase-1)	-1.66	-1.29	-0.36
Cox7c	Cytochrome c oxidase subunit 7C,	-1.66	-1.24	-0.31

Cotl1	Coactosin-like protein	-1.62	-1.23	-0.30
Ppt1	Palmitoyl-protein thioesterase 1	-1.60	-1.23	-0.30
Tppp3	Tubulin polymerization-promoting protein family member 3	-1.60	-1.27	-0.34
Bcap29	B-cell receptor-associated protein 29	-1.58	-1.29	-0.37
Vdac1	Voltage-dependent anion-selective channel protein 1	-1.57	-1.30	-0.38
Ldhd	Probable D-lactate dehydrogenase,	-1.49	-1.29	-0.37
Arf6	ADP-ribosylation factor 6	-1.48	-1.27	-0.34
Eif4g2	Eukaryotic translation initiation factor 4 gamma 2)	-1.45	-1.27	-0.35
Ywhaq	14-3-3 protein theta (14-3-3 protein tau)	-1.45	-1.29	-0.36
Krt10	Keratin, type I cytoskeletal 10 (56 kDa cytokeratin)	-1.44	-1.25	-0.32
Fabp5	Fatty acid-binding protein 5 (Epidermal-type fatty acid-binding protein)	-1.41	-1.24	-0.31
Hnmt	Histamine N-methyltransferase (HMT)	-1.40	-1.25	-0.32
Cox7	Cytochrome c oxidase subunit 7A2,	-1.35	-1.19	-0.25
Crip2	Cysteine-rich protein 2 (CRP-2) (Heart LIM protein)	-1.34	-1.22	-0.29
Cox7a1	Cytochrome c oxidase subunit 7A1,	-1.33	-1.18	-0.24
Myl2	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	-1.32	-1.25	-0.32
Vdac3	Voltage-dependent anion-selective channel protein 3 (	-1.32	-1.25	-0.32

Anxa1	Annexin A1 (Annexin I) (Phospholipase A2 inhibitory protein) (p35)	-1.32	-1.20	-0.26
Cmbl	Carboxymethylenebutenolidase homolog (EC 3.1.)	-1.31	-1.20	-0.26
Dhodh	Dihydroorotate dehydrogenase (quinone),	-1.30	-1.18	-0.23
Mmab	Corrinoid adenosyltransferase (EC 2.5.1.17)	-1.30	-1.23	-0.30
Copb1	Coatomer subunit beta (Beta-coat protein) (Beta-COP)	-1.29	-1.23	-0.30
Fis1	Mitochondrial fission 1 protein (FIS1 homolog)	-1.28	-1.17	-0.22
Asah1	Acid ceramidase (AC) (ACDase) (Acid CDase) (EC 3.5.1.23)	-1.28	-1.20	-0.26
Khsrp	Far upstream element-binding protein 2	-1.27	-1.20	-0.26
Aamdc	Mth938 domain-containing protein (LI2)	-1.26	-1.19	-0.26
Dtymk	Thymidylate kinase (EC 2.7.4.9) (dTMP kinase)	-1.24	-1.23	-0.30
Grpel1	GrpE protein homolog 1, mitochondrial (Mt-GrpE#1)	-1.23	-1.17	-0.22
Cox5b	Cytochrome c oxidase subunit 5B,	-1.23	-1.23	-0.29
	Protein C19orf12 homolog	-1.23	-1.22	-0.28
Atl2	Atlastin-2 (EC 3.6.5.-) (ADP-ribosylation factor-like protein 6-interacting protein 2)	-1.20	-1.19	-0.25
Tmem43	Transmembrane protein 43 (Protein LUMA)	-1.20	-1.20	-0.26
Gmps	GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2	-1.20	-1.20	-0.27

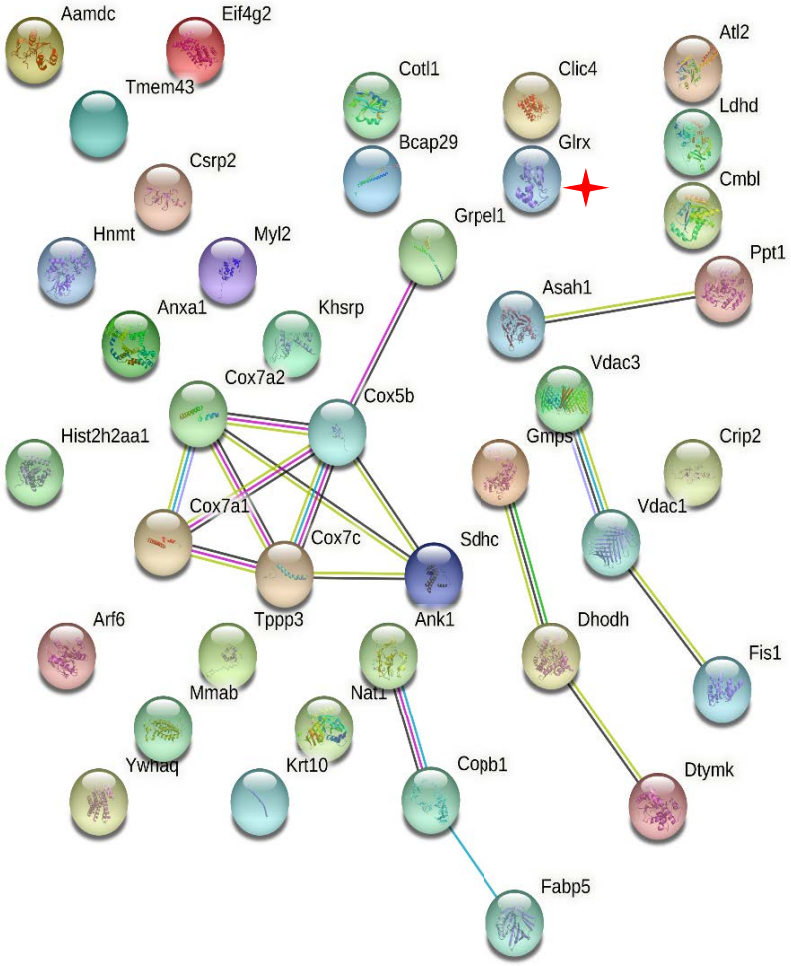


Figure 4.10: Network nodes represent proteins, which significantly downregulated in Caveolae subfractions 4 and 5 in FXYD1 KO mice's heart

Table 4.8: The 25 most relevant pathways sorted by p-value, (downregulated in caveolae subfractions 4 and 5)

Pathway name	P-Value
Triglyceride catabolism	0.111886704
Formation of the Editosome	0.121458627
Interleukin-33 signalling	0.121458627
mRNA Editing: C to U Conversion	0.165818313
Activation, translocation, and oligomerization of BAX	0.2079449
Interleukin-36 pathway	0.2079449
mRNA Editing	0.2079449
Mitochondrial protein import	0.228206028
MET receptor recycling	0.247950466
Hypusine synthesis from eIF5A-lysine	0.267191348
Interleukin-21 signalling	0.267191348
Sterols are 12-hydroxylated by CYP8B1	0.285941479
TP53 Regulates Transcription of Genes Involved in G2 Cell Cycle Arrest	0.285941479
Chk1/Chk2(Cds1) mediated inactivation of Cyclin B: Cdk1 complex	0.304213335
Other semaphorin interactions	0.356279347
Triglyceride metabolism	0.371497268
Cristae formation	0.372756683
Regulation of localization of FOXO transcription factors	0.419708384
Activation of BAD and translocation to mitochondria	0.44904621
Formation of ATP by chemiosmotic coupling	0.44904621
Presynaptic function of Kainate receptors	0.44904621
Receptor-type tyrosine-protein phosphatases	0.44904621
Formation of the cornified envelope	0.459690298
G beta: gamma signalling through CDC42	0.463155847
Growth hormone receptor signalling	0.463155847

P< 0.05 is significant

Due to the lack of experimental evidence for the listed pathways in Table 4.8, those pathways which are relevant to downregulated proteins in FXYD1 KO mice, computationally were inferred from an event demonstrated in another species by Reactome online software. At this stage, there is no firm evidence that shows those pathways are part of biological systems in mice.

4.3.3.3. Caveolae Subfractions Six:

It has been shown that the isolated caveolae from the heart lysates using discontinuous sucrose gradient ultracentrifugation techniques are mostly concentrated in fractions 4, 5 and 6 (Lisanti et al. 1994; Sargiacomo et al. 1993), therefore, as with fractions four and five quality and quantity of proteins in fraction 6 in both WT and FXYD1 KO mice were determined and compared.

The results showed that 43 proteins significantly up-regulated (Table 4.9), and 31 proteins were significantly downregulated (Table 4.10) in FXYD1 KO mice. Table 4.11 shows the 25 most relevant pathways sorted by p-value for proteins that were significantly up-regulated in caveolae subfraction 6 and Figure 4.11 shows the network nodes represent proteins, which were significantly up-regulated caveolae subfraction 6 in FXYD1 KO mice' heart.

Table 4.10 shows the 25 most relevant and significant pathways sorted by p-value for proteins significantly downregulated in caveolae subfraction 6.

Table 4.9: List of proteins significantly up-regulated in caveolae subfraction 6 FXYD1 KO mice

Gene	Protein Name	N-fold changes	z-Score	Log2 KO vs WT
Dlst	Dihydrolipoyllysine-residue succinyl transferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	3.18	1.67	10.51
Ugp2	UTP--glucose-1-phosphate uridylyltransferase	2.96	1.57	10.1
Fasn	Fatty acid synthase	2.71	1.44	7.85
Gss	Glutathione synthetase	1.84	0.88	4.84
Vcp	Transitional endoplasmic reticulum ATPase	1.68	0.74	4.6
Cat	Catalase	1.66		
Hba	Haemoglobin subunit alpha	1.62	0.7	4.07
Hbb-b1	Haemoglobin subunit beta-1	1.57	0.65	3.78
Phb	Prohibitin	1.54	0.62	3.76

Phb2	Prohibitin-2	1.54	0.63	3.72
Camk2d	Calcium/calmodulin-dependent protein kinase type II subunit delta	1.54	0.62	3.37
Cav1	Caveolin-1	1.53	0.61	3.92
Flot2	Flotillin-2	1.51	0.6	3.14
Ca2	Carbonic anhydrase 2	1.5	0.58	3.56
Nutm1	NUT family member 1	1.48		
Ighg1	Ig gamma-1 chain C region, membrane-bound form	1.46	0.54	2.86
Slc4a1	Band 3 anion transport protein	1.46	0.54	3.17
Rab5c	Ras-related protein Rab-5C	1.43	0.52	2.78
Ca1	Carbonic anhydrase 1	1.41	0.5	3.06
Sugt1	Protein SGT1 homolog	1.41	0.5	2.53
Rpl4	60S ribosomal protein L4	1.4	0.49	2.56
Lipe	Hormone-sensitive lipase	1.37	0.46	2.54
Plin1	Perilipin-1	1.36	0.44	2.41
Kif5b	Kinesin-1 heavy chain	1.36	0.45	2.41
Vta1	Vascular protein sorting-associated protein VTA1 homolog	1.35	0.43	2.22
Cfb	Complement factor B	1.34	0.54	2.85
Ndufa13	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	1.34	0.42	2.67
Me3	NADP-dependent malic enzyme, mitochondrial	1.34	0.42	2.63
Cbr2	Carbonyl reductase [NADPH] 2	1.34	0.42	2.6
Ca3	Carbonic anhydrase 3	1.33	0.41	2.6
Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9	1.3	0.38	2.31

Ndufa2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	1.3	0.38	2.4
Ndufv1	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	1.27	0.34	2.08
Echdc2	Enoyl-CoA hydratase domain-containing protein 2, mitochondrial	1.25	0.32	1.66
Ryr2	Ryanodine receptor 2	1.24	0.31	1.85
Ndufs2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	1.22	0.29	1.77
Mdp1	Magnesium-dependent phosphatase 1	1.22	0.28	1.5
Ndufb7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	1.21	0.27	1.67
Samm50	Sorting and assembly machinery component 50 homolog	1.2	0.26	1.54
Ndufs3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	1.19	0.25	1.55
Pak2	Serine/threonine-protein kinase PAK 2	1.19	0.25	1.33
Dync1li1	Cytoplasmic dynein 1 light intermediate chain 1	1.17	0.23	1.42
Fn3k	Fructosamine-3-kinase	1.1	0.14	0.68

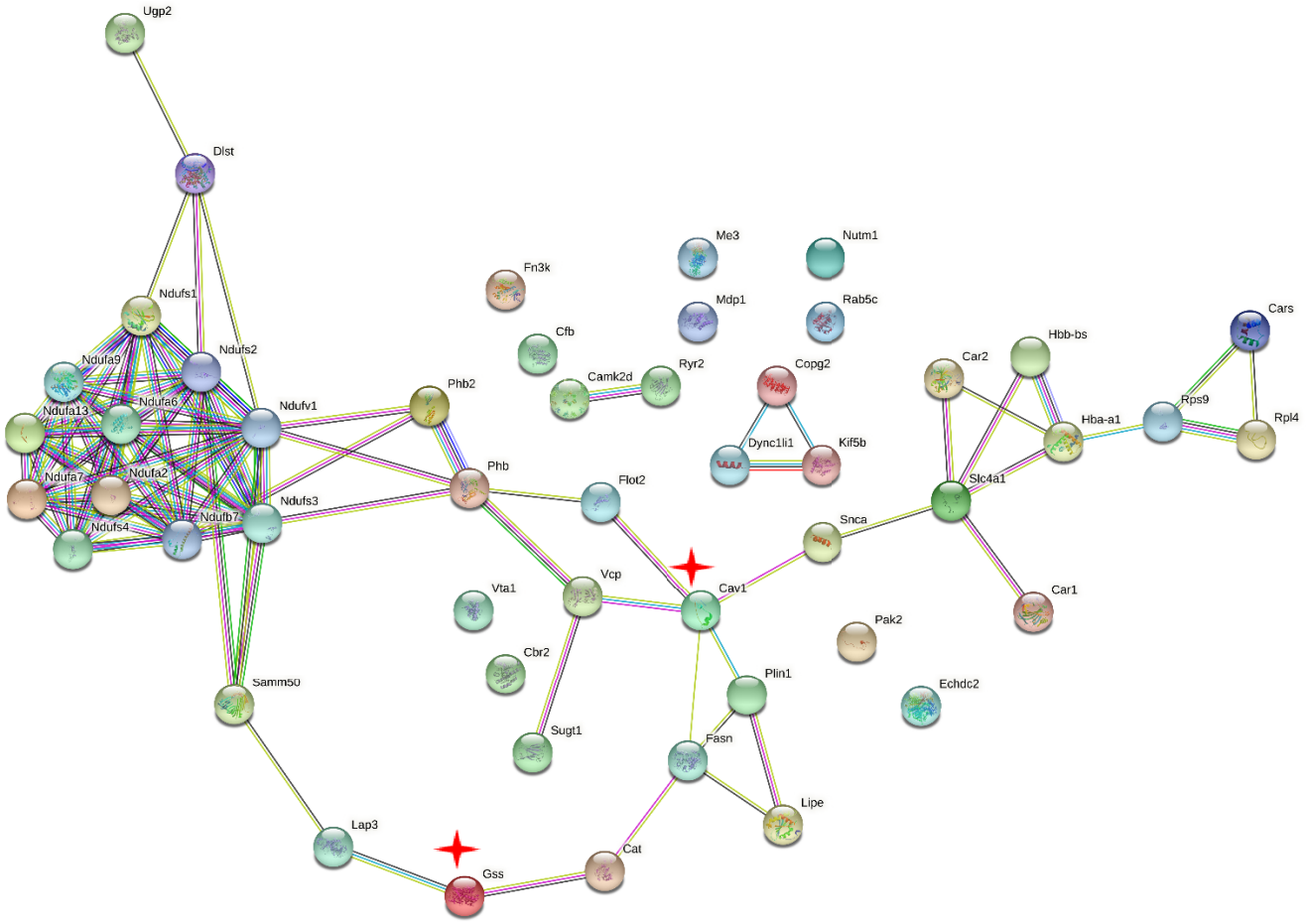


Figure 4.11: Network nodes represent proteins, which significantly up-regulated in caveolae subfraction 6 in FXYD1 KO mice's heart

One of the important parts of the defence system against oxidative stress and free radicals in the eukaryotic organisms is the tripeptide glutathione (GSH) (L-g-glutamyl-L-cysteinyl glycine). GSH is synthesised in a two-step procedure, which in the final step is catalysed by glutathione synthetase (GSS) (Njalsson 2005; Zitka et al. 2012). The proteomics results revealed that GSS is one of the proteins whose expression was increased by 1.84-fold in the FXYD1 KO mice's heart tissue in caveolae subfraction 6. Also, expression of cavolin1 (Cav1), which is a caveolae's core structural protein (Parton 2018), increased in FXYD1 KO mice by 1.53-fold.

Table 4.10: The 25 most relevant and significant pathways sorted by p-value, up-regulated in caveolae subfraction 6

	Pathway name	P-Value
1	Complex I biogenesis	9.44E-07
2	Respiratory electron transport	8.02E-05
3	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	8.66E-04
4	Erythrocytes take up oxygen and release carbon dioxide	0.001114576
5	O2/CO2 exchange in erythrocytes	0.004093888
6	Reversible hydration of carbon dioxide	0.01219874
7	The citric acid (TCA) cycle and respiratory electron transport	0.020394347
8	Processing of SMDT1	0.081381795
9	RAF activation	0.142968968
10	Mitochondrial calcium ion transport	0.162713431
11	Alternative complement activation	0.176281541
12	Activation of C3 and C5	0.176281541
13	ChREBP activates metabolic gene expression	0.176281541
14	HSF1-dependent transactivation	0.192266662
15	GRB7 events in ERBB2 signalling	0.198795735
16	Unblocking of NMDA receptors, glutamate binding and activation	0.232587556
17	HSF1 activation	0.262722988
18	RHO GTPases activate KTN1	0.282881051
19	Interferon-gamma signalling	0.283554554
20	Synthesis of dolichyl-phosphate mannose	0.302489553
21	Formation of the active cofactor, UDP-glucuronate	0.302489553
22	Josephin domain DUBs	0.302489553
23	Bicarbonate transporters	0.321563434
24	Type I hemidesmosome assembly	0.321563434
25	NOSTRIN mediated eNOS trafficking	0.358165077

P< 0.05 is significant

As with up-regulated protein in caveolae subfractions 4 and 5, some of the relevant pathways to up-regulated proteins in subfraction 6 involve in electron transportation such as “complex I (NADH-ubiquinone oxidoreductase)”, “respiratory electron transport”, “respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins” and “the citric acid (TCA) cycle and respiratory electron transport”, which were all discussed previously. Noteworthy that these pathways have been computationally inferred from an event that has been demonstrated in another species by Reactome online software.

Table 4.11: List of proteins significantly downregulated in caveolae subfraction 6 FXYD1 KO mice

Gene	Protein Name	n-Fold changes	z-Score	Log2 KO vs WT
Mb	Myoglobin	-1.54	-0.62	-3.67
Cox7b	Cytochrome c oxidase subunit 7B,	-1.48	-0.57	-3.41
Cav3	Caveolin-3 (M-caveolin)	-1.41	-0.50	-3.14
Cox7a1 Cox7a Cox7ah	Cytochrome c oxidase subunit 7A1, 7A, 7 Ah	1.41	0.49	-3.13
Lcp1	Plastin-2 (65 kDa macrophage protein))	-1.38	-0.46	-2.85
Lum	Lumican (Keratan sulfate proteoglycan	-1.40	-0.49	-2.83
Ppa2	Inorganic pyrophosphatase 2, mitochondrial (	-1.39	-0.47	-2.72
Asah1	Acid ceramidase (AC)	-1.36	-0.44	-2.63
Phpt1	14 kDa phosphohistidine phosphatase	-1.36	-0.44	-2.56
Cox5a	Cytochrome c oxidase subunit 5A,	-1.27	-0.34	-2.24
Macrod1 Lrp16	ADP-ribose glycohydrolase	-1.26	-0.33	-2.12
Gga1	ADP-ribosylation factor-binding	-1.26	-0.34	-2.03
Glrx3	Glutaredoxin-3	-1.24	-0.31	-2.01
Stk26	Serine/threonine-protein kinase 26	-1.24	-0.31	-1.99
Tppp	Tubulin polymerization-promoting protein	-1.24	-0.31	-1.96
Nutf2 Ntf2	Nuclear transport factor 2 (NTF-2)	-1.23	-0.30	-1.95
Cox7c Cox7c1	Cytochrome c oxidase subunit 7C, mitochondrial (Cytochrome c)	-1.23	-0.29	-1.89
Myl2 Mylpc	Myosin regulatory light chain 2,	-1.23	-0.30	-1.88

	ventricular/cardiac muscle isoform			
Tardbp Tdp43	TAR DNA-binding protein 43 (TDP-43)	-1.22	-0.29	-1.83
Anp32a Anp32 Lanp	Acidic leucine-rich nuclear phosphoprotein 32 family member A	-1.22	-0.29	-1.83
Alb Alb-1 Alb1	Serum albumin	-1.23	-0.30	-1.81
Psme1	Proteasome activator complex subunit 1	-1.22	-0.28	-1.80
Capg	Macrophage-capping protein (Actin regulatory protein CAP-G)	-1.22	-0.29	-1.78
Cox6c	Cytochrome c oxidase subunit 6C (Cytochrome c oxidase)	-1.22	-0.28	-1.75
Atp5mpl Mp68	ATP synthase subunit ATP5MPL, mitochondrial	-1.21	-0.27	-1.73
Rmdn1 Fam82b	Regulator of microtubule dynamics protein 1	-1.20	-0.27	-1.72
Ywhab	14-3-3 protein beta/alpha (Protein kinase C inhibitor protein 1)	-1.21	-0.28	-1.71
Armc1	Armadillo repeat-containing protein 1	-1.21	-0.27	-1.70
Txn Txn1	Thioredoxin (Trx)	-1.21	-0.27	-1.70
Copa	Coatomer subunit alpha	-1.21	-0.28	-1.64
Cox4i1 Cox4 Cox4a	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial IV isoform 1)	-1.20	-0.27	-1.64

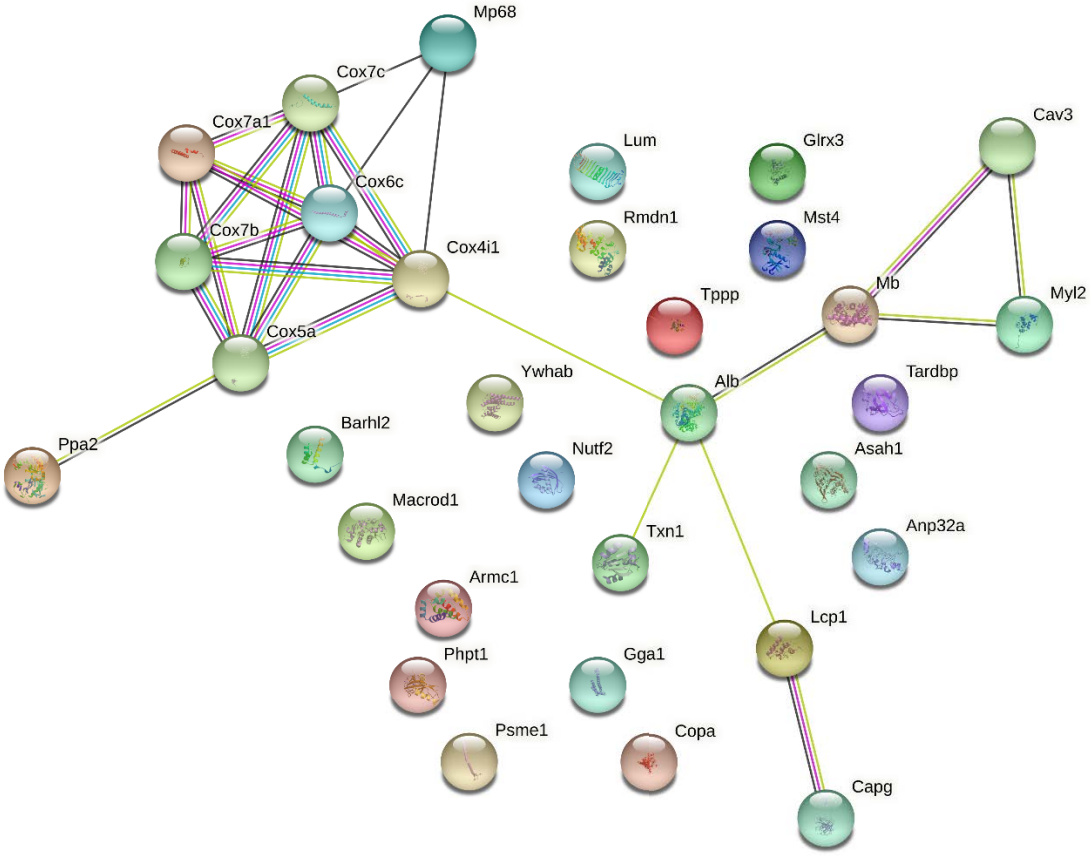


Figure 4.12: Network nodes represent proteins, which significantly downregulated in caveolae subfraction 6 in FXYD1 KO mice' heart

The caveolae subfraction 6 proteomics results showed that the absence of FXYD1 KO was associated with significant downregulation of Cav-3 in fractions 6 by 1.41-fold. Also, the results showed that Thioredoxin 1 (TXN1) was downregulated in FXYD1 KO mice heart by 1.21. In addition, the results showed that the expression of a few member of Cytochrome c oxidase family (Cox7a, Cox7b, Cox5a, Cox7c) was downregulated. These enzymes remove an electron from their substrate to an electron acceptor. The reaction is usually coupled with a reduction reaction.

Table 4.12: The 25 most relevant pathways sorted by p-value, for proteins which significantly downregulated in caveolae subfraction 6

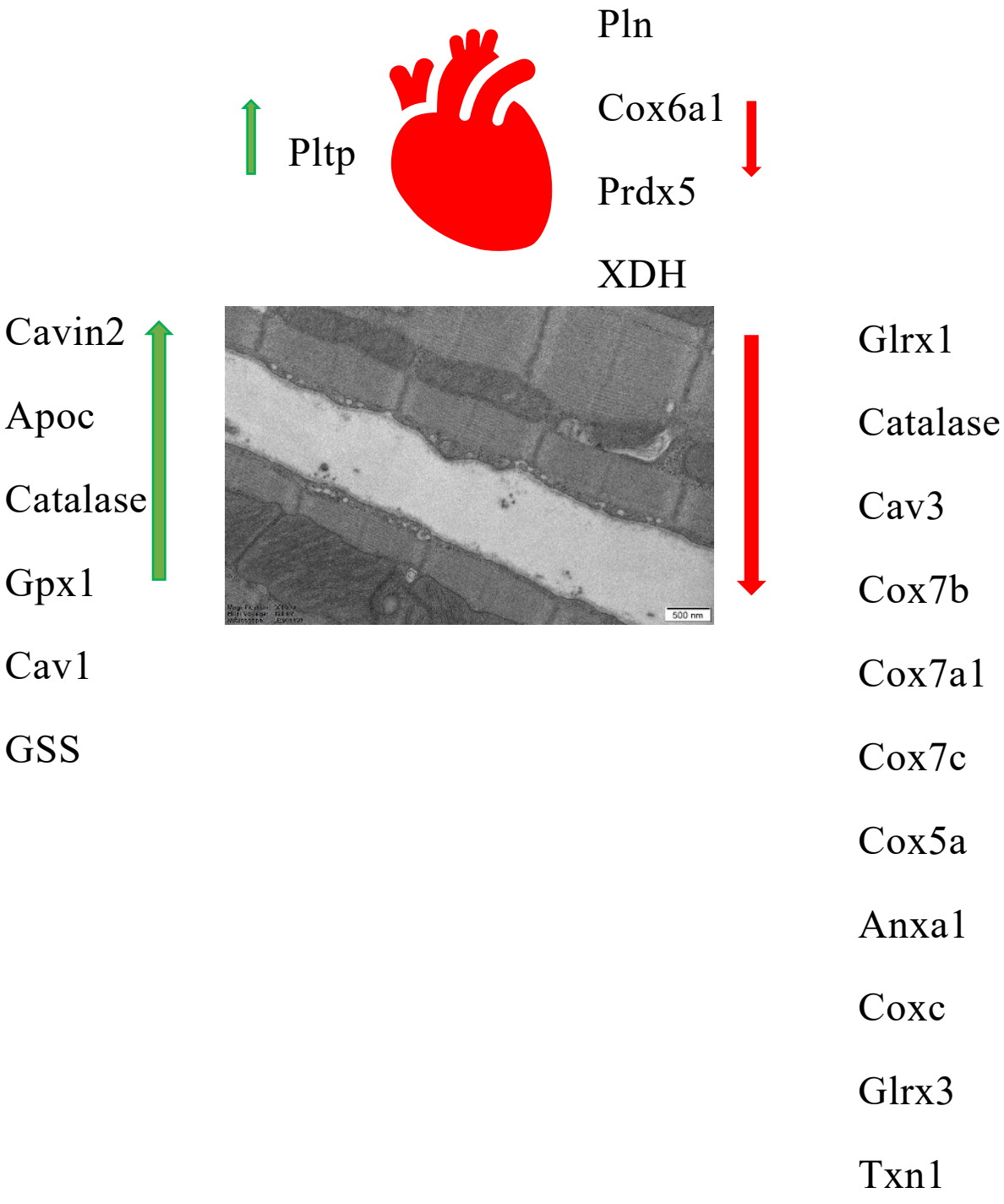
	Pathway name	P-Value
1	Interleukin-21 signalling	0.026216452
2	Interleukin-15 signalling	0.039349143
3	Interleukin-9 signalling	0.044176739
4	TP53 Regulates Metabolic Genes	0.056817669
5	Interleukin-20 family signalling	0.065451642
6	Respiratory electron transport	0.073713173
7	Interleukin-37 signalling	0.07964696
8	Interleukin-7 signalling	0.095743293
9	MET activates STAT3	0.098551234
10	Mitochondrial tRNA aminoacylation	0.098551234
11	Intracellular oxygen transport	0.098551234
12	Pyrophosphate hydrolysis	0.117068849
13	tRNA Aminoacylation	0.117068849
14	PTK6 Activates STAT3	0.117068849
15	Interleukin-10 signalling	0.13520768
16	Downstream signal transduction	0.164775874
17	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	0.171199332
18	Interleukin-23 signalling	0.204127142
19	Eukaryotic Translation Termination	0.2204847
20	Interleukin-35 Signalling	0.2204847
21	Sterols are 12-hydroxylated by CYP8B1	0.23650748
22	Protein repair	0.23650748
23	Interleukin-27 signalling	0.23650748
24	Interleukin-2 family signalling	0.239768005
25	Interleukin-21 signalling	0.026216452

$P<0.05$ is significant

The listed pathways in Table 4.12 mostly are involved in the immune system, cell metabolism and signal transduction.

Table 4.13 is the summary of all the proteins that were upregulated and downregulated proteins in the absence of FXYD1 in heart and caveolae of heart, which have direct or indirect impact on CVD or ROS.

Figure 4.13: Summary of proteins which up or downregulate in FXYD1 KO mice's heart and plasma membrane caveolae that directly or indirectly impact oxidative stress or caveolae structure and subsequently contribute to CVD



4.4. Discussion:

The role and function of caveolae and FXYD1 protein, which is one of the localised proteins in caveolae, on redox signalling have been reviewed. To study the impact of FXYD1 on the size, number, and protein components of caveolae, electron microscopy and proteomic approaches were utilised.

It has been shown that some stimuli have impacts on dynamic entities of caveolae (Bubb et al. 2017; Patel & Insel 2009). Tsutsumi (2008) showed the number of caveolae increases acutely in response to cardiac ischemia (Tsutsumi et al. 2008), interestingly our electron microscopy results showed that the number of caveolae in FXYD1 KO heart tissues was significantly increased. However, the results of diameter and circumference measurements showed that the diameter and the circumference of caveolae in FXYD1 KO mice's heart tissues were significantly decreased. Caveolin 3 (Cav-3), one of main caveolae proteins, is an essential caveolin isoform involved in caveolae morphogenesis (Rothberg et al. 1992; Tang et al. 1996; Way & Parton 1996). Our caveolae subfractions proteomics results showed that FXYD1 KO was associated with significant downregulation of Cav-3 in fractions 6 by 1.41 fold. Given the crucial function of Cav-3 in morphogenesis of caveolae, our observation that FXYD1 KO was associated with reduced caveolae size and downregulation of Cav-3 is of interest. It is suggestive that FXYD1 protein is playing an important role in Cav-3 dependent regulation of caveolae formation and or stability and structure. It may be a compensatory response of the myocardial tissue to increase the number of caveolae, as we report for the first time in response to FXYD1 KO.

In protein discovery part of this study, we first quantified the extracted proteins from heart tissues to determine whether FXYD1 gene had an impact upon the quantity of protein in heart tissues. The results of total protein quantification showed no significant differences between genotypes, which can be concluded that the absence of FXYD1 gene did not impact upon the total protein synthesis in heart tissues. Besides, after caveolae isolation using discontinuous sucrose gradient ultracentrifugation method, the protein quantification results did not show significant differences between WT and FXYD1 KO samples. As previously explained in the chapter 4 introduction, caveolae and its associated proteins mostly concentrated in the subfractions 4,5 and 6 of sucrose gradient. Due to low level of proteins in sections 4 and 5, these two subfractions were pooled to

increase the level of proteins that could be detected by liquid chromatograph and tandem mass spectrometer.

Proteomics techniques provided a complementary approach to examining FXYD1 KO's impact on caveolar structure and protein content. Table 4.1 shows the FXYD1 KO resulted in significant upregulation of Pltp, a protein which stimulates the development of atherosclerosis (Lie et al. 2004; van Haperen et al. 2000; Yang et al. 2003) and which has a close connection to the proteins that facilitates transferring a range of different lipid molecules, including diacylglycerol, phosphatidic acid, sphingomyelin, phosphatidylcholine, phosphatidylglycerol, cerebroside and phosphatidylethanolamine (Nishida et al. 1997; Rao et al. 1997).

As shown in Table 4.3, FXYD1 KO resulted in significant downregulation of Prdx5 in whole heart tissues, which acts as a cytoprotective antioxidant enzyme in inflammation (Knoops et al. 2016), and Pln that has an essential role in calcium homeostasis in the heart muscle (Koss & Kranias 1996). In addition, Cox6a1, which was downregulated 1.33-fold in the whole heart, is an enzyme that catalyses the electrons transportation from cytochrome *c* to oxygen, leading to the synthesis of ATP (Grossman & Lomax 1997). Downregulation of this enzyme may interrupt ATP production in the biological system, which may lead to interruption of activity of various pumps including Ca^{2+}/ATPase and heart contraction (Suga 1990). In addition, XDH which is involved in oxidative stress and cardiovascular disease (Berry & Hare 2004) significantly downregulated. The reduction of XDH can reduce the oxidative stress and risk of CVD.

In the absence of FXYD1 a large number of proteins in the caveolae subfractions significantly upregulated. As shown in the results section in caveolae subfractions 4 and 5 expression of Cavin 2, Apoc1, Gpx1 and Apoa1 were significantly increased (Table 4.5). Cavin 2, a caveolae structural protein (Hansen et al. 2009), plays key roles in regulating eNOS stability and activity in angiogenesis (Boopathy et al. 2017). Angiogenesis plays a fundamental role in wound healing (Adams & Alitalo 2007). During the term of this study, some of the mice developed wounds that led to culling the mouse in most cases. From the issue with wound healing and the role of Cavin 2 in angiogenesis, it can be inferred that significant upregulation of Cavin 2, which happened in FXYD1 absence, may disturb wound healing in FXYD1 KO mice. The role and function of Apoc1 and Apoa1 in lipoprotein binding to LDL and cholesterol transportation show the significance of these proteins in cholesterol handling (Gautier et al. 2007; Westerterp et

al. 2007) and their impact on the development of atherosclerosis. Gpx1, another upregulated protein in caveolae subfractions 4 and 5, is an antioxidant enzyme that counteracts oxidative stress (Meng et al. 2018). It should be considered that significant upregulating of a protein may lead to converse effect and cause damages to the biological system.

In the caveolae subfraction, 6 Cav-1 and GSS significantly increased (Table 4.9). It has been demonstrated that the expression of Cav1, which is one of the coat proteins of caveolae (Parton 2018), negatively correlated to eNOS and NO production (Ju et al. 1997; Michel et al. 1997; Razani et al. 2001). Significant upregulation of Cav-1 may lead to a significant reduction of NO, which increase the risk of CVD. Expression of GSS, an enzyme that catalyses GSH production (Njalsson 2005; Zitka et al. 2012), was significantly increased which may lead to increased production of GSH that would increase the GSH: GSSG ratio and thus boost the cell antioxidant defences (Carelli et al. 1997; Chai et al. 1994; Locigno & Castronovo 2001; Noctor & Foyer 1998; Townsend, Tew & Tapiero 2003).

The absence of the FXYD1 also leads to the significant downregulation of a number of key proteins in the caveolae subfractions of the heart. These included: the cardioprotective enzyme GLRX1 (Malik et al. 2008) and GLRX3, which plays as a vital negative regulator of cardiac hypertrophy and a positive inotropic regulator (Cha et al. 2008; Jeong et al. 2006; Jeong et al. 2008); and Txn1that have a role in the reversible S-nitrosylation of cysteine residues in target proteins, and in that way contributes to the response to intracellular NO (Andoh, Chiueh & Chock 2003; Andoh, Chock & Chiueh 2002; Ohashi et al. 2006). Besides, FXYD1 KO downregulated expression of proteins from the Cox family (Cox6a1, Cox7b, Cox7a1, Cox7c, Cox5a, Cox6c), which plays a role in electron transport and subsequently in oxidation and reduction systems in organisms.

In addition, the subfraction caveolae proteomics results showed Anxa1, which has vial role in inflammation and wound healing (D'Acquisto et al. 2007; Leoni et al. 2015), has significantly decreased.

In summary, we showed that the absence of FXYD1 in animal model has vital impacts on structure and protein contents of cardiomyocytes, which may lead to interruption of pathways that modulate the heart function and increases the risk of CVD.

This study has some limitations that overcoming those could improve our understanding of the role and function of FXYD1. The electron microscopic techniques are great tools for investigating the subcellular details of cells and their components in the samples used in this study. Despite the great advantages of the electron microscopic methods (SEM and TEM) that I used to visualise the caveolae, there are limitations with the methods that I applied. There is another component similar to caveolae in the plasma membrane, called clathrin-coated pits (Kirchhausen, Owen & Harrison 2014). These components are easily distinguished by their characteristic electron-dense outer coating; however discriminating between caveolae and similarly structured vesicles by morphological criteria alone is extremely difficult (von Ruhland et al. 2004). An immunocytochemical approach is a better technique to identify the caveolae in the samples to overcome this issue. However, due to instrument availability and other technical issues, the immunocytochemical analysis was not able to be performed.

In addition to electron microscopy limitation, the online proteomics analytical software limits the analysis of the collected data. The Reactome software that was used currently has limitation that is likely to be overcome with some datasets being obtained from pre-clinical models.

CHAPTER FIVE

The Impact of FXYD1 on development of Atherosclerosis

In this chapter, the role and function of the FXYD1 gene in the development of atherosclerosis, with regards to unstable plaques using a mouse model, were investigated.

5.1. Introduction:

Atherosclerosis is a progressive disease, characterised by the deposition of lipids and fibrous elements in the large and medium-sized arteries and is the most common pathological process that leads to cardiovascular diseases (Galkina & Ley 2009).

Atherosclerosis, which is a leading cause of death and disability globally (Murray & Lopez 2013), is a complicated disease of the arterial wall in which a large number of mediators have been implicated in lesion development. A broad range of factors including smoking, hypertension, diabetes mellitus, lack of exercise, male sex and genetics (Lim et al. 2012; Yusuf et al. 2004) contribute to the development of atherosclerosis, and there is firm evidence that hypercholesterolaemia is one of the key risk factors for the development and progression of atherosclerosis (Lu & Daugherty 2013). The accumulation of LDLs in the arterial intima, where the LDLs could be modified by oxidation or aggregation, has a crucial role in developing atherosclerosis (Steinberg & Witztum 2010). It has been shown that individuals with extremely low LDL typically do not develop clinically relevant atherosclerosis regardless of the presence of other risk factors (Steinberg, Glass & Witztum 2008). Additionally, the role and function of ROS in developing CVD have been demonstrated (Sugamura and Keaney 2011) as has the impact of the lipoprotein oxidation in the maturation of atherosclerosis (Steinberg & Witztum 2010). The starting step in developing an atherosclerotic lesion is commonly believed to be damage to the endothelium (Hadi, Carr & Al Suwaidi 2005). This monolayer of cells is indispensable for regulating vascular tone and the maintenance of vascular homeostasis and function (Tschudi et al. 1996).

Atherosclerosis plaques mainly form in parts of the arterial tree with low or oscillatory endothelial shear stress, which are located near branch areas and on inner curvatures of arteries and form atherosclerotic plaques (Wentzel et al. 2012). Atherosclerotic plaques are shaped by intimal thickening with excessive build-up of oxidised and non-oxidised low-density lipoprotein-derived cholesterol accompanied by inflammatory cell infiltration, smooth muscle proliferation, and extracellular matrix accumulation (Bentzon

et al. 2014; Stary et al. 1994). Some of these plaques, which are prone to rupture and called vulnerable plaques, are responsible for the majority of acute coronary cases (Falk, Shah & Fuster 1995). The vulnerable plaques are characterised by the presence of a large necrotic core covered by an inflamed thin fibrous cap (Schaar et al. 2004).

Previously, understanding of the relationship between endothelial shear stress and plaque formation relied on the autopsy materials (Moore et al. 1994); however, three-dimensional reconstruction techniques for coronary arteries *in vivo* (Slager et al. 2000) opened a new path in the investigation of the role of endothelial shear stress in atherosclerosis studies (Giannoglou et al. 2006). Despite all the advantages of the models mentioned above, lack of an appropriate mouse model that demonstrates plaque rupture and lesion features of vulnerable and unstable plaques was obvious (Finn et al. 2010). Chen et al. (2013) for the first time introduced tandem stenosis (TS) in the carotid arteries of ApoE KO mouse to overcome this issue (Chen et al. 2013). This model was applied to ApoE KO/FXYD1 KO and FXYD1 wild type mice and those mice were used for one part of this study.

The results of the proteomic study (chapter 4) showed the significant changes in the quantity of proteins such as phospholipid transfer protein (Pltp), which facilitates the transfer of a range of different lipid molecules, including diacylglycerol, phosphatidic acid, sphingomyelin, phosphatidylcholine, phosphatidylglycerol, cerebroside and phosphatidylethanolamine (Nishida et al. 1997; Rao et al. 1997). The protective role of FXYD proteins in protecting the ß1 subunit of Na^+-K^+ ATPase from oxidative inhibition has been studied and demonstrated (Bibert et al. 2011). The protective role of FXYD proteins in protecting the β1 subunit of Na^+-K^+ ATPase from oxidative S-glutathionylation and inhibition is clear (Bibert et al. 2011; Liu & Shapiro 2007) and this Na^+-K^+ ATPase/FXYD1 complex co-localises with eNOS in caveolae. In addition, we have postulated a functional interaction of FXYD1 with eNOS (Bubb, K. et al 2021 submitted paper Appendix1). Therefore, I hypothesised that FXYD1 might have a broader role beyond that of the Na^+-K^+ ATPase in the caveolae, such as impacting circulating lipid profile and circulating cytokines. This may result in a greater risk of atherosclerotic burden and plaque instability in mice lacking FXYD1.

This study aimed to investigate the role and function of FXYD1 in the formation and progression of atherosclerosis and the impact of this protein on stable and unstable atherosclerosis plaques. To achieve this aim, we have developed a unique mouse model

of global FXYD1KO on a background of atherosclerosis-prone (Tamminen et al. 1999) apolipoprotein E (ApoE) KO. We have measured inflammation, cholesterol handling and progression of aortic plaque formation.

5.2. **Method**:

5.2.1. Mouse Model:

The following mouse models were used in this study.

5.2.1.1. Development of a novel mouse model

All animal studies performed were approved by the Northern Sydney Local Health District Animal Ethics Committee (approval numbers RESP/17/96, RESP/17/56, RESP/17/88) in confirmation with the National Health and Medical Research Council of Australia's *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

In order to study atherosclerosis, standard laboratory mice are not suitable due to the resistance to plaque development (von Scheidt et al. 2017). We utilised a strain of mice that are susceptible to atherosclerosis, apolipoprotein knockout mice.

Despite many differences between humans and mice, the ApoE KO mice are a powerful experimental model for studying atherosclerosis (Piedrahita et al. 1992). For this part of the study, ApoE KO mice were used to determine the role and function of FXYD1 gene in an atherosclerosis mouse model. FXYD1 KO and wild type (WT) mice and their generation has been previously described (Jia et al., 2005). FXYD1 KO mice were interbred with ApoE KO mice to produce a colony of FXYD1 heterozygous/ ApoE KO mice, allowing the supply of ApoE KO/FXYD1 WT and ApoE KO/FXYD1 KO littermates. As all mice are ApoE KO, they will be referred to as FXYD1 WT and KO from this point on.

5.2.1.2. Development of atherosclerosis

At the age of 6-8 weeks, male and female mice were randomised to experimental groups and housed individually or in groups of up to 5 in standard laboratory conditions (0700–1900-hour (hr) light, 1900-0700hr dark) at a constant temperature (21°C) and humidity (40%). All mice were fed a high fat and high cholesterol diet (Cat#SF04-027, Specialty Feeds, WA, Australia) from week 6-8 of age for 16 weeks and food and tap water were available *ad libitum*.

5.2.1.3. Body Mass

At the age of 6-8 weeks, mice's body mass was recorded as day zero. Then the standard chow mixed with a high-fat chow (Cat#SF04-027, Specialty Feeds, WA, Australia) to the ratio of 50/50, was provided, to give the mice opportunity to adapt themselves to the new high-fat diet, after three days, the remaining of standard chow was removed and replaced

with the high-fat/cholesterol diet chow. Then after, every two-week, the mice were weighed, and the body masses were recorded.

5.2.1.4. Endpoint non-fasted blood glucose

After inducing anaesthesia, the tail was pricked, and the blood glucose was determined using a blood glucose monitoring system (See 2.7.1 for more details).

5.2.1.5. Metabolic Cage Measurements

Mice were placed in metabolic cages to measure food and water intake and urinary and faecal excretions over 24 hours (See 2.6 for more details). Data presented are an average from 3 x 24-hour periods.

5.2.1.6. Total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride

Anaesthesia was induced, then blood was collected and transferred to Eppendorf tubes. Subsequently, the samples were spun down, and plasma separated (See 2.8).

5.2.1.7. Circulating inflammatory markers

The collected plasma in 5.2.1.6 was used to determine the circulating inflammatory markers' level see chapter 2, section 4, for comprehensive details.

5.2.1.8. Plaque formation

5.2.1.8.1. Aorta collection

The mice were anesthetised, and the aortas were collected 16 weeks after commencing the high fat/high cholesterol diet. Subsequently, the collected aortas were immersed in formalin solution, neutral buffered (10% v/v) and stored at 4°C for further experiments. The details were explained in chapter 2, section 9.

5.2.1.8.2. Detection of plaques using oil red O

The 10% (v/v) formalin solution was decanted, and each aorta was washed with 1mL of 1X Phosphate Buffered Saline (PBS) (Product ID #:09-2051-100, Astral Scientific, Australia) twice each time 5 min on a rotor. The PBS was discarded, and 1mL of 60% (v/v) isopropanol (Cat# I9516, Sigma-Aldrich, Australia) was transferred to the tube, which was then incubated at RT for 5 min on a rotor, and this step was repeated. The 60% (v/v) isopropanol was replaced with 1.5mL of 0.05% w/v Oil Red O (Cat # O1391- Sigma -Aldrich, Australia) subsequently and the sample was then incubated at RT for 60min on a rotor. The oil red O was replaced with 1mL of 60% (v/v) isopropanol and incubated at RT for 5min on a rotor. The aorta was cut and pined onto a wax tablet and images were taken using a camera (Sony cyber shot exmor r 20.4)

5.2.1.9. Tandem Stenosis (TS)

5.2.1.9.1. Surgery

TS surgery performed to the right carotid artery of ApoE KO/FXYD1 KO and FXYD1 wild type male and female mouse, the details of surgery have comprehensively explained in chapter 2 section 10.

5.2.1.9.2. Tissue Collection

Animals were euthanised at 7 weeks after surgery. The carotid arteries were dissected out carefully cut into five sections (Figure 5.1) (Chen et al. 2013) and immersed in tissue-Tek OCT compound (Product code: IA018, ProSciTech). The sections and OCT compound were placed on dry ice to freeze and stored at -80°C. Section one displayed the characteristics of atherosclerotic plaque instability/rupture, section 2 demonstrated unstable atherosclerosis plaque, section three represented a stable atherosclerosis plaque phenotype, section 4 presented plaque-free healthy vasculature and section 5 showed stable atherosclerotic plaque.

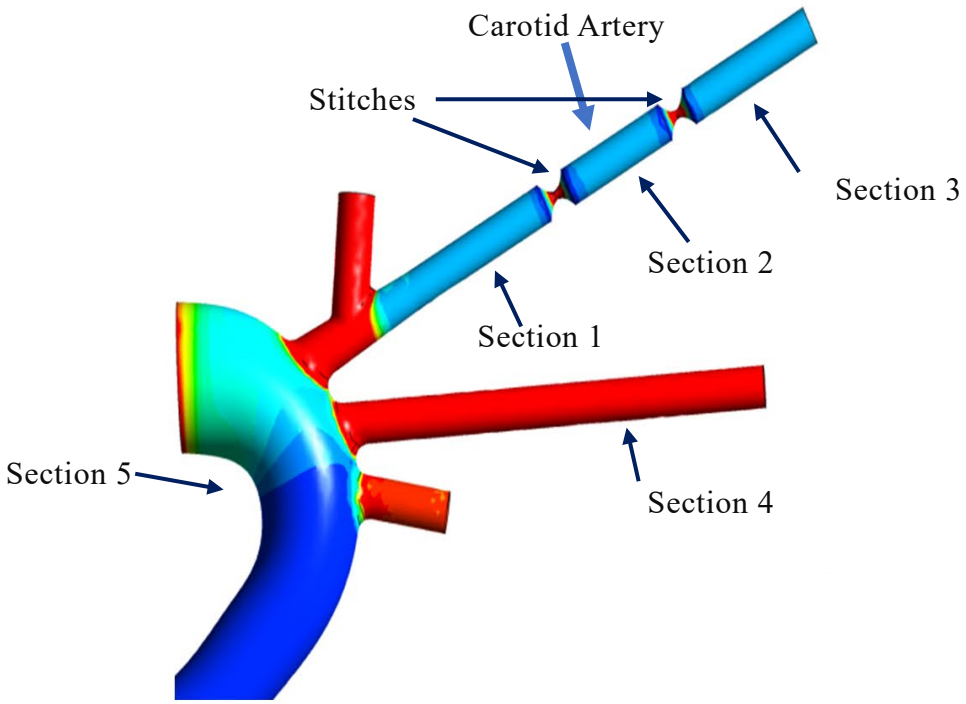


Figure 5.1: Carotid Artery Tandem Stenosis (TS) Model (Chen, Y.C. et al, 2013)

5.2.1.9.3. Histochemistry

The methods which were used for sectioning and staining the carotid arteries were described in chapter 2, section 11. Briefly, the five frozen sections of carotids, which are shown in Figure 5.1, were cut using cryostat microtome (Lecia, CM3050 S) into 5-7μM sections and transferred onto glass slides. The slides were placed in slide holders and immersed in ice-cold acetone (Cat#179124-2.5L, Sigma Aldrich) for 10 minutes. The slides were then air-dried in the fume hood overnight and stained.

5.3. Results ApoE KO/ FXYD1 KO and WT samples

5.3.1. Atherosclerosis

5.3.1.1. Body Mass

The results of measuring the body mass of mice showed that regardless of sex or genotype, body mass increased in all samples from mice that were treated with the high fat/cholesterol diet (Figure 5.2). However, when the body mass increases were calculated, and the samples were discriminated based on sex, the results showed a significant difference in male mice body mass gain between WT and KO (Figure 5.3 A). In contrast, the same analyses for the female mice showed no significant differences in ApoE KO/FXYD1KO female mice mass gain compared with ApoE KO/WT female mice (Figure 5.3 B). Table 5.1 shows the details of the mice that were used in this study.

Table 5.1: Details of the mice were used in atherosclerosis study

Number of samples	Genotype	SEX	Average baseline weight (g)
21	ApoE KO/FXYD1 WT	Male	25.7
27	ApoE KO/FXYD1 WT	Female	19.9
22	ApoE KO/ FXYD1 KO	Male	23.6
21	ApoE KO/ FXYD1 KO	Female	19.3

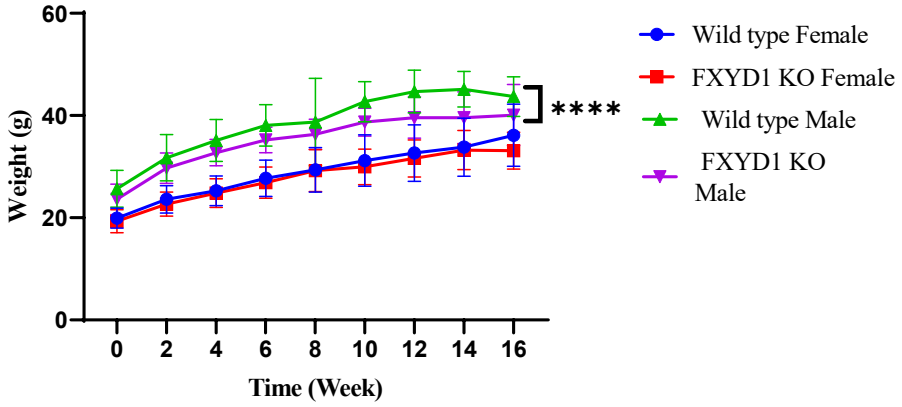


*Figure 5.2: Body mass changes on the high-fat diet. Male and female ApoE KO, FXYD1 WT and KO mice were fed a high-fat diet for 16 weeks, and mice were weighed fortnightly. Data are shown as mean ± SEM, N= 21-27 and statistical analysis was performed by two-way ANOVA, ****p<0.0001*

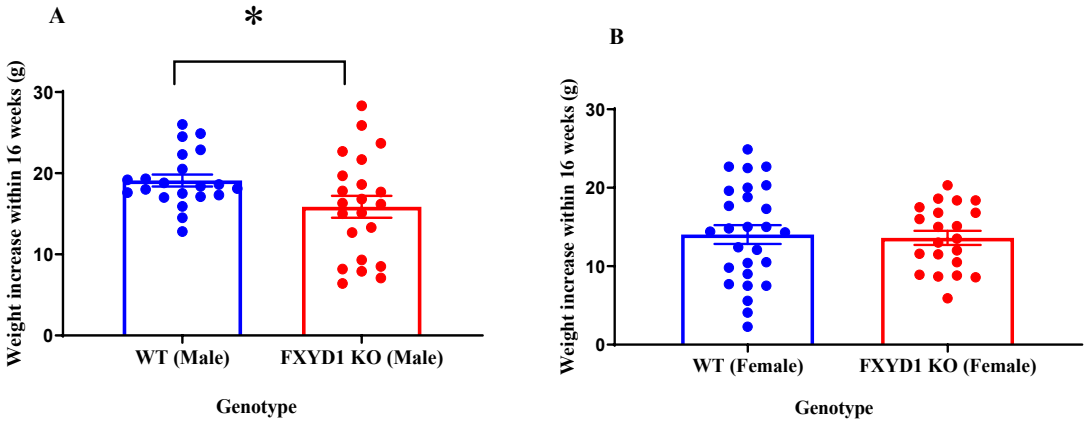


*Figure 5.3: Weight changes in WT and FXYD1 KO male (A, n=21-27) and female (B, n=21-22) mice. All mice are ApoE KO. Data are presented as mean ± SEM. Statistical analysis was performed by the Mann-Whitney non-parametric test to determine differences between WT and FXYD1 KO (*P<0.05).*

5.3.1.2. Non-fasted blood glucose

The results of measuring the blood glucose test showed that the FXYD1 gene did not have an impact upon the level of blood glucose in ApoE KO mice (Figure 5.4).

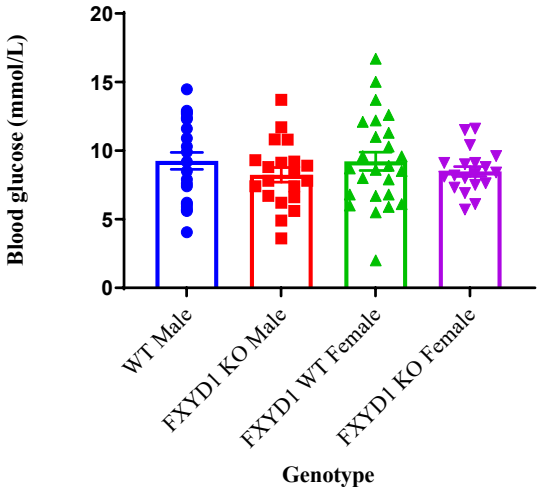


Figure 5.4: Non-fasted blood glucose data are shown as mean ± SEM (n = 20-25), all mice are ApoE KO, statistical analysis was performed by two-way ANOVA

5.3.1.3. Metabolic Cage study

The metabolic cage study results illustrated that genotype or sex did not have a significantly impact upon water or food consumption in mice (Figure 5.5). The results also showed that genotype and sex did not significantly affect urinary and faecal excretion (Figure 5.6).

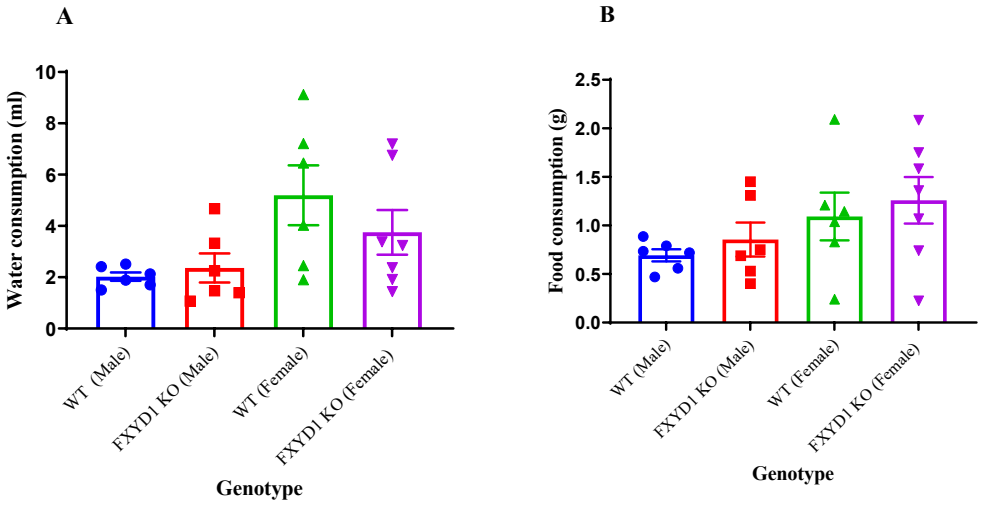


Figure 5.5: Water (A) and Food (B) intake in mice inhabiting metabolic cages, data are shown as mean ± SEM (n = 6-7), all mice are ApoE KO, statistical analysis was performed by two-way ANOVA

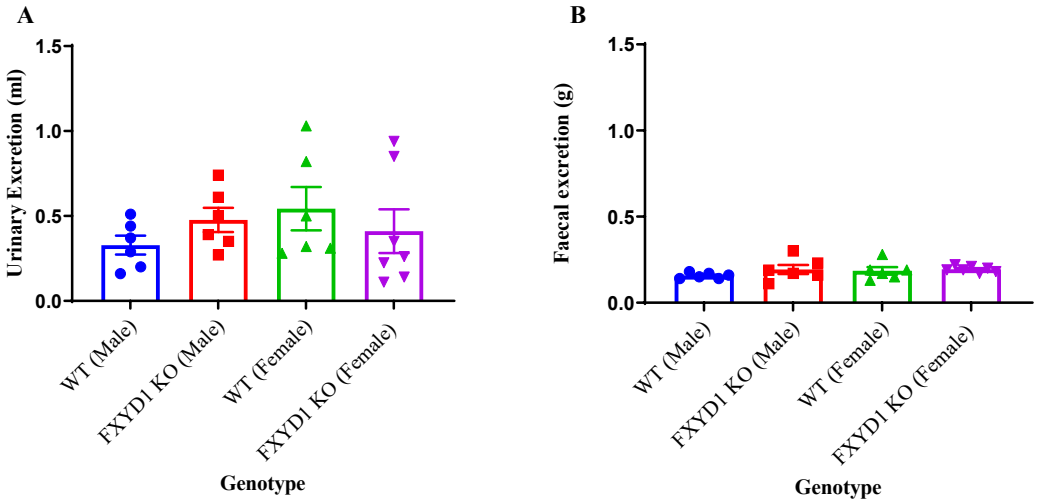


Figure 5.6: Urinary (A) and Faecal (B) excretion in mice inhabiting metabolic cages, data are shown as mean ± SEM (n = 6-7), all mice are ApoE KO, statistical analysis was performed by two-way ANOVA

5.3.1.4. Total cholesterol, HDL, LDL-cholesterol, and triglyceride

The results of measuring total cholesterol in plasma samples showed that the cholesterol level in FXYD1 KO mice were significantly higher than the total cholesterol

level in WT mice (Figure 5.7 A). When the results separated based on the sex, results showed that only in FXYD1 KO female mice was the level of total cholesterol significantly higher than for female WT mice (Figure 5.7 C). In contrast, the differences of total cholesterol in male mice were not significant (Figure 5.7 B).

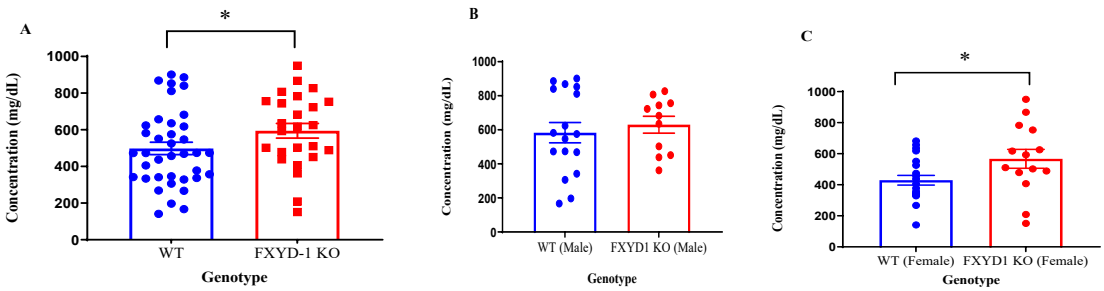


Figure 5.7: Total cholesterol concentration in FXYD1 WT and FXYD1 KO male and female mice (A, n=25-38), male (B) and female(C) mice (n=11-21, all mice are ApoE KO. Data are presented as mean ± SEM. Mann-Whitney was used to determine differences between FXYD1 WT and FXYD-1 KO mice (P<0.05).*

The results of measuring the HDL-C showed the same trend as total cholesterol, in other words, the level of HDL-C in FXYD1 KO mice was significantly higher than the level of HDL-C in WT mice (Figure 5.8 A) and when results of HDL-C were separated based on sex, the results showed that the level of HDL-C in female FXYD1KO mice was significantly higher than for the female WT (Figure 5.8 C). However, the results of HDL in male mice did not show significant differences (Figure 5.8 B).

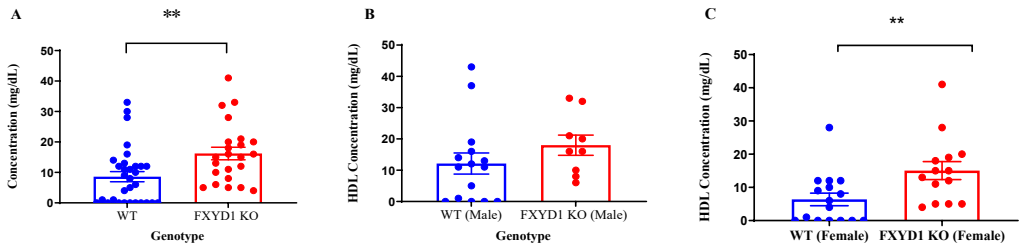


*Figure 5.8: Levels of HDL-C in FXYD1 WT and FXYD1 KO male and female mice (A, n=23-31), male (B) and female(C) mice (N=9-16) All mice are ApoE KO. Data are presented as mean ± SEM. Mann-Whitney was used to determine differences between FXYD1 WT and FXYD-1 KO mice (** P<0.01)*

The results of LDL-C quantification did not show any differences between the different genotypes (Figures 5.9, all samples (A)). However, as shown in Figure 5.9 B the level of LDL-C in females is significantly lower than male.

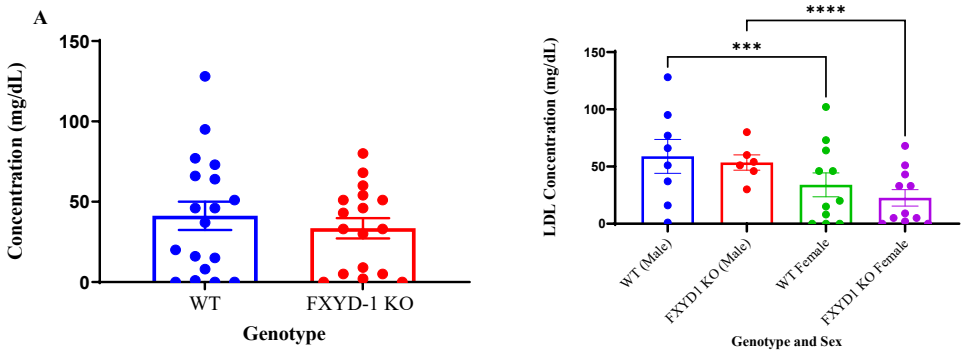


*Figure 5.9: Levels of LDL-C in FXYD1 WT and FXYD1 KO male and female mice (A, n=17-18), male and female (B) mice (n=6-11). All mice are ApoE KO. Data are presented as mean ± SEM. Mann-Whitney (A) and two-way ANOVA (B) were used to determine differences between FXYD1 WT and FXYD-1 KO mice, ***p<0.001, ****p<0.0001*

Within contrast to the HDL and LDL-C, the results of triglyceride quantification did not show significant differences in different genotypes or sexes (Figures 5.10, all samples (A), male (B), female (C)).

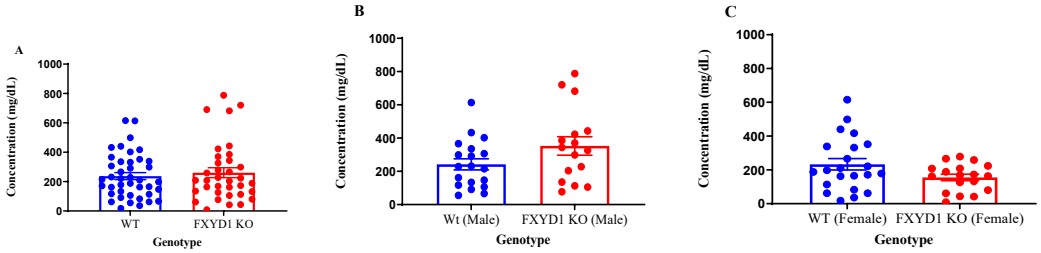


Figure 5.10: Levels of triglyceride in FXYD1 WT and FXYD1 KO male and female mice (A, n=35-41), male (B) and female (C) mice (n=18-22). All mice are ApoE KO. Data are presented as mean ± SEM. Mann-Whitney was used to determine differences between FXYD1 WT and FXYD-1 KO mice

5.3.1.5. Circulating inflammatory markers

The plasma levels of 3 pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and one anti-inflammatory cytokine (IL-10) were measured. IL-6, IL-10 and TNF-α were not detected. However, the results of IL-1β showed that in female ApoE KO/FXYD1KO mice the level of that pro-inflammatory cytokine was significantly increased compared with female ApoE KO/FXYD1WT mice (Figure 5.11 B). The results showed no differences in pooled sexes (Figure 5.11 A) or male mice (Figure 5.11 B). It is interesting to find out that the level of IL-1β in ApoE KO/FXYD WT female is significantly less than male (Figure 5.11 B).

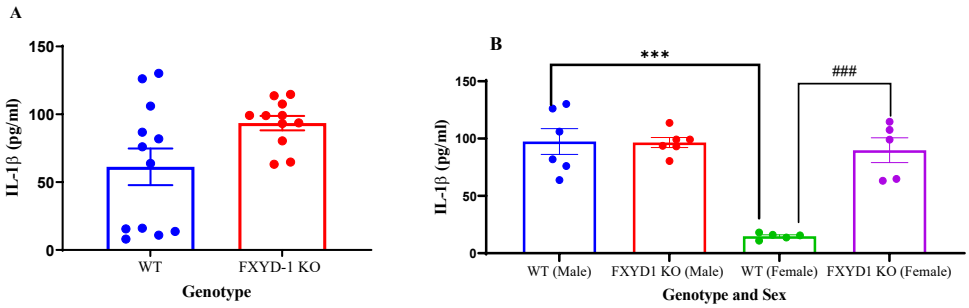


*Figure 5.11: Levels of IL-1β in FXYD1 WT and FXYD1 KO male and female mice (A, n=11-12), male and female (B) mice (N=5-6). All mice are ApoE K.O Data are presented as mean ± SEM. Mann-Whitney (A) and two-way ANOVA (B) were used to determine differences between FXYD1 WT and FXYD-1 KO mice, ***p<0.001, ###p<0.001*

5.3.1.6. Plaque Formation

The impact of FXYD1 gene expression upon the size of plaques in the aorta of mice was studied. The figure 5.12 shows the representative images of aortas, which were stained with oil red O in order to detect the plaques.

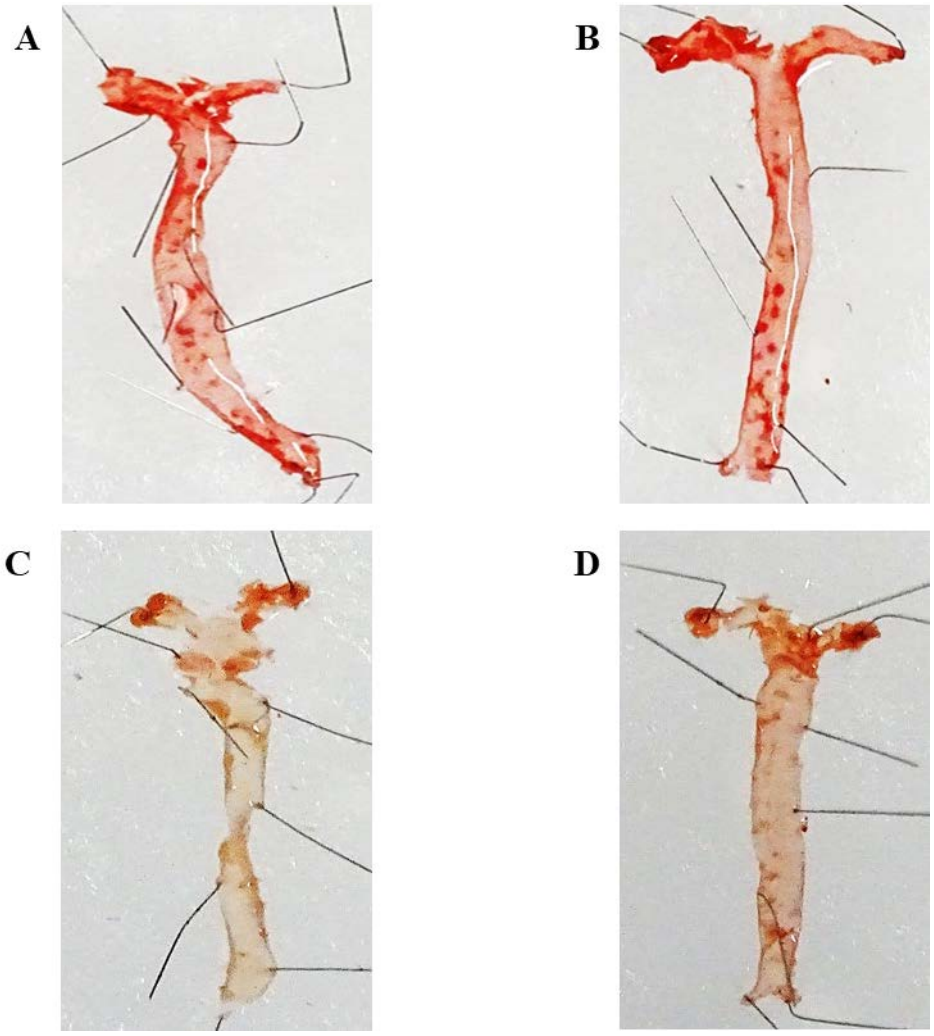


Figure 5.12: Collected aorta from WT and FXYD1 KO mice stained with Oil Red O procedure, all mice are ApoE KO. (A) Male FXYD-1 WT, (B) Male FXYD-1 KO, (C) Female WT, (D) Female FXYD-1 KO

The results showed that absence of the FXYD1protein did not significantly affect the aortic plaques' coverage (Figure 5.13 A). The results also showed that sex did not have impact the total area of the plaques, which were formed in the aorta (Figure 5.13 B and C).

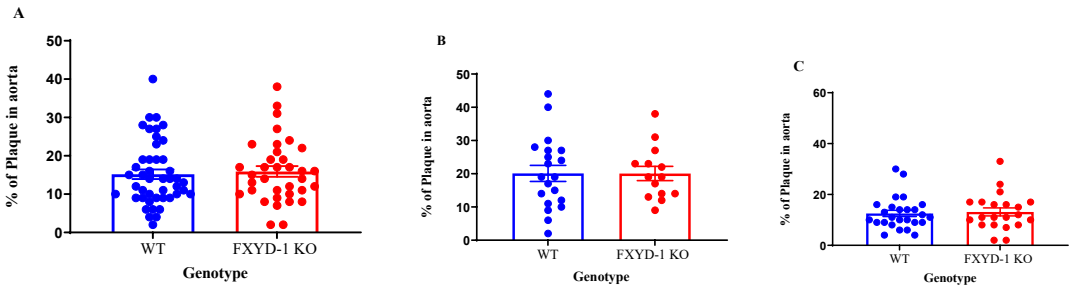


Figure 5.13: Percentage of the aorta covered by plaques in FXYD-1 WT and FXYD-1 KO male and female (A, n=35-46) mice, male (B, n=15-20) and female (C, n=20-26) mice. All mice are ApoE KO. Data are presented as mean ± SEM. Mann-Whitney test was used to determine differences between FXYD-1 WT and FXYD-1 KO (P>0.05)

5.3.2. Results, Tandem Stenosis (ApoE KO/ FXYD1KO and WT)

5.3.2.1. Body Mass

The mice weighed at the commencement of the study. After that, every two weeks, mice weighed, and the results were recorded. The results showed (Figure 5.14) that the mice gradually gained body mass. TS surgery did not have an impact upon the female mice body mass gain. However, after surgery, male mice started to lose weight, but after two weeks recovered. Table 4.4 shows the details of the mice used in this study.

Table 5.2: Details of mice used in the TS study

Number of Samples	Genotype	Sex	Avg Baseline Weight (g)
10	ApoE KO/FXYD1 WT	Male	27.6
6	ApoE KO/FXYD1 WT	Female	22.5
12	ApoE KO/ FXYD1 KO	Male	29.5
6	ApoE KO/ FXYD1 KO	Female	22.5

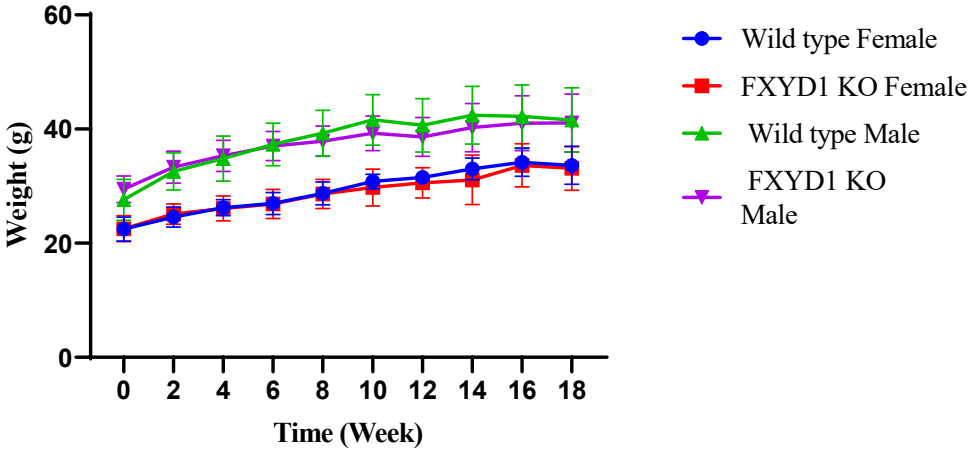


Figure 5.14: Body mass changes on the high-fat diet. Male and female ApoE KO, FXYD1 WT and KO mice were fed a high-fat diet for 9 weeks, and mice were weighed fortnightly. TS surgery performed at week 10 and the high-fat diet continued for 7 more weeks. Data are shown as mean ± SEM, N=6-12, and statistical analysis was performed by two-way ANOVA

5.3.2.2. Histochemistry

The slides were stained with Hematoxylin and Eosin (H&E) and analysed using ImageJ 1.53C" (Schneider, Rasband & Eliceiri 2012). In sections 1-4, plaques, media, and necrotic cores' area were measured and the ratio of the plaques, media, and necrotic cores to the total area of the section were determined.

Segment I represented the characteristic of atherosclerotic plaque instability/rupture characterised by disruption of fibrosis cap and luminal thrombosis. Figure 5.15 represents the segment I in ApoE KO/FXYD1 KO and ApoE KO/ FXYD1 WT samples.

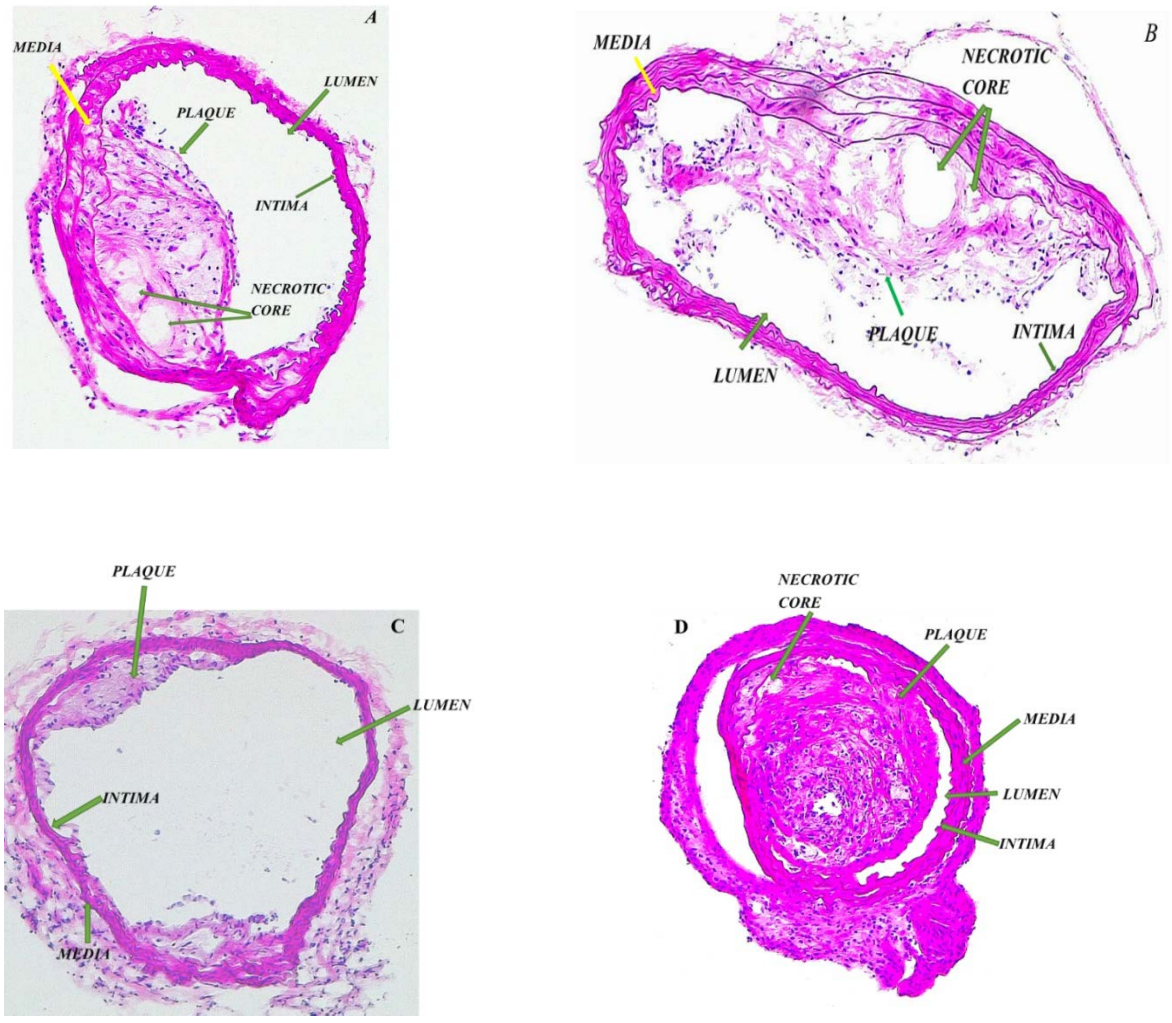


Figure 5.15: Section 1 of collected carotids from WT and FXYD1 KO mice stained with H&E procedure, all mice are ApoE $^{-/-}$ and TS surgery performed. (A) Male FXYD-1 KO, (B) Male FXYD-1 WT (C)Female FXYD-1KO, (D) Female FXYD-1 WT

Figure 5.16 shows the ratio of formed plaques in the segment I to the whole section. The results show that the absence of FXYD1 did not have an impact upon the size of plaques. Also, the sex of samples had no effect on plaque size.

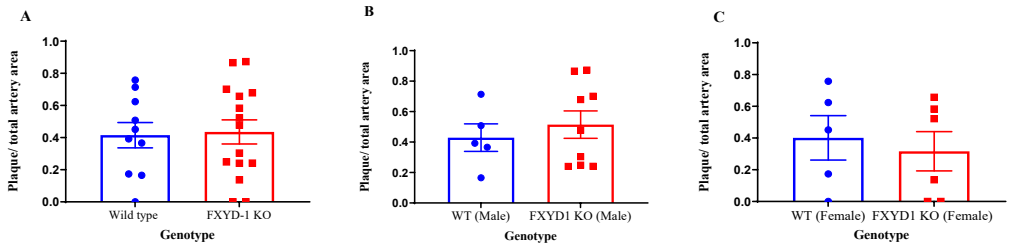


Figure 5.16: Figure shows the ratio of plaques to the whole section area in section 1 in FXYD1 WT and FXYD1 KO male and female (A, n=10-15), male (B, N=5-9) and female (C, n=5-6) mouse carotid All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Figure 5.17 shows the results of media ratio to the whole section in segment I. These results show that FXYD1 protein or different sexes did not have a significant impact upon size of media.

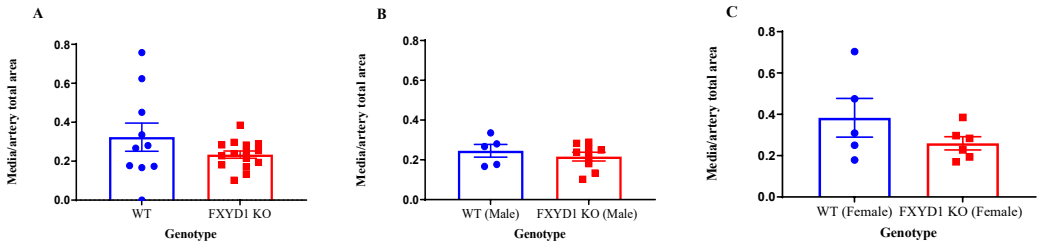


Figure 5.17: Figure shows the ratio of media to the whole section area in section 1 in FXYD1 WT and FXYD1 KO male and female (A, n=10-15), male (B, N=5-9) and female (C, n=5-6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

The size of necrotic cores correlates to the vulnerability plaques, our results showed that the sex or absence of FXYD1 protein did not have an impact upon the size of necrotic core in plaques (Figure 5.18).

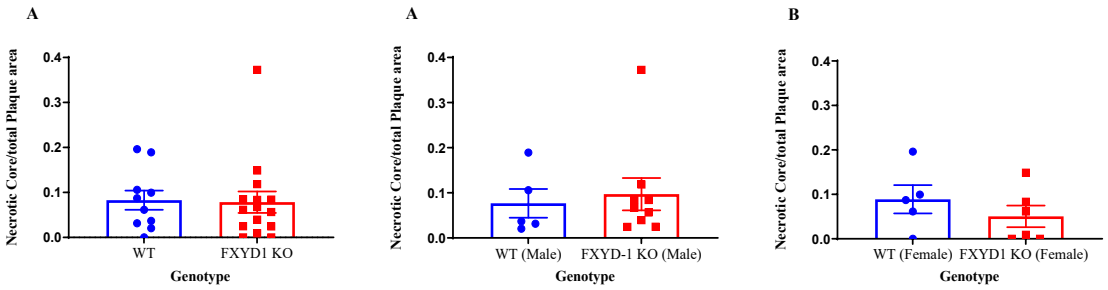


Figure 5.18: Figure shows the ratio of necrotic cores to the whole plaque area in section 1 in FXYD1 WT and FXYD1 KO male and female (A, n=10-15), male (A, N=5-9) and female (B, n=5-6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Figure 5.19 shows the representative images of section II of carotids, which in most cases contain intact thin fibrous caps with a highly cellular contents. The size of plaques, media and necrotic core were measured in segment II.

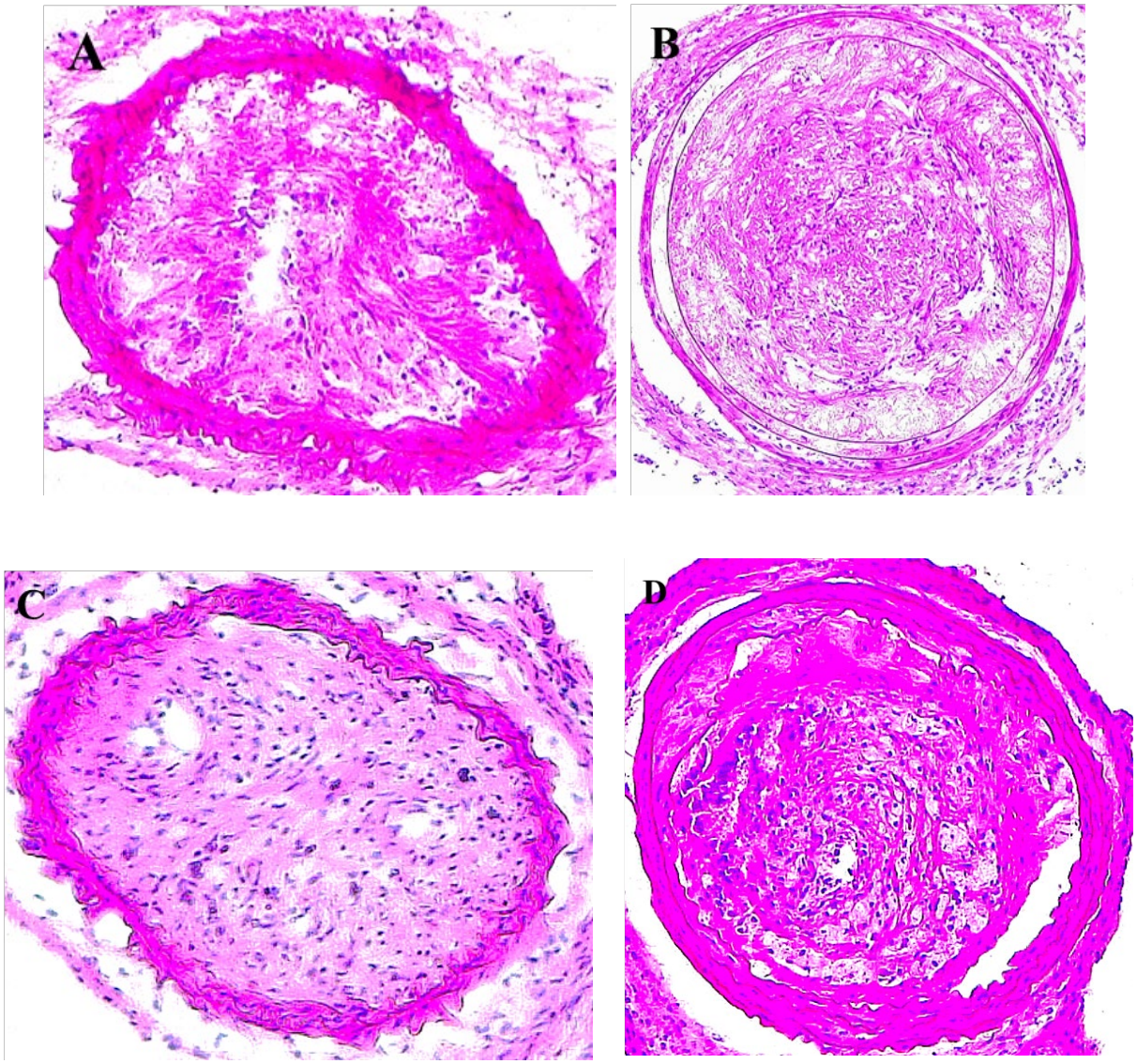


Figure 5.19: Section 2 of collected carotids from WT and FXYD1 KO mice stained with H&E procedure, all mice are ApoE KO and TS surgery performed. (A) Male FXYD-1 KO, (B) Male FXYD-1 WT (C)Female FXYD-1KO, (D) Female FXYD-1 WT

Figure 5.20 shows the ratio of plaques area to the whole section in segment II of carotids. The analysed results did not show any differences between male and female samples. Also, the results did not show differences between FXYD1 KO and FXYD1 WT samples.

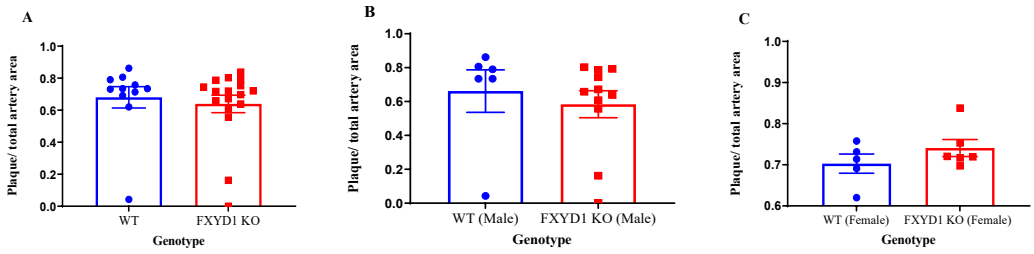


Figure 5.20: Figure shows the ratio of plaques to the whole section area in section 2 in FXYD1 WT and FXYD1 KO male and female (A, n=11-17), male (B, N=6-11) and female (C, n=5-6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Figure 5.21 shows the results of the media ratio to the whole section of segment II. The analysed data showed that the genotype and sex did not have an impact upon the size of media in segment II.

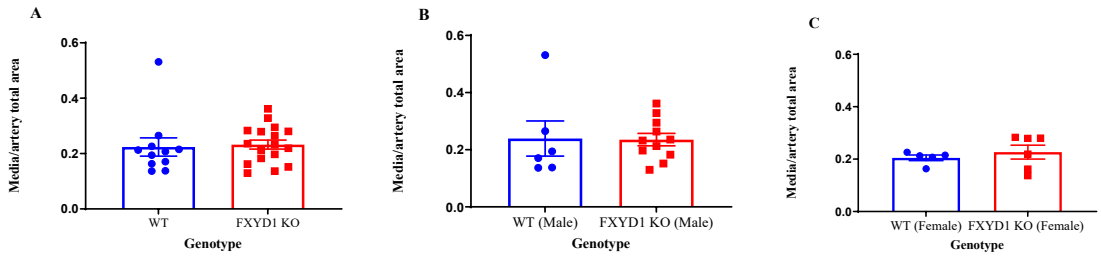


Figure 5.21: Figure shows the ratio of media to the whole section area in section 2 in FXYD1 WT and FXYD1 KO male and female (A, n=11-17), male (B, N=6-11) and female (C, n=5-6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Figure 5.22 shows the results of necrotic core detection in segment II of carotid and results did not show any differences between genotype or sex.

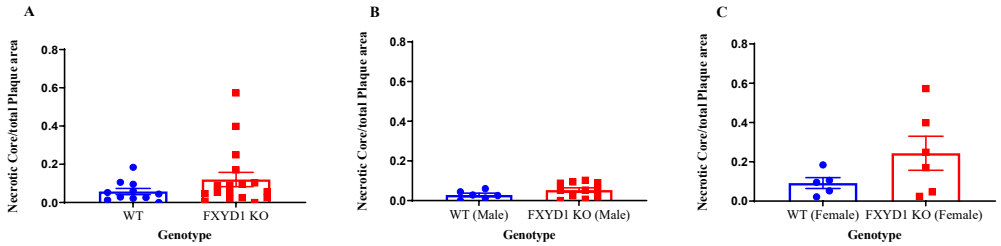


Figure 5.22: Figure shows the ratio of necrotic cores to the whole plaque area in section 2 in FXYD1 WT and FXYD1 KO male and female (A, n=11-17), male (B, N=6-11) and female (C, n=5-6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Figure 5.23 demonstrates the represents images of segment IV of carotids. Segment IV represents plaque free healthy vasculature.

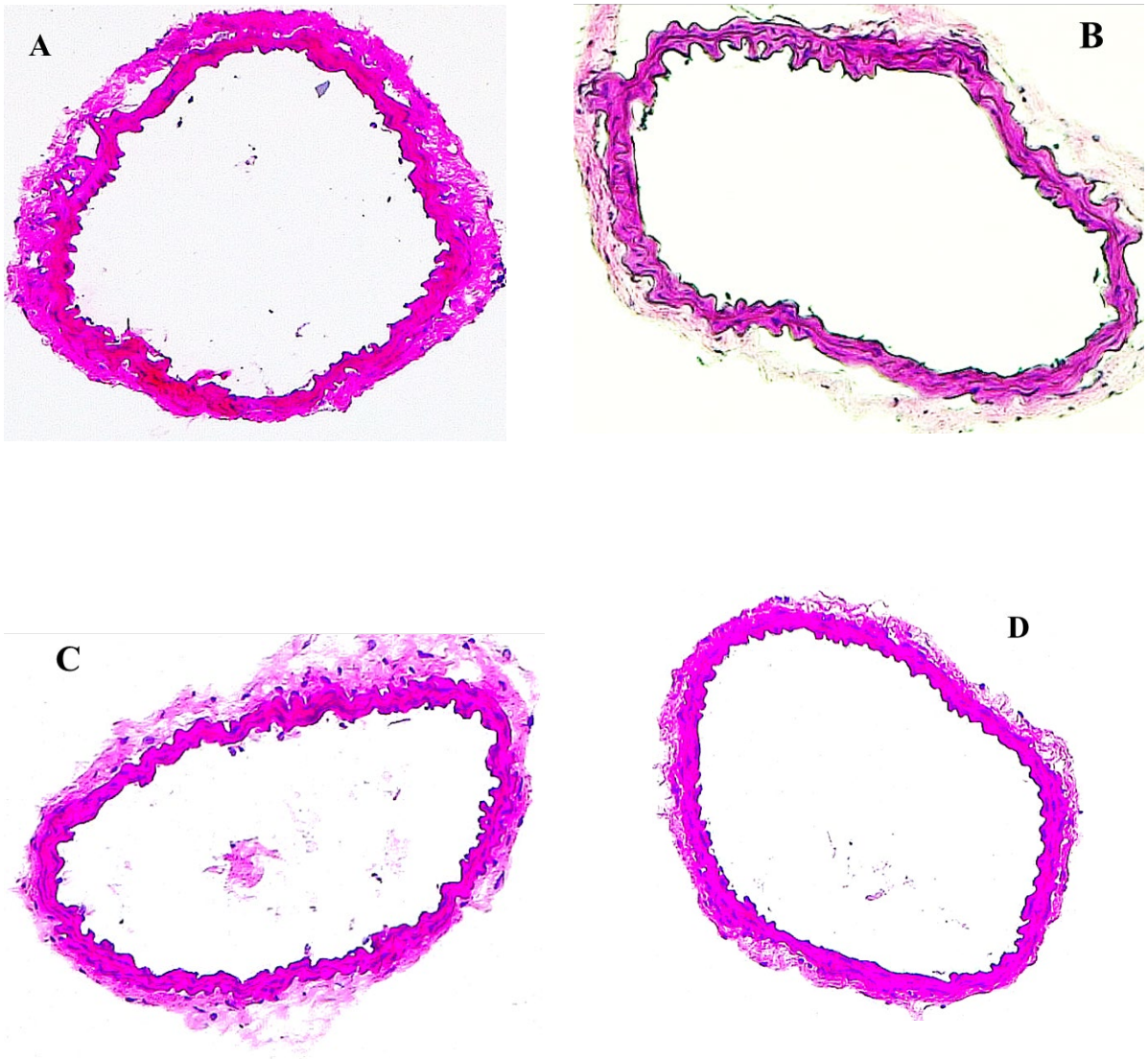


Figure 5.23: Section 4 of collected carotids from WT and FXYD1 KO mice stained with H&E procedure, all mice are ApoE KO and TS surgery performed. (A) Male FXYD-1 KO, (B) Male FXYD-1 WT (C)Female FXYD-1KO, (D) Female FXYD-1 WT

The image analysis results for section 4 of the collected carotid did not show any plaque formation or necrotic cores. Figure 5.24 show the ratio of media to the surface area of segment IV. The results showed that absence of FXYD1 or different sexes did not have an impact upon the size of media in segment IV.

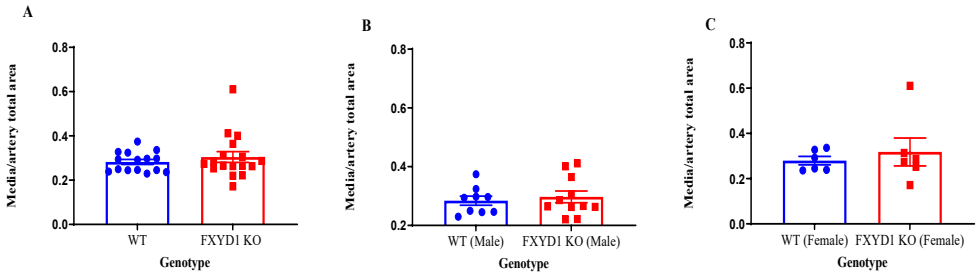


Figure 5.24: Figure shows the ratio of medial to the whole section area in section 4 in FXYD1 WT and FXYD1 KO male and female (A, n=15-17), male (B, N=9-11) and female (C, n=6) mouse carotid. All mice are ApoE KO and TS surgery performed. Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney test to determine differences between FXYD1 WT and FXYD1 KO

Due to some technical issues, sections 3 and 5 could not be collected from all samples, and the collected samples were not enough to match the analytical method requirements, so the analysed data have not been presented. Hence, I could not investigate the impact of FXYD1 protein on stable plaques which mainly localise in segment III and V of carotids.

5.4. Discussion

The proteomics results (Chapter 4) showed that in the absence of the FXYD1 gene, the expression level of some of the proteins such as Pltp significantly increased. Pltp protein facilitates transferring a range of different lipid molecules, including diacylglycerol, phosphatidic acid, sphingomyelin, phosphatidylcholine, phosphatidylglycerol, cerebroside and phosphatidylethanolamine (Nishida et al. 1997; Rao et al. 1997). Moreover, it has been shown that Pltp transfers the antioxidant α-tocopherol, which evokes the hypothesis that Pltp plays a role in regulating the oxidation of lipoproteins and tissues that stimulates atherosclerosis (Desrumaux et al. 1999; Kostner et al. 1995). We decided to investigate the impact of the FXYD1 gene on the development and progression of atherosclerosis.

Also, the proteomics results showed that the absence of FXYD1 impacts many metabolic pathways such as carbohydrate, glucose, triglyceride metabolism pathways. Hence, to investigate the impact of FXYD1 on the metabolism of ApoE KO mice, first, the body mass was monitored. The body mass measurements showed that the ApoE KO/FXYD1 KO and ApoE KO/FXYD1 WT mice gained weight regardless of genotype or sex. However, results showed that FXYD1 KO male mice did not gain weight in the same way as WT. These results inferred FXYD1 gene might have some impact on gaining weight. In addition, the differences in body mass gain in males and females imply that FXYD1 impacts the body weight in males and females differently. Besides, metabolic cage study results revealed that in the ApoE mouse model, the absence of FXYD1 did not have any impact on water or food consumption and urine and faecal excretion. Also, the results showed that metabolic rate was not different in different sexes.

The plaque formation results showed that despite using high fat and high cholesterol diet, the percentages of plaques in the aorta do not offer many differences. The plaque formation showed the same trend for male and female mice. The impact of FXYD1 on pathways such as HDL remodelling, plasma lipoprotein remodelling, assembly, and clearance pathways, as revealed by the proteomic results, suggested that the absence of FXYD1 may have an effect on the plaque formation in ApoE KO samples.

Atherosclerosis is known as a chronic inflammatory disease; hence it is important for our study to investigate the impact of FXYD1 on the circulating inflammatory markers. Thus far, there is no evidence to show an impact of the absence of FXYD1on inflammatory markers of the immune system. Therefore, it is crucial to investigate this.

The results of circulating inflammatory markers experiments showed that IL-1β in female FXYD1 KO was significantly increased, which means the absence of FXYD1 gene may cause inflammation in female mice but not in males and maybe increase the FXYD1 protein in female mice can help to reduce the inflammation in females.

Interestingly, the IL-1β ELISA results showed that IL-1β in female ApoE KO/FXYD1 WT is significantly less than males, which maybe because of the hormonal differences between males and females (Kim et al. 2020; Manfredini et al. 2019).

The tandem stenosis surgery results showed that surgery was successful in line with previous studies which used the same tandem stenosis technique (Chen et al. 2013).

There is substantial evidence showed that hypercholesteraemia plays a major function in developing and progression atherosclerosis and plaque formation (Lu & Daugherty 2013). In line with this, our results support modulation of cholesterol metabolism by FXYD1. However, the histology results of stained carotid did not show significant differences between WT and FXYD1KO mice in plaque area, necrotic core formation or media size. In addition, due to limited carotid tissues it was not possible to use other staining methods such as Pico Sirius Red and Trichrome to investigate the impact of FXYD1 on formation or alteration of smooth muscle cells and collagen in carotide tissues, which is the limitaion of this part of study.

In summary, FXYD1 appears to have an anti-inflammatory role and may also be involved in cholesterol metabolism or handling in female mice. This could provide a novel therapeutic angle for females.

Here we have shown for the first time that the absence of FXYD1 is associated with higher circulating lipids and pro-inflammatory cytokines in females, but this does not appear to alter plaque development in the short term. Ongoing studies will determine the effect on plaque stability and the role of FXYD1 in lipidomics. Moreover, in chapter 3, we showed that in the absence of FXYD1 protein, the expression level of eNOS in male mice heart was significantly decreased.

CHAPTER SIX

Discussion

This thesis was designed to investigate the role, function, and impact of FXYD1 protein on development and progression of atherosclerosis, diabetes, and hypertension in mouse models and subsequently on the structure and proteomics of caveolae, which is a centre of cell signalling including redox signalling.

It has been shown that CVD is the leading cause of death and disability worldwide (Feigin et al. 2018; WHO-Group 2019). Despite some declines, CVD remains the principal cause of death in developing and developed countries (Rosamond et al. 2007; Roth 2018; WHO-Group 2019). Notwithstanding major declines in CVD mortality, there is still significant residual risk of major cardiovascular events, so it is essential to look for a novel therapeutic approach to prevent mortality and morbidity caused by CVD. Antioxidant therapy is one method that has been attractive for the treatment and prevention of CVD.

Due to protective role and function of FXYD1, which is a caveolae plasma membrane resident protein, against oxidative stress (Bubb et al. 2021; Bubb et al. 2016), in this project my aim was to determine whether FXYD1 within caveolae could impact diseases.

In the first instance I examined global knockout of FXYD1 and the impact on cardiovascular redox signalling in different disease models, different tissues, and between the sexes. The NADPH oxidases (NOX) are a group of plasma membrane enzymes that can catalyse the reaction to transfer one electron of NADPH or NADH to oxygen to produce superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which are ROS. The results of NOX enzymes detection in the FXYD1 KO atherosclerosis mouse model showed, the expression of NOX2 significantly decreased in the male heart tissues, but not in the females. In addition, in the FXYD1 KO diabetes mouse model the expression of NOX4 was significantly decreased in male heart tissue but not in female samples. The reduction of NOX2 and NOX4 in atherosclerosis and diabetes mouse models implies that less ROS would be generated in the absence of FXYD1 in male mice's heart tissue, which contrasts with the hypothesis of a protective role for FXYD1. From these studies I also showed that in the absence of FXYD1 gene in heart tissues of atherosclerosis female mice the level of endothelial nitric oxide synthase (eNOS) significantly decreased. This may lead to the reduction of nitric oxide (NO) and thereby increase the risk of CVD due to

dysfunction of NO, which is vital for cardiovascular health. Whilst there were some minor changes in protein expression across the different models, overall, it seemed that FXYD1 did not have a major or consistent role in protecting or worsening CVD in mice. Therefore, I moved to try to examine FXYD1 specifically in the caveolae. In order to achieve this, an unbiased proteomics analysis of hearts from WT and FXYD1 KO mice was undertaken to identify cellular networks that are dysregulated in the absence of FXYD1. As part of this, I focused my interpretation of the results on investigating the impact of the absence of FXYD1 upon proteins involved with weight gain, blood glucose homeostasis, lipid metabolism, and expression of redox enzymes in the hope of subsequently identifying potential therapeutic targets. Interestingly, in line with protein expression analysis in models of CVD, where NOX expression was decreased in FXYD1 KO mice, the proteomics results showed that the expression of a few members of cytochrome c oxidase family, including Cox6a1, Cox7b, Cox7a1, Cox7c, Cox 5a and Cox6c in heart and caveolae subfraction was significantly decreased in FXYD1 KO samples. It has been shown reduction or loss of cytochrome c oxidase led to mitochondrial dysfunction and ROS production (Leadsham et al. 2013). In addition, the cytochrome c oxidase enzymes contribute to transfer of electron to molecular oxygen and production of ATP (Chicherin et al. 2019), the reduction of these enzymes may disturb the ATP production and subsequently have an impact upon ROS production.

It has been shown that the subfraction 6 of isolated caveolae plasma membrane has the equal importance as fraction 4 and 5, therefore, any changes in protein content of this subfraction directly related the caveolae (Lisanti et al. 1994; Sargiacomo et al. 1994). In caveolae subfraction 6, the protein caveolin 1 (Cav1) was significantly increased. It has been shown that Cav1 negatively correlated to eNOS and NO production, which support the results of chapter 3, however the proteomics was performed only for male mice.

The redox enzymes' detection study showed that the absence of FXYD1 did not impact the expression level of NOX2, NOX4, PRDX6, GLRX-1 and eNOS in the mesentery tissues in atherosclerotic and diabetic mice models. Also, the results showed that expression of NOX2, NOX4, GLRX-1 and eNOS in the mesentery tissues in Ang II induced hypertension remained the unchanged in FXYD1 KO mice. However, the results of PRDX6 enzyme detection in the mesentery of Ang II induced hypertension FXYD1 KO male mice showed a significant decrease. PRDX6 is a bifunctional enzyme that is

able to play a role as an antioxidant or oxidant generator (Chatterjee et al. 2011), yet the function of PRDX6 in Ang II induced hypertension FXYD1 male mice remains unclear.

The glucose blood test results of STZ induced diabetes samples revealed that in male FXYD1 KO mice the level of glucose was significantly lower than WT diabetes samples. The proteomics results showed that the level of Glucose-6-phosphate isomerase (G6pi) in subfraction 4 and 5 of FXYD1 KO mice significantly increased. It has been demonstrated that G6pi contributes to glycolysis pathway (Cordeiro et al. 2003) and the increasing of G6pi in FXYD1 KO mice may lead to lowering the blood glucose in STZ induced diabetes male samples.

The electron microscopy results showed that in the absence of FXYD1 the diameter and circumferences of caveolae was significantly decreased. The proteomics results showed that concentration of caveolin 3 (Cav3), which is an essential structural protein in caveolae formation, in caveolae subfractions was also significantly decreased. The reduction of Cav 3 concentration in absence of FXYD1 may lead to the reduction of diameter and circumference of caveolae. In addition, Tsutsumi et al, (2008) showed that in cardiac ischaemia the number of caveolae increased (Tsutsumi et al. 2008), similar to what we found in the hearts of FXYD1KO mice. Taken together the absence of FXYD1, which may mimic the cardiac ischaemia, leads to the reduction of diameter and circumferences of caveolae in heart tissues. Or loss of FXYD1 may predispose to worsened cardiac injury post-ischemia, which is being followed up in current and future experiments in our laboratory. In response to the reduction of diameter and circumferences of caveolae, the heart increases the number of caveolae possibly to compensate this condition. Figure 6.1 summarized our results and compare that with published study.

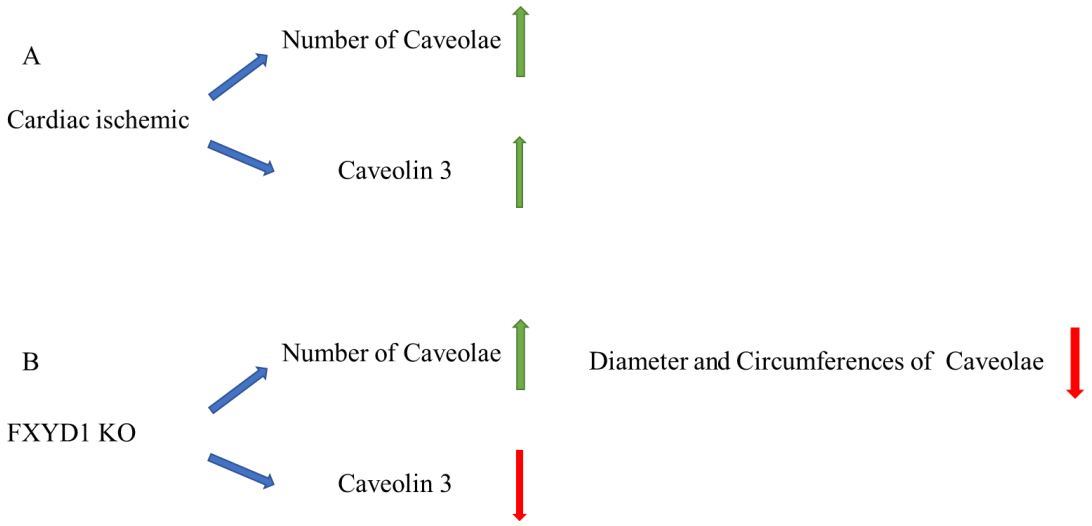


Figure 6.1; A) shows the results of Tsutsumi 2008 study and B) the results of this study. The number of caveolae in both condition increased, but absence of FXYD1 reduces the Cav 3 concentration and the size of caveolae

It has been shown that long-term high fat diet intake causes dysregulated bile acid signalling and dermatitis (Jena et al. 2019). In our FXYD1 KO atherosclerosis mouse model, where mice received a high-fat diet, dermatitis developed, causing substantial irritation which led to development of epidermal scar tissue. Despite applying the veterinary-prescribed ointment, the wounds did not heal, and the mice had to be euthanised on humane grounds. This identifies a potential novel role of FXYD1 in angiogenesis or wound healing. I then examined proteins involved in these pathways from my proteomics analysis. In caveolae subfractions (4-5), the caveolae structural protein Cavin 2 was upregulated 1.46 fold in FXYD1 KO mice. It has been shown that Cavin2 plays a key role in angiogenesis and subsequently wound healing (Adams & Alitalo 2007; Boopathy et al. 2017). Also, fibrinogen alpha (FGA) chain and fibrinogen beta (FGB) chain, which were upregulated 1.66 and 1.55 fold respectively in FXYD1 KO samples, play roles in blood clotting and wound healing (Fish & Neerman-Arbez 2012; Vilar et al. 2020). In addition, the level of Anxa1 (Annexin A1), which has a crucial impact on wound healing (Leoni et al. 2015), significantly decreased.

In our atherosclerosis study, 10.46% of the tested mouse needed to be culled before the end of research due to sickness, from that 6.3 % of the mouse developed scars, which were FXYD1 KO animals. Taken together, significant reduction of Anxa1 and significant upregulation of Cavin2, FGA and FGB proteins in FXYD1 KO mice could disturb the

process of wound healing, which may make the FXYD1 a possible therapeutic target for wound healing.

The proteomic results also showed that Pltp in FXYD1 KO mice's heart was tissue upregulated by 1.21-fold. It has been demonstrated that Pltp acts as a pro-atherogenic factor (van Haperen et al. 2002) although, in some circumstances, it has been shown that Pltp plays as an atheroprotective agent (Vikstedt et al. 2007). The ApoE KO/FXYD1 KO and ApoE KO/FXYD1WT aortas and carotids histology results did not show significant differences in plaque formation in those tissues. Therefore, our results do not endorse the atheroprotective role of Pltp and contribution of FXYD1 protein in this process. However,, it has been shown that the Pltp level correlates with HDL-C (Cheung et al. 1999), and we did see that ApoE KO/FXYD1 KO mice had a higher level of HDL-C in plasma samples compared with WT mice. Although, when these results were separated based on the sex of animals, it showed the HDL-C level in female ApoE KO/FXYD1 KO was significantly higher than WT samples, however in male samples no differences detected; strengthening our need to apply proteomics assessment to female mice in future.

Also, it has been shown that Pltp deficiency promotes accumulation of vitamin E that protects circulating lipoprotein from oxidation (Jiang et al. 2002). Our proteomic results showed that in the absence of FXYD1, Pltp increased. Hence, overexpression of Pltp disturbs vitamin E accumulation which leads to oxidation of lipoprotein and stimulates atherosclerosis, which is in contrast with our results of plaque formation experiments in the atherosclerosis mice model. Nevertheless, it may explain why the results of vitamin E clinical trial was disappointing if the level of Pltp and its associate proteins including Apoa I, ApoB and ApoC-I could be determined in the samples.

The association between chronic inflammation and CVD has been widely investigated. Also, it has been shown that inflammation plays a crucial role in endothelial dysfunction and early atherosclerosis onset (Libby 2006; Libby et al. 2019). Our proteomics results revealed that some of proteins which were downregulated in caveolae subfractions are contributing to several immune system pathways. Hence, the impact of FXYD1 on inflammation in atherosclerosis mice models were investigated. The detection of inflammatory cytokines in ApoE KO/ FXYD1KO and ApoE KO/FXYD1 WT showed IL-1β in FXYD1 KO female mice increased. However, no changes were detected in male mice. Also, it should be noted that the level of IL-1β in ApoE KO/ FXYD1 WT female is significantly less than male, which may happen due to hormonal differences in male and

female. Morishita et al, (1999) showed that estradiol and progesterone suppressed the production of IL-1 from human peripheral monocytes (Morishita, Miyagi & Iwamoto 1999), which may be the cause of lower level of IL-1β in our female WT samples. Moreover, it has been shown that high level of IL-1β leads to endothelial dysfunction (Vallejo et al. 2014), our result showed that the expression of eNOS significantly decreased in the heart of atherosclerotic female mice, which may cause endothelial dysfunction due to the high level of IL-1β in those samples. Audo et al. (2018) showed that in rheumatoid arthritis (RA), which is a chronic inflammatory rheumatic disease, the Pltp activity in synovial fluid correlates with the level of IL-1β (Audo et al. 2018), which supports our proteomics results for Pltp and inflammatory cytokine detection for IL-1β. However, there are still question about sex differences that need to be determined. It will be suggested to collect bone marrow-derived macrophages from atherosclerosis male and female mice model and determine the level of IL-1β in both male and female in those tissues (Zajd et al. 2020).

Analysing the proteomics results revealed that the 11 proteins that were upregulated in FXYD1 KO mouse whole hearts are involved in the metabolism pathways of carbohydrate, transporting small molecules, plasma lipoprotein assembly, remodelling and clearance of plasma lipoprotein, immune system pathway and haemostasis. These results imply that the absence of FXYD1 gene, which causes the upregulation of 11 protein, impacts many biological pathways that may improve the cardiovascular system's condition or make it worse. High-Density Lipoprotein (HDL) remodelling pathway is one of the most relevant paths to the upregulated proteins in FXYD1 KO heart tissue as it facilitates converting the HDL to steroid hormones (Tall et al. 2008). Due to the overactivation of HDL remodelling pathway, there is the possibility of overproduction of steroid hormones such as glucocorticoids that may lead to hyperglycaemia (Opherk et al. 2004; Perez et al. 2014), fat deposits redistribution and insulin resistance (Beaupere et al. 2021; Bruno et al. 1994), which could increase the risk of CVD.

The analyses of downregulated proteins in FXYD1 heart tissue from mice showed that important pathways are involved with these proteins, such as metabolism of biological oxidation, the immune system, haemostasis, cellular response to external stimuli, protein metabolism, transport of small molecules and vesicle-mediated transport. The “cellular response to external stimuli” pathway is one of the pathways relevant to the

downregulated proteins in FXYD1 KO samples. The “Reactome” online software showed that this pathway impacts cellular responses to stress, hypoxia, and detoxification of ROS.

Interestingly, some of the pathways are common in both upregulated and downregulated proteins such as “transport of small molecules” which means the absence of FXYD1 KO may disturb this pathway.

In summary, from the results of this study it can be concluded the FXYD1 protein, a localised protein in caveolae plasma membrane, is a multifunctional protein that in some circumstances is able to protect the cardiovascular system by maintaining the levels of proteins, which have antioxidant capacity, including GLRX1, Anxa1, PRDX5, PRDX6, GLRX3, Pln and TXN1. In contrast, FXYD1 is able to stimulate CVD by modulating proteins that increase the risk of CVD such as Pltp and Cavin 2. At the same time, the absence of FXYD1 reduces the concentration of proteins that increase the oxidative stress such as cytochrome c oxidase family, XDH and caveolin 1. Moreover, absence of FXYD1 increases the concentration of proteins that protect cardiovascular system such as catalase and Gpx1. More importantly, the absence of FXYD1 leads to increase in the levels of IL-1β and reduction of eNOS in female mice.

In addition, this study highlighted the substantial degree of sex differences in the function of FXYD1 and that FXYD1 has varying roles, dependent on specific disease model. All these characteristics make FXYD1 a potential therapeutic target for treatment CVD. I have clearly identified a role and function of FXYD1 beyond what has been thought previously.

For better understating of the role and function of FXYD1 and caveolae in human disease in general and specifically in cardiovascular disease, it is crucial to use both sexes in all disease models and compare the results. Also, it is crucial to use other tissues such as liver and skeletal muscles parallel to the heart tissues. In addition, using a large animal model or investigating caveolae from human vascular samples is desirable.

From this series of this studies, I concluded that FXYD1 has a role against oxidative stress in CVD and more specifically atherosclerosis, along with an evident role in regulation of cholesterol and lipoprotein metabolism and inflammation. Therefore, it is important to conduct future studies to investigate the potential therapeutic functional of FXYD1. These should be focused on atherosclerosis with particular attention paid to biological differences between males and females in their response to FXYD1.

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Appendix 1

Adobe Acrobat Document

Appendix 2

Adobe Acrobat Document

Appendix 3

[Product Note: If required, contact the author for appendix 3.]

Hypertension

OXIDATIVE STRESS

FXYD1 Is Protective Against Vascular Dysfunction

Kristen J. Bubb,* Owen Tang,* Carmine Gentile, Seyed M. Moosavi, Thomas Hansen, Chia-Chi Liu, Belinda A. Di Bartolo, Gemma A. Figtree

ABSTRACT: Nitric oxide (NO) production by eNOS (endothelial NO synthase) is critical for vascular health. Oxidative stress-induced uncoupling of eNOS leads to decreased NO bioavailability, compounded by increased superoxide generation. FXYD1 (FXYD domain containing ion transport regulator 1), a caveolar protein, protects against oxidative inhibition of the Na^+-K^+-ATPase. We hypothesized that FXYD1 may afford a similar inhibition of oxidative dysregulation of eNOS, providing a broader protection within caveolae. FXYD1-eNOS colocalization was demonstrated by co-immunoprecipitation in heart protein and by proximity ligation assay in human umbilical vein endothelial cells. The functional nature of this partnership was shown by silencing FXYD1 in human umbilical vein endothelial cells, where 50% decreased NO and 2-fold augmented superoxide was shown. Three-dimensional cocultured cardiac spheroids generated from FXYD1 knockout mice were incapable of acetylcholine-induced NO production. Overexpression of FXYD1 in HEK293 cells revealed a possible mechanism, where FXYD1 protected against redox modification of eNOS cysteines. In vivo, vasodilation in response to increasing doses of bradykinin was impaired in knockout mice, and this was rescued in mice by delivery of FXYD1 protein packaged in exosomes. Bloods vessels extracted from knockout mice exhibited increased oxidative and nitrosative stress with evidence of reduce eNOS phosphorylation. Impaired vascular function and augmented superoxide generation were also evident in diabetic knockout mice. Despite this, blood pressure was similar in wildtype and knockout mice, but after chronic angiotensin II infusion, knockout of FXYD1 was associated with a heightened blood pressure response. FXYD1 protects eNOS from dysregulated redox signaling and is protective against both hypertension and diabetic vascular oxidative stress. (***Hypertension*. 2021;77:2104–2116. DOI: 10.1161/HYPERTENSIONAHA.120.16884.)**
• **Data Supplement**

Key Words: blood pressure ■ exosomes ■ nitric oxide ■ superoxide ■ vasodilation

There is an enormous personal and global socioeconomic burden of cardiovascular disease (CVD).[1,2] Oxidative stress is recognized to play a key role in mediating the pathophysiological effects of diabetes, hypertension, and aging on the arterial wall, all key risks factors for CVD. Yet, clinical trials of antioxidants have been disappointing.[3,4] This is partially attributable to the compartmentalization of redox signaling, and redox-sensitive proteins within the cell,[5] which dietary antioxidants fail to effectively penetrate. Caveolae are important examples of such compartmentalization that may be impossible to influence with dietary antioxidants. These specialized invaginated plasma membrane domains are ≈50 to 100 nm in diameter and defined by positive caveolin expression. They can act as functional signalosomes where membrane receptors, signal transduction molecules, membrane channels, and transporters are housed in a concentrated space.[6,7] A large number of the ≈250 identified caveolar proteins are redox sensitive,[8] with many of these redox modifications shown to be functionally important for the molecule and downstream signaling in the cell.[9–11]

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.120.16884.
For Sources of Funding and Disclosures, see page 2115.

Hypertension is available at www.ahajournals.org/journal/hyp

Novelty and Significance

What Is New?

- The current study demonstrates for the first time, a key protective role of the small protein, FXYD1, in vascular health.
- FXYD1 knockout is associated with dysregulated oxidative and nitrosative stress and increased blood pressure following angiotensin II infusion.

What Is relevant?

- The prevention of nitric oxide dysregulation has long been a key strategy in preventing or reversing vascular disease, yet major cardiovascular events are still common.
- The current work sets the foundations for exploring targeted therapy at the sub-cellular level of the caveolae, a so-called redox hot-spot, with a small molecule that can protect against redox dysregulation.

Summary

We have demonstrated, for the first time, that FXYD1 is involved in the regulation of vascular health due to an intimate relationship with eNOS (endothelial nitric oxide synthase)-derived nitric oxide and NADPH-dependent superoxide generation. This is relevant in the setting of both hypertension and diabetes, and further work should target FXYD1-based therapeutics for these conditions.

Nonstandard Abbreviations and Acronyms

Ang II	angiotensin II
BP	blood pressure
CVD	cardiovascular disease
eNOS	endothelial nitric oxide synthase
HUVEC	human umbilical vein endothelial cell
NO	nitric oxide

eNOS (endothelial nitric oxide [NO] synthase), a functional homodimer protein, is a well-known resident in the caveolae. In its healthy coupled state, eNOS catalyzes the generation of NO. This vasoprotective molecule regulates vascular tone and attenuates both platelet aggregation and neutrophil-endothelium interaction.[12] However, in diseases such as diabetes and hypertension, the elevated oxidative stress exacerbates eNOS dysfunction, resulting in a shift from NO production to superoxide generation.[13] This eNOS uncoupling can occur via a number of mechanisms, including inhibition of phosphorylation (at serine 1177) and oxidization of the critical cofactor, tetrahydrobiopterin.[14] More recently, the discovery that this uncoupling can be mediated by S-glutathionylation of cysteine residues in the eNOS reductase domain[15] has led to a paradigm shift in our understanding of eNOS regulation. In this oxidative reaction, a reversible disulfide bond is formed between glutathione and the reactive cysteine residues of the enzyme. Protecting eNOS from S-glutathionylation and uncoupling during pathophysiological insults is predicted to halt the amplification process of reactive oxygen species (ROS)-induced eNOS uncoupling and ROS production in this critical signaling microdomain.

The FXYD protein family are type I membrane proteins and caveolae residents,[8] known to colocalize with the Na^+-K+ ATPase and play a role in kinase-dependent pump regulation.[16] FXYD1 (FXYD domain containing ion transport regulator 1) (also known as phospholemman), expressed in both the heart and vasculature, is the only member of the family to have serine residues susceptible to phosphorylation. In contrast, 2 cytoplasmic cysteine residues are highly conserved. We have demonstrated a new role for FXYD proteins, dependent on one of these cysteine residues, in protecting the β1 subunit of Na^+-K^+ ATPase from oxidative inhibition.[17] Given the protective effect of FXYD1 against oxidative S-glutathionylation and inhibition of the Na^+-K^+ ATPase, as well as the known colocalization of the Na^+-K^+ ATPase/FXYD complex[17] with eNOS in caveolae, we postulated that FXYD1 may have a broader protective role beyond that of the Na^+-K^+ ATPase in the caveolae. Here, we identify a novel role for FXYD1 as an endogenous protector of eNOS from oxidative uncoupling both in vitro using human-derived cells and in vivo, in mouse models of redox stress. This has implications for all vascular diseases mediated by oxidative stress.

METHODS

The authors declare that all supporting data are available within the article (and in the Data Supplement).

In Vivo Animal Studies

All animal studies performed were approved by the Northern Sydney Local Health District Animal Ethics Committee (approval numbers RESP/14/278, RESP/17/96, RESP/17/55) and conform to the National Health and Medical Research Council of Australia's Code of Practice for the Care and Use of Animals for Scientific Purposes, and all procedures conform to the guidelines from Directive 2010/63/EU of the European

Parliament on the protection of animals used for scientific purposes. FXYD1 wildtype and knockout mice were derived as previously described.[17,18] FXYD1 hemizygous mice were interbred within the Kearns Facility, Kolling Institute of Medical Research, to produce FXYD1 wildtype and knockout littermates. For all studies, mice were randomized to groups, and blinded analysis of end point experiments was performed.

Mouse Models

For baseline measures, male mice were used at 14 to 20 weeks of age. To investigate the role of FXYD1 under increased oxidative stress, several models were used. (1) Aged male mice were kept until 50 to 52 weeks of age; (2) a sub-pressor dose of Ang II (angiotensin II) was used to activate redox signaling in male mice aged 14 to 20 weeks; and (3) hyperglycemia was induced in male mice aged 10 to 12 weeks of age, with a model of insulin resistance that we have previously described.[19] For the latter 2, mice were anesthetized using isoflurane (1.5%, in 100% O_2 at 0.4 L/min) and implanted with subcutaneous osmotic minipumps (Alzet; model 1004) containing Ang II (0.72 mg/kg per day, 4 weeks) or insulin-receptor antagonist, S961 (0.3 mg/kg per day in dimethyl sulfoxide, 4 weeks, KareBay Biochem Inc) to induce hyperglycemia.[19] Of the total mice enrolled in the study, 2 mice (wildtype) died after telemetry pump implantation.

Blood Pressure Measurements by Radiotelemetry

Mice were implanted with radiotelemetric transmitters (TA11PA-C10, Data Sciences International) in the aortic arch via the left carotid artery while anesthetized (isoflurane, 1.5%–2%, in 100% O_2 at 0.4 L/min). Battery packs were tunneled under the skin to sit in the abdominal region. Mice were allowed to recover from surgery for 10 days before blood pressure (BP) measurement, as described previously.[20] BP was recorded continuously for 24 hours and average systolic, diastolic and mean arterial pressures, and heart rate and activity were determined. At the end of the recording period, all mice were euthanized by cervical dislocation while under isoflurane anesthesia (3%–4% in oxygen), following a blood sample and saline perfusion via the vena cava.

Acute BP Measurements

Mice were maintained under anesthesia (isoflurane, 1.5%, in 100% O_2 at 0.4 L/min) throughout the experiments, and body temperature was kept constant at 37 °C. Catheters filled with heparinized saline (20 U/mL, polyvinyl tubing, 0.61 mm outside diameter) were placed in the left carotid arteries for arterial BP measurements recorded with LabChart 6 using a bridge amplifier connected to a Powerlab system (AD Instruments, Australia).[21] Similar catheters containing saline only were placed in jugular veins for intravenous injections of vasoactive substances and changes in pressor responses were recorded. Drugs used were bradykinin acetate (0.3–10 µg/kg), sodium nitroprusside (0.1–10 µg/kg), and Ang II (0.01–10 µg/kg) all sourced from Sigma Aldrich, Australia and were administered into the jugular vein in ≈50 µL bolus doses.[22] Mice were euthanized as above at the end of the recording period, and tissue was collected and snap-frozen in liquid nitrogen and stored at −80 °C for analysis.

In Vitro Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) or human coronary artery smooth muscle cells (commercially available, Lonza) were cultured in EGM+ culture media (Lonza, Australia) under standard tissue culture conditions, in 6 or 12-well plates (on glass coverslips as needed) and underwent the following experiments (detailed in full in the Data Supplement): (1) overexpression of FXYD1 by transfection of FXYD1 plasmid; (2) FXYD1 knockdown by siRNA transfection; (3) treatment with acetylcholine (1 mmol/L); (4) Ang II (500 nmol/L); (5) NO reactive fluorescent dye, 4-amino-5-methylamino-2′,7′-difluorofluoroscein diacetate (2.5 µmol/L); or (6) dihydroethidium (2.5 µmol/L) for O_2^-; or (7) fixed for proximity ligation assay (Duolink). Vascularized cardiac spheroids were generated from isolated hearts of neonatal FXYD1 wildtype and knockout mice (bred from homozygous parents) as described previously,[23] using Perfecta 3D 96-well hanging drop plates (3D Biomatrix, Ann Arbor, MI).

Delivery of FXYD1 Using Exosomes

To effectively deliver the protective FXYD1 protein into the vasculature without being cleared away or degraded, we packaged FXYD1 protein into exosomes by over-expressing myristoylation-palmitoylation tagged FXYD1 in HEK293T cells,[24] outlined in detail in the Data Supplement.

Tissue and Cell Analysis

Immunoblotting

Protein was extracted from cultured HUVECs and from mesenteric and aortic vessels isolated from mice. Ten to fifty micrograms of protein lysate was denatured and run under reducing conditions on SDS-PAGE as described previously.[25] Membranes were incubated in primary antibodies directed at determining expression of proteins as described in each figure. For co-immunoprecipitation of eNOS and FXYD1 protein, lysate was extracted from rabbit hearts and incubated with protein G dynabeads coated with anti-eNOS (1 mg) overnight at 4 °C.[25] The eNOS bound fraction was extracted using a magnet, and immunoblotting for FXYD1 and eNOS was performed as above.

Free Cysteine Assay

Cleared cell lysate was labeled with 1 µmol/L freshly made IRDye 800CW Maleimide (Licor, the United States in lysis buffer for 2 hours at room temperature in dark). Free labeling reagent was then removed by passing the lysate through the Zeb Spin Desalting Columns, 7K MWCO (Thermofisher) 3×. Lysate was then incubated with 2 µg anti-eNOS antibody overnight at 4 °C with rotation. Twenty-five microliters Protein G Dynabeads (Thermofisher) was used to immune-precipitate labeled eNOS which was eluted under reducing condition and resolved by Western blotting. The resulting membrane was probed with anti-eNOS and secondary antibody conjugated to IRDye 680RD.

Detection of Intracellular NO and Superoxide Generation

The intracellular NO level was measured 4-amino-5-methylamino-2′,7′-difluorofluoroscein diacetate, 2.5 µmol/L and intracellular levels of O_2^- were detected using dihydroethidium; 2.5 µmol/L according to manufacturer's instructions (Thermofisher Scientific, Australia). Samples were prepared for confocal analysis (Leica SP5) as described in the Data Supplement.[23,25] For mouse vascular tissue, superoxide anion production was determined using lucigenin-enhanced chemiluminescence.[26]

Statistical Analysis

Data are expressed as mean±SEM. Student *t* test was used for comparison between 2 groups. For multiple comparisons, 1- or 2-way ANOVA was used with Bonferroni post hoc analysis for multiple comparisons. A P value <0.05 was considered statistically significant. For all animal studies, power calculations were performed before the study to determine the appropriate group size.

RESULTS

FXYD1 Physically Associates With eNOS

Although FXYD1 is known to be a caveolar protein, its physical association with eNOS has not been investigated. Given the known caveolar localization of both eNOS and FXYD1, and the functional effect of FXYD1 to protect against redox modification of the vicinyl β_1 subunit of the Na^+-K^+ ATPase in the caveolae, we examined specifically for their physical interaction. As shown in Figure 1A, FXYD1 co-immunoprecipitated with eNOS in freshly isolated rabbit hearts. A close physical interaction of the 2 proteins was supported by the proximity ligation assay results, with the Duolink staining of eNOS and FXYD1 in HUVECs showing a close proximity of <40 nm of the 2 proteins (Figure 1B).

FXYD1 Regulates eNOS Activity and Functional Coupling

Given FXYD1/eNOS were closely associated physically, we examined for any functional effect of FXYD1 on eNOS activity. NO bioavailability was initially assessed using the NO-sensitive fluorescent dye 4-amino-5-methylamino-2′,7′-difluorofluoroscein diacetate under basal conditions, and after stimulation with acetylcholine in HUVECs. FXYD1 knockdown resulted in lower NO bioavailability versus nonspecific control siRNA under basal conditions and when stimulated with acetylcholine (Figure 1C). S-glutathionylation of eNOS occurs at cysteine residues 689 and 908, in the reductase domain of the enzyme, and has been shown to mediate uncoupling.[15] As an indirect measure of redox modification, we measured total free cysteines in HEK293 cells that were transiently transfected with either empty vector or recombinant FXYD1 protein. Conditions of high oxidative stress were simulated by exposing cells to hydrogen peroxide (H_2O_2). The co-immunoprecipitation of free cysteines and eNOS were detected in a series of individual experiments, and each immunoblot is shown in Figure 1D, with quantification performed by calculating the fold change of FXYD1 relative to the empty vector control in each experiment, after normalization to account for the considerable differences in intensity of bands detected on each occasion. Overexpression of FXYD1 increased the proportion of eNOS free cysteines compared with empty vector (Figure 1D) suggesting that FXYD1 protects against oxidative modification of these residues.

We then used tissue from mice deficient in FXYD1, with global knockout of FXYD1. To further validate our findings using monolayer cultures of HUVECs, we measured intracellular NO synthesis in 3-dimensional in vitro vascularized cardiac spheroids containing the diverse cell types found in vivo (endothelial, myocytes, and fibroblasts[23]). These were generated from cells isolated from hearts of wildtype and FXYD1-deficient mice. We found a similar pattern in 3-dimensional cultures, where acetylcholine-stimulated NO production was lower in FXYD1-deficient mice compared with wildtype mice (Figure 2A). We next investigated eNOS enzyme expression in the vasculature of mice. Despite similar expression levels of the enzyme, eNOS phosphorylation at serine 1177, involved in activation of the enzyme, was lower in FXYD1 knockout mice than their wildtype controls (Figure 2B). This may contribute to lower NO bioavailability.

FXYD1 Protects Against Superoxide Generation and Nitrosative Stress

Given the emerging evidence that FXYD1 had a protective role in eNOS coupling suggested by enhanced NO bioavailability and proportion of eNOS free cysteines, we examined for any associated change in oxidative and nitrosative stress. In mesenteric tissue of mice, FXYD1 knockout was associated with an increase in nitrotyrosine levels (Figure 2C). We next investigated superoxide production, first in a real-time cell culture model, and then in freshly extracted tissue lysate from mice. HUVECs were incubated with dihydroethidium for 10 minutes before Ang II was added and incubated for an additional 50 minutes. Under basal conditions, FXYD1 knockdown had no significant effect on superoxide production (Figure 3A). However, under conditions of Ang II exposure, FXYD1 knockdown substantially augmented superoxide production by 67% compared with nonspecific siRNA (Figure 3A). We then measured superoxide generation in tissue from FXYD1 wildtype and knockout mice using a NADPH-dependent lucigenin assay. In the aorta from FXYD1 knockout mice, we saw higher generation

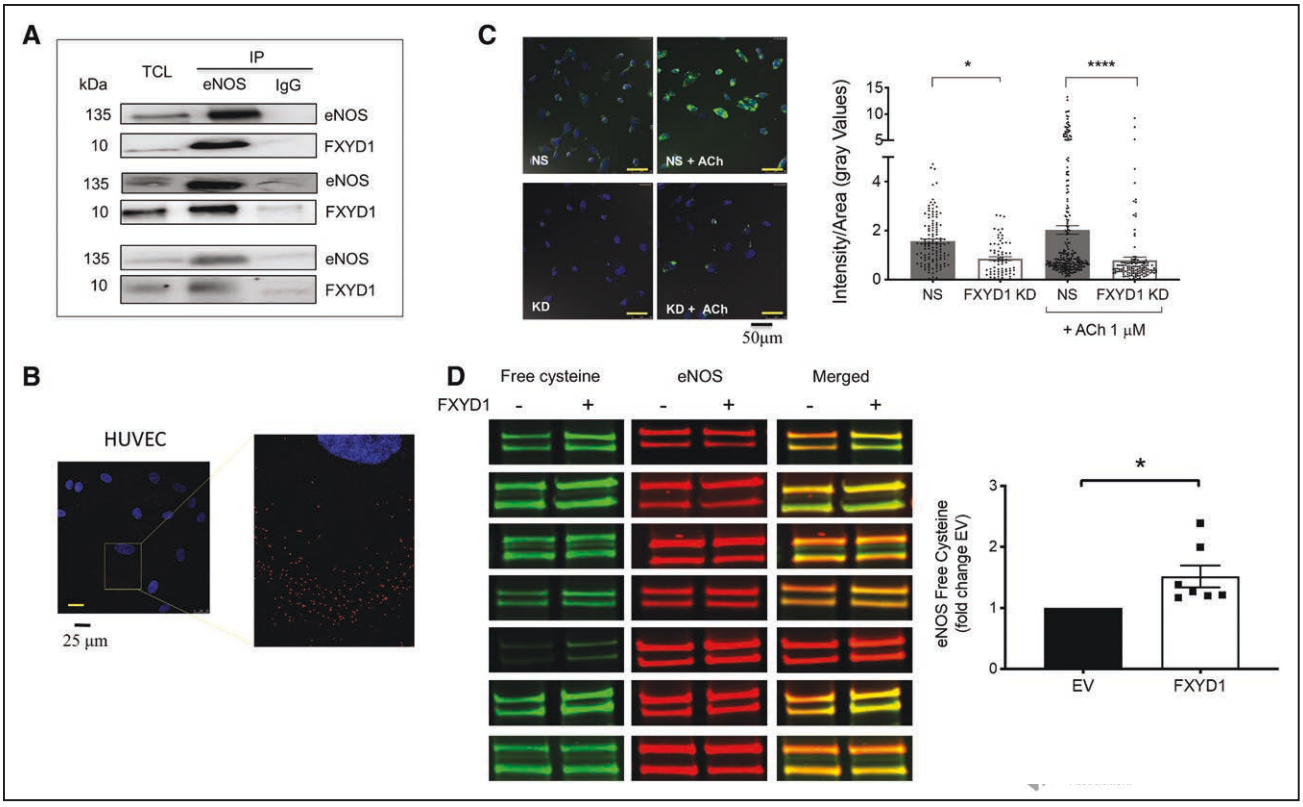

Figure 1. FXYD1 (FXYD domain containing ion transport regulator 1) and eNOS (endothelial nitric oxide synthase) share a functional partnership.

A, Co-immunoprecipitation studies in protein lysate from rabbit hearts show an association between FXYD1 and eNOS. Immunoprecipitation (IP) of eNOS (mouse anti-eNOS, 610297, BD Biosciences, 1:1000) or immunoglobulin G (IgG) was performed, with the acquired sub-fraction then immunoblotted (IB) for eNOS (rabbit anti-eNOS, 32027, Cell Signalling Technologies, 1:1000) and FXYD1 (rabbit anti-FXYD1 ab76597, Abcam, 1:1000). Results are shown compared with the expression in total cell lysate (TCL) from the hearts (n=3). **B**, Physical association of FXYD1 and eNOS in HUVECs was shown by proximity ligation assay. Each red dot is indicative of FXYD1 and eNOS co-expression within 40 nm. DAPI stain for cell nucleus is shown in blue. **C**, DAF staining (green) of NO with DAPI (blue) of cell nuclei in representative confocal images from HUVECs with nonspecific (NS) or FXYD1 knockdown (KD) by siRNA. Summary data demonstrated significantly reduced bioavailability of NO in FXYD1 silenced HUVECs at both basal level and after acetylcholine (ACh; 1 µmol/L) stimulation (n=70–240 cells from 3 separate experiments). **D**, eNOS free cysteines were measured by labeling free cysteines on eNOS using fluorophore-conjugated maleimide in HEK293 cells transfected with either empty vector (EV) or FXYD1 and stimulated with hydrogen peroxide (H_2O_2, 200 µmol/L) >all 7 immunoblots of the co-immunoprecipitation are shown in the **left**, with free cysteines in the green channel (800 nm), eNOS in the red channel (680 nm), and merged channels shown to the **right**. The far-**right** shows quantification of free cysteines/eNOS expression as a fold change relative to the control of each individual experiment. Data mean±SEM. $P<0.05$, $P<0.0001$ vs NS by 1-way ANOVA with Bonferroni multiple comparison analysis or Student *t* test.

of superoxide (Figure 3B). Yet, superoxide production in mesenteric resistance vessels was not different between the mice under baseline conditions (Figure 3C). To examine for additional enzymatic contributors to the superoxide levels, we studied NADPH oxidase isoform expression. Neither Nox 1 (Figure S1 in the Data Supplement) or Nox 2 (Figure 3D) expression were altered in association with FXYD1 knockout, and Nox4 isoform of NADPH oxidase was decreased ≈50% in the FXYD1 knockout compared with wildtype (Figure 3E).

FXYD1 Plays a Key Protective Role in Vascular Function In Vivo

Given the functional importance of protecting against ROS-induced eNOS uncoupling in cardiovascular health, we investigated the effect of FXYD1 absence on blood pressure in vivo. BP was monitored using radiotelemetry in freely moving, undisturbed mice, and normal circadian rhythms were observed. There were no differences in systolic or diastolic BP under basal conditions in FXYD1 knockout mice compared with wildtype controls (Figure 4A, Table S1). Heart rate was also similar in wildtype and knockout mice (Table S1). We next measured mean arterial BP in cannulated, anesthetized mice, and determined responses to the endothelial-dependent vasodilator bradykinin and the NO donor, sodium nitroprusside. Both of these drugs evoked substantial, concentration-dependent reductions in mean arterial BP in wildtype mice. FXYD1 knockout mice had substantially impaired BP responses to bradykinin (Figure 4B and 4C) but no effect on BP reductions

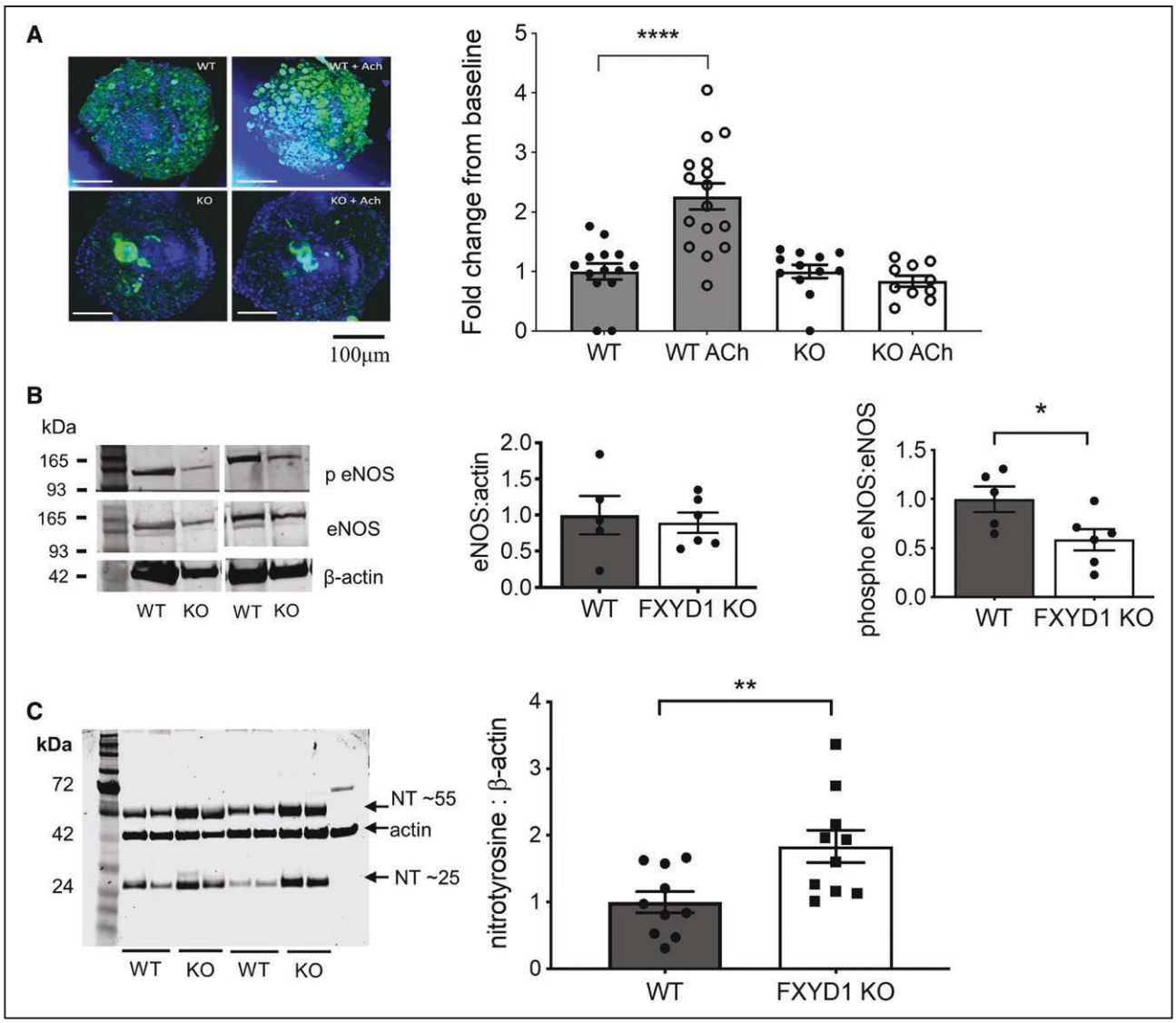

Figure 2. FXYD1 (FXYD domain containing ion transport regulator 1) deficiency is associated with reduced nitric oxide (NO) bioavailability and nitrosative stress.

A, Representative confocal images and summary data from vascularized cardiac spheroids from wildtype (WT) and FXYD1 knockout (KO) mice (n=10–16 spheroids from 3 separate isolations of 3–6 pooled neonates). Data mean±SEM. $P<0.05$, $P<0.0001$ vs NS by 1-way ANOVA with Bonferroni multiple comparison analysis. **B**, eNOS (endothelial NO synthase) expression (mouse anti-eNOS, 610297, BD biosciences, 1:1000) relative to β-actin (mouse anti-beta actin, MAP1501, Merck Millipore, 1:5000) and phosphorylated eNOS (serine 1177, anti-phospho-eNOS No. 9571, Cell Signaling Technologies, 1:1000) relative to eNOS in mesenteric vessels shown by immunoblotting. **C**, Nitrotyrosine levels detected by immunoblotting (combined density of bands 25 and 55, mouse anti-nitrotyrosine, 61392, Abcam, 1:1000) in mesenteric vessels of WT and FXYD1 KO mice *$P<0.05$ and **$P<0.01$ by Student *t* test, n=6–10. ACh indicates acetylcholine.

using sodium nitroprusside (Figure 4D). These data suggest that FXYD1 plays an important role in endothelial-dependent relaxation, but signaling downstream of NO was normal.

Potential Therapeutic Opportunity—Delivery by Exosomes

To explore the therapeutic potential related to our findings, we developed a novel delivery method of FXYD1. FXYD1 was packaged into exosomes by over-expressing myristoylation-palmitoylation (MyrPalm) tagged FXYD1 in HEK293T cells and purifying exosomes from the HEK293T culture medium. The precipitant from harvested cell effluent was resuspended in PBS. FXYD1-exosomes, or control exosomes were administered intravenously to anesthetized FXYD1 knockout mice, following baseline bradykinin response measurements. Ninety minutes after FXYD1-exosome delivery, bradykinin responses were repeated. Delivery of FXYD1-exosomes to the knockout mice resulted in detectable FXYD1 expression in mesenteric tissue 90 minutes after delivery, although this was substantially

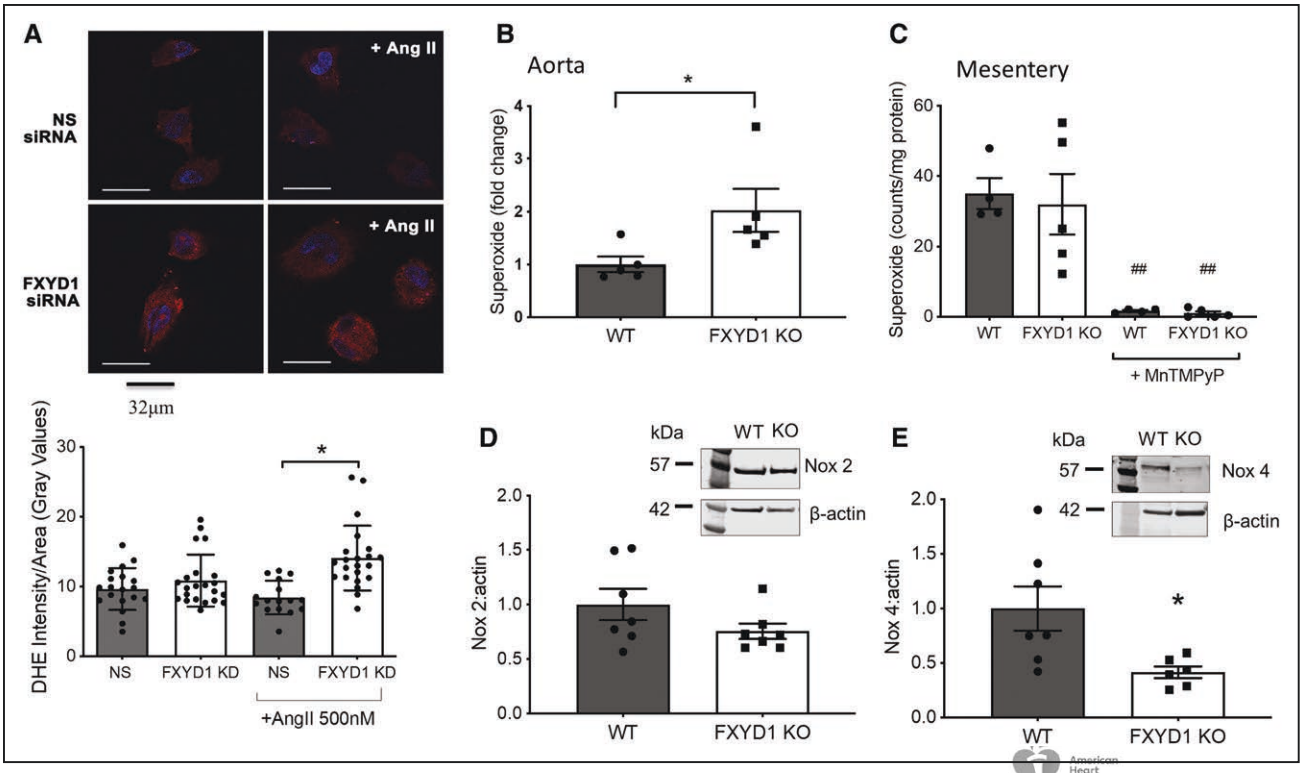

Figure 3. FXYD1 (FXYD domain containing ion transport regulator 1) deficiency contributes to excessive superoxide generation. **A**, dihydroethidium (DHE) staining demonstrating increased superoxide production after FXYD1 silencing and Ang II (angiotensin II) 500 µmol/L (+Ang II), compared with nonspecific (NS) siRNA. Red is indicative of DHE while blue shows the cell nucleus stained with DAPI. Data mean±SEM. n=16–22 cells from 3 experiments, ****P<0.0001 vs NS, #P<0.05 vs basal (−Ang II) by 1-way ANOVA with Bonferroni multiple comparison. **B**, Superoxide generation measured by 20 µmol/L lucigenin-enhanced chemiluminescence was higher in aortae of FXYD1 knockout (KO) mice but was not different in mesentery (**C**) and this was confirmed to be superoxide as superoxide dismutase mimetic MnTMPyP 30 µmol/L completely abolished the lucigenin response. Data summarized as mean±SEM. *P<0.05 vs wildtype (WT) by 1-way ANOVA; n=4–6. **D**, Expression of NADPH oxidase isoforms 2 (rabbit anti-NOX2/gp91phox, ab129068, 1:5000) and (**E**) expression of NADPH oxidase isoform 4 (rabbit anti-NADPH oxidase 4, ab133303, 1:5000) in mesentery vessels by immunoblotting from FXYD1 WT and KO mice. *P<0.05 vs WT by Student t test, (n=6–7).

less than the expression of that from tissues in wildtype animals (Figure 5A and Figure S2). Delivery of control exosomes (empty vector) did not significantly alter BP, or bradykinin-induced response (Figure 5B). However, despite the low expression, FXYD1-exosome delivery was associated with significantly augmented bradykinin-induced vasodilatation compared with knockout control (Figure 5B). The result was an almost complete rescue of the vascular dysfunction by FXYD1-exosomes in FXYD1 knockout mice. Our evidence of increased phosphorylated eNOS in the mesenteric vessels from these FXYD1 exosome-treated mice compared with empty vector controls, (Figure 5C) leads us to speculate that there may be increased eNOS activity after FXYD1 delivery.

FXYD1 Protects Against Redox-Mediated Vascular Dysfunction

Despite FXYD1 knockout resulting in reduced bradykinin-induced decreases in BP, reflecting endothelial dysfunction, we did not detect any major changes in superoxide generation in the resistance vasculature under baseline conditions. We next investigated the effects of FXYD1 knockout on vascular function under conditions of elevated oxidative stress. Ang II, well known to activate NADPH oxidase and shown in Figure 3 to increase superoxide generation in HUVECS, was infused in both an acute and chronic protocol in mice. FXYD1 knockout was associated with an exaggerated pressor response to acute bolus dosing of Ang II in both young and middle-aged mice compared with wildtype (Figure 6A). Baseline BPs from FXYD1 knockout mice measured by direct cannulation were not different between wildtype and knockout, similar to what was found using invasive radiotelemetry (Figure 4A and 6B). However, FXYD1 knockout mice had substantially increased BP in response to subpressor doses of Ang II infused over 4 weeks (Figure 6B). This does not appear to be due to increased Ang II receptor density, as expression of AT1R was not different between wildtype and knockout aged mice (Figure 6D). Stimulation of an endothelium-dependent vasodilator response using bradykinin was

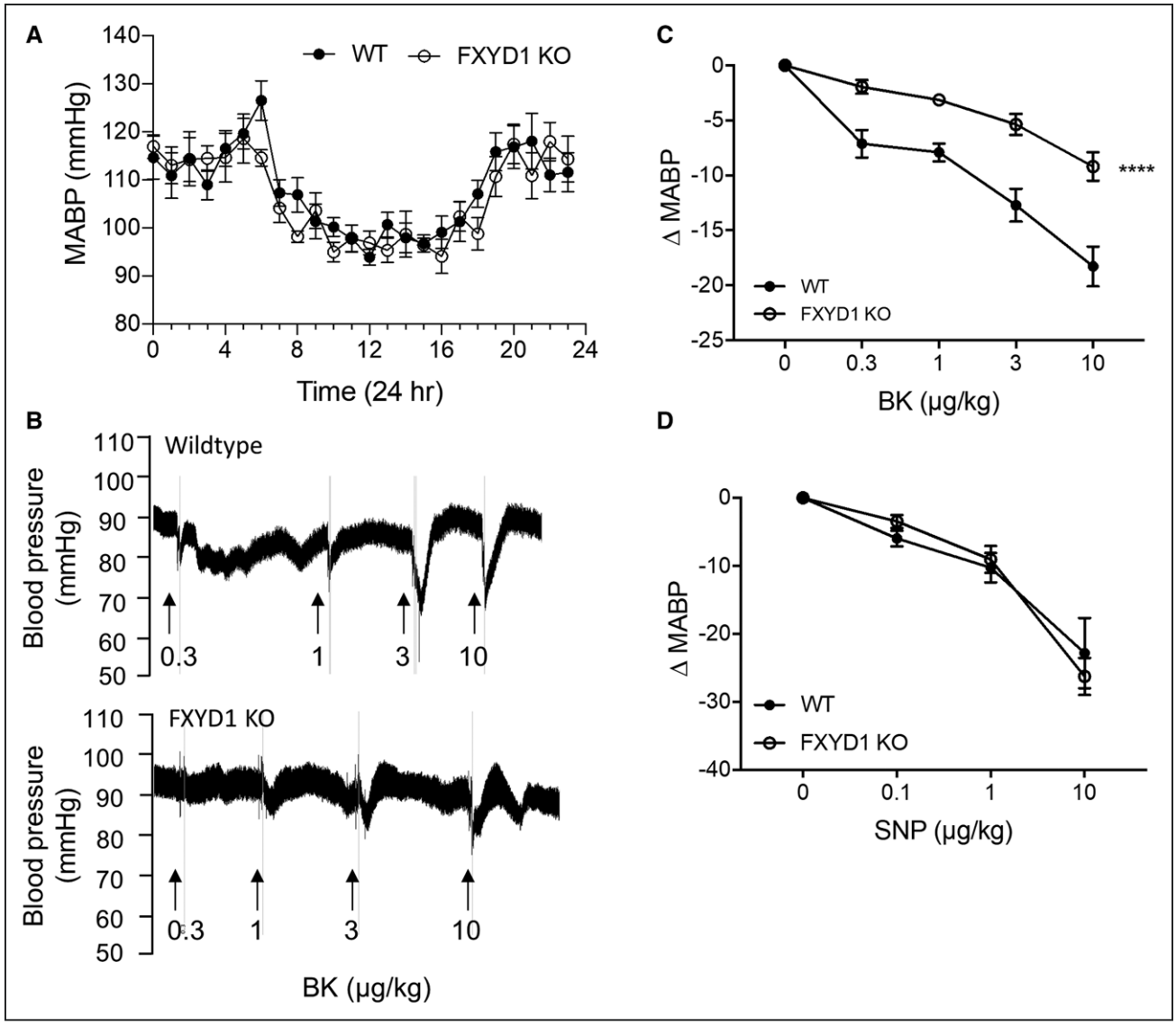

Figure 4. FXYD1 (FXYD domain containing ion transport regulator 1) plays a key protective role in vascular function in vivo. Blood pressure was measured in chronically instrumented mice using radiotelemetry and recorded over 48 h. **A**, Mean arterial blood pressure (MABP) data is shown for the middle 24 h period when mice (n=5) were undisturbed. Blood pressure was also measured in anesthetized mice using an indwelling catheter in the carotid arteries. Jugular veins were cannulated for the intravenous administration of bolus doses of vasodilators. Acute BP changes in response to bolus doses were recorded. **B**, Representative images of change in BP evoked by BK (bradykinin) in vivo in Wildtype (wildtype [WT], n=10) and FXYD1 knockout (KO; n=7) mice. **C**, Summary data of BP changes after BK. **D**, BP changes evoked by sodium nitroprusside (SNP) in vivo. Data summarized as mean±SEM. ***P<0.001 and ****P<0.0001 vs WT by ANOVA.

abolished in mice treated with Ang II, but this was not worsened in FXYD1 knockout mice (Figure 6C). We also determined whether aging or diabetes affected BP responses but found that anesthetized MABP responses were not different in FXYD1 knockout mice versus wildtype in either middle-aged or diabetic conditions, and in both cases, BP was comparable to young, normoglycemic control mice (Table S2). Despite the lack of effect of FXYD1 knockout on baseline BP under these conditions, acute responses to the endothelium-dependent vasodilator bradykinin were impaired in knockout diabetic (Figure 6E) and aged (Figure S3) mice compared with wildtype. Like the nondiabetic control mice in Figure 3C, mesenteric vessels from FXYD1 knockout normoglycemic mice showed no differences in mesenteric superoxide generation, but superoxide levels were elevated 5-fold by FXYD1 knockout in mice under diabetic conditions (Figure 6F).

DISCUSSION

Despite our longstanding knowledge regarding the role of dysregulated oxidative signaling in many forms of CVD, we have made little progress in therapeutically targeting this.[27] Furthermore, we have limited

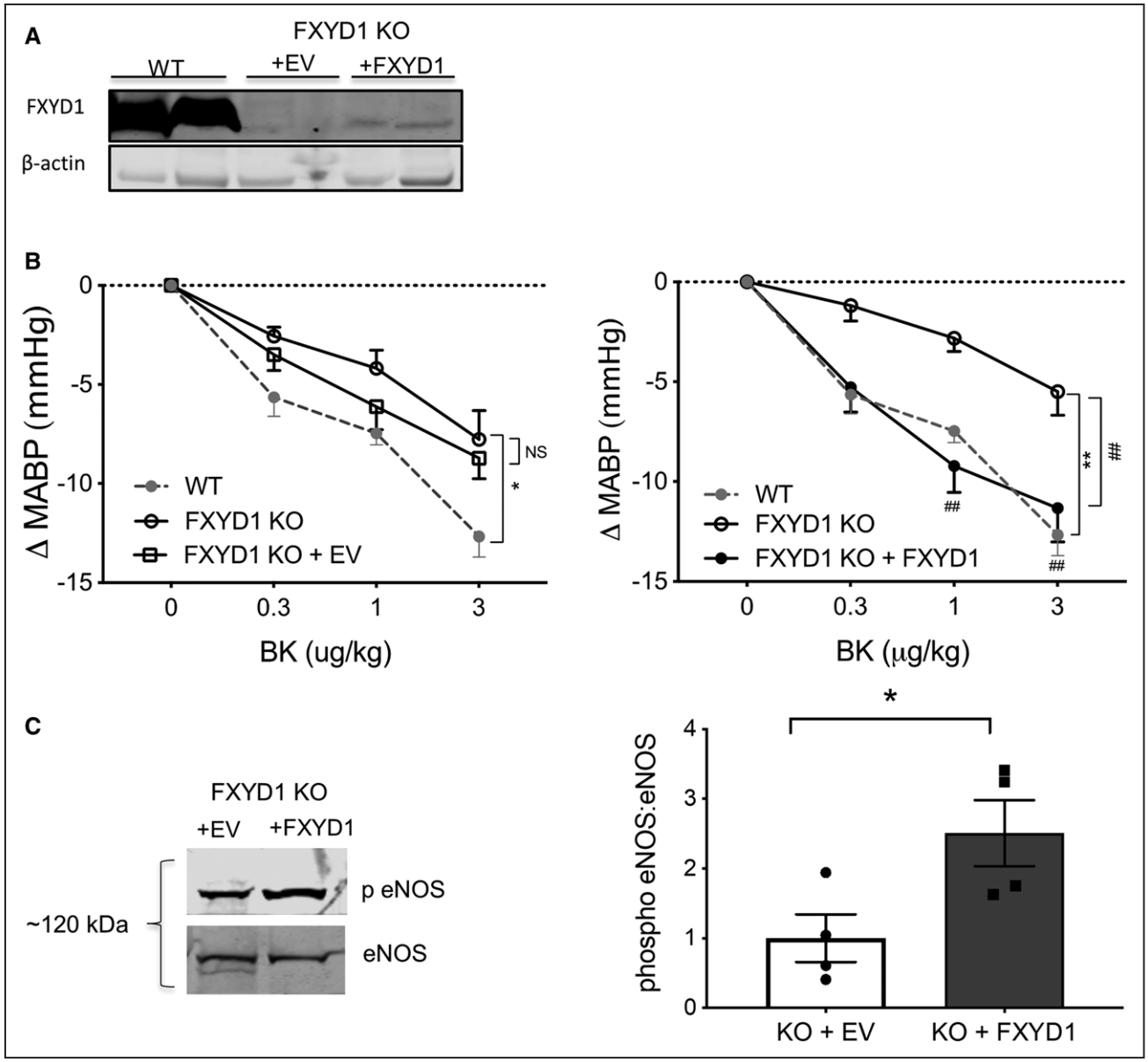

Figure 5. Potential therapeutic opportunity of FXYD1 (FXYD domain containing ion transport regulator 1) protein delivery. FXYD1 was delivered to the blood vessel lumen of FXYD1 knockout (KO) mice by injection (intravenous) of empty vector (EV) or FXYD1 transfected exosomes. **A**, FXYD1 expression (rabbit anti-FXYD1 ab76597, Abcam, 1:1000) was not evident in mesenteric vessels of FXYD1 KO EV-treated mice, but faint expression was detected in the mesenteric vessels of FXYD1 exosome-treated mice. **B**, Reductions in blood pressure (BP) in response to BK (bradykinin) were recorded before and 75 min after injection of exosomes. Compared with wildtype (WT) mice (n=15, 5 new, and 10 from Figure 4B), FXYD1 KO mice had impaired bradykinin-induced pressor responses and these were not altered 75 min after injection of empty vector (EV) exosomes (n=7). Exosomes containing FXYD1 caused increased BP vasodilator responses compared to pre-FXYD1 control responses (n=7) and were almost superimposed with WT mice. **C**, Phosphorylated eNOS (endothelial NO synthase) expression relative to total eNOS expression in mesenteric vessels was increased in response to FXYD1 exosome delivery (mouse anti-eNOS, 610297, BD biosciences, 1:1000; anti-phospho-eNOS serine 1177 No. 9571, Cell Signalling Technologies, 1:1000). Data summarized as mean±SEM. MABP indicates mean arterial blood pressure. **P*<0.05, *****P*<0.0001 vs WT, #*P*<0.01, ##*P*<0.01 by Student *t* test or 2-way ANOVA.

understanding of the cell's own mechanism of redox protection in the important signaling domain of the caveolae. By harnessing naturally occuring protein interactions to inhibit oxidative dysregulation, or by fixing disturbed redox signaling within these sub-cellular redox hotspots, we may be able to overcome some of the challenges with translation of antioxidant therapy, that we have faced previously.[10] Our findings from this study demonstrate that the small membrane protein FXYD1 has a novel functional partnership with eNOS, protecting it from uncoupling in the endothelium, and re-establishing NO/ROS balance in a number of different disease-relevant models. Harnessing this knowledge may have important therapeutic implications for redox-dependent vascular disease, including hypertension and atherosclerosis.

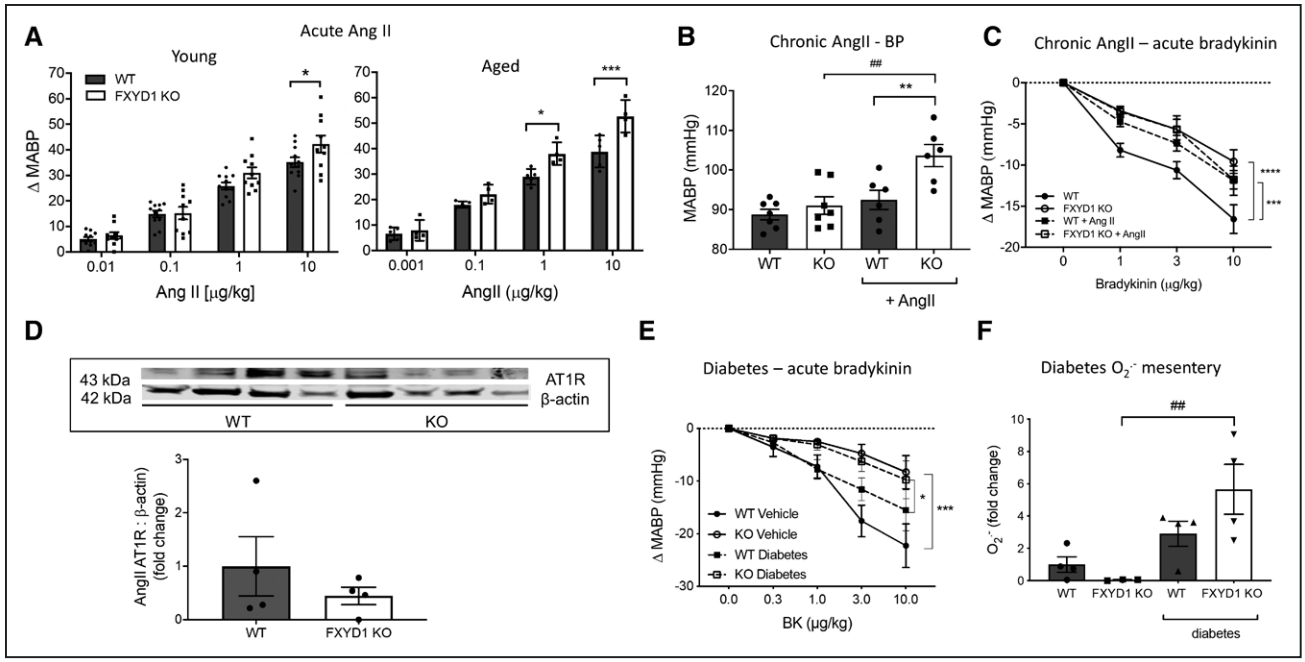

Figure 6. FXYD1 (FXYD domain containing ion transport regulator 1) protects against vascular dysfunction under conditions of pathophysiological redox stress.

A, Acute bolus doses of Ang II (angiotensin II) were administered to mice by intravenous injection and mean arterial blood pressure (MABP) responses were recorded. In young mice, Ang II caused mildly elevated MABP in FXYD1 knockout (KO) vs wildtype (WT) mice at the highest dose. In middle-aged mice, Ang II caused greater increases in MABP in FXYD1 KO vs WT mice. **B**, Chronic infusion of a sub-pressor dose of Ang II over 4 wk did not cause an increase in MABP in WT mice but substantially increased MABP in FXYD1 KO mice. **C**, MABP responses to BK (bradykinin) were impaired in KO Ang II-infused hypertensive mice vs WT Ang II-infused mice. **D**, There was no change in Ang II receptor expression (AT1R) relative to β-actin (rabbit anti-Ang II type 1 receptor, ab124743, 1:1000; mouse anti-beta actin, MAP1501, Merck Millipore, 1:5000), in KO mice after Ang II infusion. **E**, MABP responses to bradykinin were impaired in KO diabetic mice vs WT diabetic mice, and (**F**) superoxide generation was higher in diabetic KO mice. **P*<0.05, ***P*<0.05, ****P*<0.001 vs WT, #*P*<0.01, ##*P*<0.01 by Student *t* test or 2-way ANOVA.

FXYD1 is associated with a reversal of S-glutathionylation of β_1 subunit the Na^+-K^+-ATPase and, therefore, protection against oxidative inhibition.[17] Being colocated with the Na^+-K^+-ATPase in the caveolae, we speculated that FXYD1 might possess a broader role and protect eNOS in a similar manner. This is particularly important given the relative paucity of redox protective mechanisms identified in the caveolae, despite housing 2of the cell's greatest producers of ROS.[11] Here, we have shown that eNOS and FXYD1 colocalize with each other in caveolae, using the proximity ligation assay, which supports a physical distance of <40 nmol/L. However, we need to take the size of the caveolae into consideration (≈60–80 nm as measured by electron microscopy),[28] and recognize that the strongly positive results of the proximity ligation assay may simply reflect their cohabitation in the caveolar microdomain. Complementary evidence for direct physical interaction of the proteins is provided by the co-immunoprecipitation experiments (Figure 1A), although this again may be indirect. Functional evidence was key to showing the relationship between eNOS and FXYD1.

The functional effect of FXYD1 to protect eNOS from uncoupling is supported by molecular data, superoxide measures in vitro and in vivo vascular function studies. S-glutathionylation of the key cysteine residues (689 and 908) in the reductase domain of the enzyme has been implicated as a major mechanism of eNOS uncoupling, associated biochemically with a decrease in NO production by the enzyme of 70% and an increase of superoxide production by 5-fold.[15] We found an increase in eNOS free cysteines in cells overexpressing FXYD1 under simulated oxidative stress. Thus, it appears that a protection against S-glutathionylation with an associated increase in free cysteines could be contributing to the vascular protective effect observed in association with FXYD1 expression. Analysis of S-glutathionylation using immunoprecipitation in hearts from wildtype and knockout mice was attempted to further support this hypothesis; however, difficulties in distinguishing between glutathionylated eNOS and immunoglobulin G bands in the tissue lysate made the findings inconclusive.

The functional effect of this lack of NO bioavailability was clear, with a striking impairment of the vasodilation response when the endothelium-stimulating vasodilator, bradykinin was used. This was apparent even under nonstressed conditions but did not translate to a hypertensive response. However, once a physiologically relevant stress was induced (Ang II at a sub-pressor dose),

FXYD1 knockout was associated with augmented pressure response in vivo. Our interpretation of this finding is that there may have been compensatory adaptation with either upregulation of other vasodilating pathways or downregulation of constrictor activity. The latter is less likely given that we demonstrated increased vasoconstrictor responses in FXYD1 knockout mice to both Ang II (Figure 6A) and phenylephrine (data not shown).

We went on to examine the consequence of FXYD1 knockout in a model of diabetic vascular oxidative stress,[19] by infusing an insulin-receptor antagonist to simulate insulin resistance. While the diabetic mice remained normotensive, the impact of FXYD1 knockout led to impaired pressor responses in diabetics and this was accompanied by a 5-fold increase in superoxide generation in the resistance vessels. This would likely have impacted on BP if the model was taken out beyond the short period of 4 weeks. As far as we are aware, this is the first study to show a relationship between FXYD1 and vascular complications of diabetes.

A key observation from hemodynamic monitoring was that both young and middle-aged FXYD1 knockout mice had a heightened BP response to bolus doses of Ang II. We postulate that this is due to redox-NO imbalance that is exacerbated by Ang II-dependent NADPH activation. We showed that even under nonstressed conditions that NADPH oxidase-dependent superoxide generation was higher in the aortic vasculature of FXYD1 knockout mice. However, it may also be receptor-dependent, but we think that this is unlikely as AT1R expression was not altered. The effect may also be a result of modified calcium-dependent contractility via second messengers (ie, IP3-PKC mediated). Another plausible explanation is that reduced NO activity leads to increased sensitivity of smooth muscle contractile proteins to calcium, as we know that NO can decrease the calcium sensitivity and shift the myosin light chain kinase/phosphatase balance.[29] There are also hints that antioxidant activity may be altered in the presence of Ang II, and this could contribute to elevated BP seen in FXYD1 knockout mice in the setting of neurohormonal dysregulation that is a common feature driving human CVD.

FXYD1 expression was also associated with eNOS phosphorylation at serine 1177, known to be involved in enzyme activation, particularly in response to agonists such as VEGF.[30] The interdependence of post-translational modifications and their impact on the regulation of function is fascinating to consider, as has been reported for PKG1α.[31] It is feasible that S-glutathionylation of cysteines 689 and 908 may have steric impact on the enzyme, decreasing phosphorylation at serine 1177, or vice versa- that phosphorylation at serine 1177 is protective against oxidative modification of the cysteines in the reductase domain. S-nitrosylation, as we observed in the setting of FXYD1 silencing, may also play into the equation. It is known to facilitate eNOS phosphorylation at serine 1177 in an autoregulatory fashion, with denitrosylation leading to decreased serine 1177-mediated eNOS activity by a mechanism that may involve depalmitoylation.[32] The interaction of these post-translational modifications is of broad interest beyond this current study.

NO can induce serine-mediated phosphorylation of FXYD1 via a PKC-epsilon dependent mechanism, which has a regulatory role in cardiac Na^+-K^+ ATPase activity.[27] Here, we have shown a reciprocal relationship where FXYD1 regulates eNOS activity. The mechanism by which FXYD1 acts to protect eNOS from uncoupling and remains unclear. Although the co-immunoprecipitation and proximity ligation assays support a direct physical interaction with eNOS as a possible mechanism for FXYD1, it has not been shown to have direct deglutathionylating ability. There are 2 cysteines separated by a single amino acid in a C1-R-C2 motif that are highly conserved across the entire FXYD family, as well as across species.[17] The resemblance to the CXXC motif seen in the redoxin family[33] is suggestive of a similar mechanism. However, in the case of the Na^+-K^+ ATPase's β1 subunit, protection by FXYD1 against ROS-induced S-glutathionylation and inhibition of the Na^+-K^+ ATPase was shown to be dependent on just one of these—Cys40, that was flanked by basic amino acids, a classic feature of reactive cysteines.[34] Despite the clear role of Cys40 in the protective effect of FXYD1 on the β1 subunit, it is still likely that this occurs indirectly. In the crystal structure of the Na^+-K^+ ATPase,[35] it is challenging to consider how the reactive cysteine of the β_1 subunit that mediates redox inhibition of the Na^+-K^+ ATPase (Cys46)[36] could directly interact with the FXYD subunit of the complex. One possibility, given the known calcium sensitivity of eNOS, is that FXYD1 protection against redox inhibition of the Na^+-K^+ ATPase improves eNOS activity by altering sub-cellular Ca^{2+} concentrations. However, such a mechanism would be predicted to have the opposite effect than what is observed. A third possibility is that the FXYD1 effects that protect these 2 important caveolar proteins is mediated via an, as yet unknown, protein-protein interaction, for example somehow increasing the caveolar localization and activity of members of the redoxin family such as peroxiredoxin 6, or glutaredoxin 1 which are known to possess biochemical deglutathionylation capabilities.

While we have demonstrated a clear physical and functional relationship between eNOS and FXYD1 in human endothelial cells in vitro, our in vivo functional measures are limited by the lack of direct evidence of endothelium-dependency of the protective effect of FXYD1, given the systemic nature of the knockout model. While the differential protective effect of FXYD1 on bradykinin versus sodium nitroprusside is supportive of a predominant endothelial-dependent mechanism, it is feasible that FXYD1 may modulate vascular function,

in part, via effects on the Na^+-K^+ ATPase of the vascular smooth muscle cell. We have previously shown that FXYD1 is expressed in human vascular smooth muscle cells, and that its protection against redox inhibition of the Na^+-K^+ ATPase has beneficial effects on vascular tone in the setting of Ang II.[37] However, this is unlikely to explain the impaired vasodilation response to bradykinin reflected in acute decrease in BP, a known endothelial-dependent response, that was not seen in response to a direct NO donor (Figure 4).

The caveolae is an intriguing signaling microdomain, which although facilitatory for receptor-coupled, physiological redox signaling in health, has the potential to be switched to a bonfire of dysregulated ROS,[38] with severe consequences for the function of redox-sensitive proteins such as receptors, channels, pumps, and signal-transduction molecules located in the immediate vicinity. The lack of clinical success of dietary antioxidants is not surprising in this context. However, our findings that FXYD1 is protective against eNOS uncoupling has therapeutic implications. To test the therapeutic potential of FXYD1 and its derivatives, we have packaged FXYD1 protein into exosomes which were able to be delivered through injection in a proof-of-principle study. The delivered FXYD1 protein rescued the bradykinin response in FXYD1 knockout mice in acute experiments. It is an ongoing challenge to optimize the delivery of FXYD1 acute and chronic therapeutic intent.

While this article has focussed on the vascular implications of FXYD1/eNOS interactions, the functional significance of FXYD1/eNOS in cardiac myocyte physiology where both proteins are independently known to be highly expressed and key to cardiac function and pathophysiology,[39,40] has not been explored. This is an important program of work beyond the scope of the current study.

In conclusion, we have identified a novel functional partnership of FXYD1 and eNOS. We concluded that FXYD1 protects the vasculature from redox-mediated dysfunction and hypertension, with beneficial effects in the setting of neurohormonal dysregulation and diabetes.

PERSPECTIVES

NO is a critical regulator of vascular health. Oxidative dysregulation of NO bioavailability underlies most vascular pathologies. Previous work has determined that S-glutathionylation of eNOS can render it inactive, leading to 70% reduction in NO and this is accompanied by out-of-control superoxide generation. In the current study, we showed for the first time that FXYD1 knockout is accompanied by vascular dysfunction that is associated with excessive superoxide generation. Our evidence suggests that FXYD1 may protect eNOS from S-glutathionylation-dependent oxidative inhibition, and this could be beneficial in CVDs including hypertension and diabetes. Future studies should determine the wider implications of this relationship in vascular diseases and investigate the most pragmatic approaches to using FXYD1 as a therapeutic target.

ARTICLE INFORMATION

Received December 22, 2020; accepted March 29, 2021.

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Acknowledgments

G.A. Figtree is supported by a National (Australia) Health and Medical Research Council (NHMRC) Practitioner Fellowship, as well as New South Wales Office of Health and Medical Research and Heart Research Australia. This study was funded by a project grant from the NHMRC (APP1080468).

Sources of Funding

This work was supported by National Health and Medical Research Council (Australia) Practitioner Fellowship (APP1135920 to G.A. Figtree); Heart Research Australia (to G.A. Figtree).

Disclosures

None.

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REVIEW
published: 23 August 2018
doi: 10.3389/fphys.2018.01167

The Regulation of Pulmonary Vascular Tone by Neuropeptides and the Implications for Pulmonary Hypertension

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Pulmonary hypertension (PH) is an incurable, chronic disease of small pulmonary vessels. Progressive remodeling of the pulmonary vasculature results in increased pulmonary vascular resistance (PVR). This causes secondary right heart failure. PVR is tightly regulated by a range of pulmonary vasodilators and constrictors. Endothelium-derived substances form the basis of most current PH treatments. This is particularly the case for pulmonary arterial hypertension. The major limitation of current treatments is their inability to reverse morphological changes. Thus, there is an unmet need for novel therapies to reduce the morbidity and mortality in PH. Microvessels in the lungs are highly innervated by sensory C fibers. Substance P and calcitonin gene-related peptide (CGRP) are released from C-fiber nerve endings. These neuropeptides can directly regulate vascular tone. Substance P tends to act as a vasoconstrictor in the pulmonary circulation and it increases in the lungs during experimental PH. The receptor for substance P, neurokinin 1 (NK1R), mediates increased pulmonary pressure. Deactivation of NK1R with antagonists, or depletion of substance P prevents PH development. CGRP is a potent pulmonary vasodilator. CGRP receptor antagonists cause elevated pulmonary pressure. Thus, the balance of these peptides is crucial within the pulmonary circulation (**Graphical Abstract**). Limited progress has been made in understanding their impact on pulmonary pathophysiology. This is an intriguing area of investigation to pursue. It may lead to promising new candidate therapies to combat this fatal disease. This review provides a summary of the current knowledge in this area. It also explores possible future directions for neuropeptides in PH.

Keywords: substance P, calcitonin gene-related peptide, endothelial function, sensory C fibers, lung, right ventricle, pulmonary hypertension

OPEN ACCESS

Edited by:
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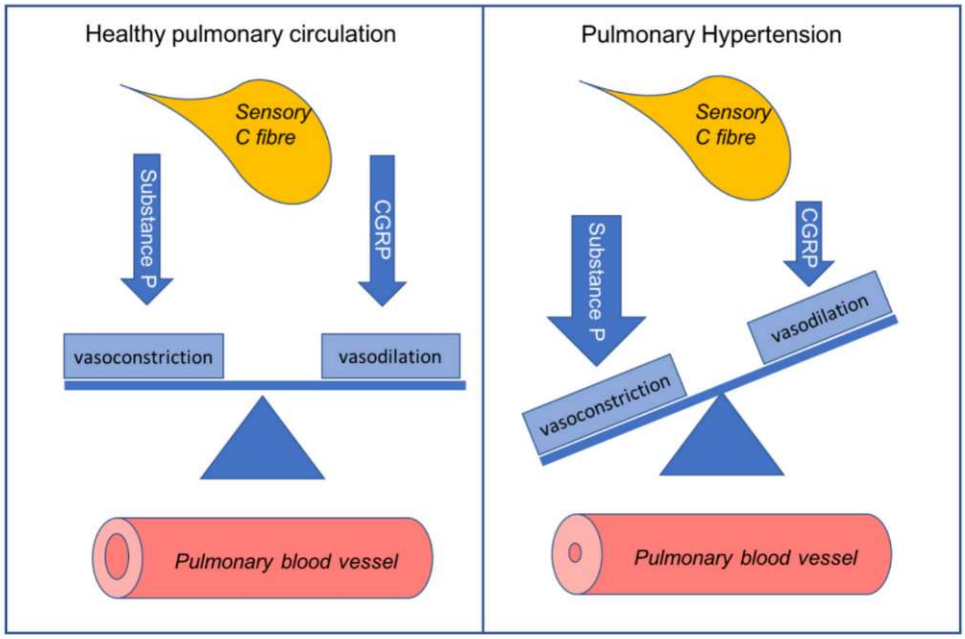
Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *30 April 2018*
Accepted: *03 August 2018*
Published: *23 August 2018*

Citation:
Lo CCW, Moosavi SM and Bubb KJ (2018) The Regulation of Pulmonary Vascular Tone by Neuropeptides and the Implications for Pulmonary Hypertension. Front. Physiol. 9:1167. doi: 10.3389/fphys.2018.01167

INTRODUCTION

Pulmonary hypertension (PH) is a rare chronic disease. Morphological changes in the pulmonary arterioles leads to increased pulmonary arterial pressure. Greater cardiac contractility occurs to overcome the higher pulmonary vascular resistance (PVR). The consequence is right heart remodeling, causing decompensating heart function (McLaughlin et al., 2015). The definition of PH is pulmonary arterial pressure >25 mmHg (or at least 30 mmHg upon exercise). Increased PVR and mean pulmonary wedge pressure occur. Patients suffering from PH show symptoms



GRAPHICAL ABSTRACT |

such as dyspnea, fatigue, chest pain, near syncope, syncope, leg or peripheral edema, angina, palpitations, and abdominal distension (Rich et al., 1987; Barst et al., 2004). PH is hard to diagnose as initial symptoms are non-specific. Fatigue, malaise, and exercise intolerance are often misdiagnosed as the patient being unfit. Asthma is also a common misdiagnosis due to the presence of dyspnoea. In fact, PH diagnosis often takes up to 2 years after symptom onset. Confirmation of PH sometimes occurs only after the progression of right heart failure.

Pulmonary Hypertension Disease Classifications

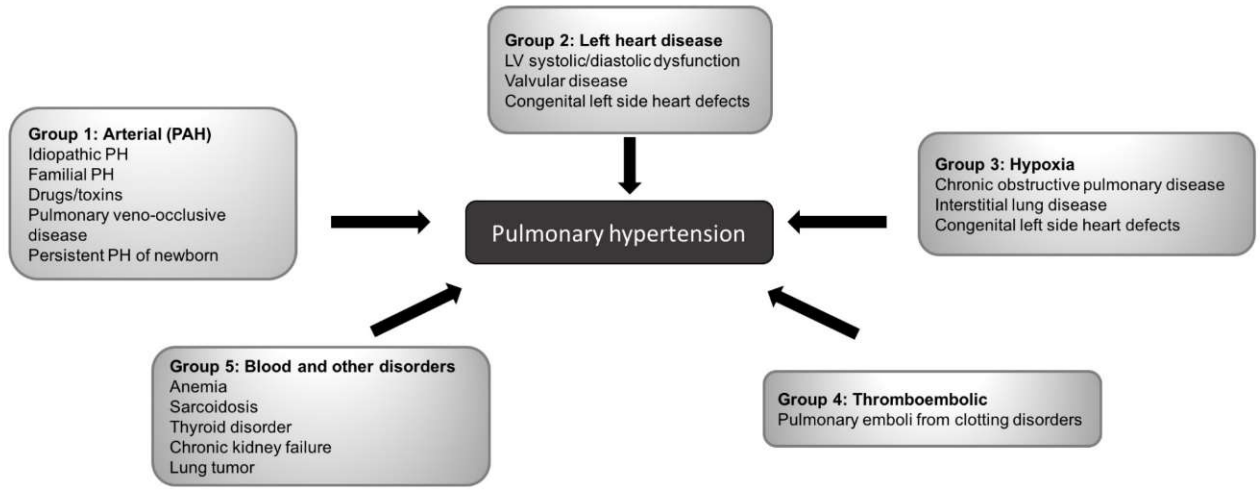
It is increasingly clear that PH has complex, multifactorial pathophysiology. In 1957 PH was categorized into five different groups based on the underlying cause of disease (Brenner, 1957), termed World Health Organization (WHO) groups. These have been further refined at subsequent World Symposiums on PH (Humbert and McLaughlin, 2009) (**Figure 1**).

Group 1 PH is pulmonary arterial hypertension (PAH). The endothelium (inner) layer of blood vessels produces both vasodilators and vasoconstrictors. These are of extreme importance in regulating vascular tone. One of the defining features of PAH is the abnormal function of the pulmonary vascular endothelium. This results in an imbalance in dilators/constrictors, resulting in predominant vasoconstriction. PAH develops from many distinct contributing mechanisms. It is often idiopathic, occurring from (as yet) unknown cause. PAH may arise from hereditary factors, such as the inactivation of the *BMPR2* gene (Cogan et al., 2006; Morrell, 2006). It may develop following human immunodeficiency virus infection (Mehta et al., 2000). PAH can also be induced by drugs. For example, some anti-depressants (Garg et al., 2017) have been implicated in causing PAH and a spate of cases was caused by appetite suppressant drugs (Abenhaim et al., 1996) that have since been withdrawn from the market. **Group 2** PH is due to left-sided heart disease, either congenital or acquired. **Group 3** PH is due to pulmonary hypoxia, which occurs secondary to chronic lung diseases. **Group 4** PH is due to thromboembolic disorders and **Group 5** PH occurs from other disorders including anemia and other blood disorders, tumors, and chronic kidney failure.

For all PH subtypes, patients are also classified into functional classes (Galie et al., 2016), indicating disease severity (**Figure 1**). Patients tend to be diagnosed in functional class II-III and progressively worsen.

The Unmet Need for Effective Treatments for Pulmonary Hypertension

Regardless of the cause of PH, there is common histopathology to all five groups. This includes hypertrophy of vascular smooth muscle cells, fibrosis, vascular wall remodeling and vessel obstruction. While the incidence of PH is low, at ∼15 cases per million people (Galie et al., 2015) the average survival time for a patient left untreated is only 2.8 years (Humbert et al., 2006; Peacock et al., 2007). Since the emergence of effective pharmaceuticals for PH, patient outcomes have substantially improved, as shown in REVEAL (Registry to Evaluate Early and Long-term PAH Disease Management) and French Consortium registries (D'Alonzo et al., 1991; McGoon et al., 2008; Frost et al., 2011; McGoon and Miller, 2012). Although there has been a substantial improvement in quality of life and longevity, the current treatments are far from ideal. For one thing, many patients become resistant to therapy (Morrell et al., 2009).



Functional Class	Symptom / Level of disease
Class I	Symptom-free when physically active or at rest
Class II	No symptoms at rest, discomfort/shortness of breath on mild exertion
Class III	Shortness of breath / tiredness with light activities
Class IV	Symptoms at rest and severe symptoms with activity

FIGURE 1 | PH World Health Organization classification and functional class. Five different major sub-types of PH according to World Health Organization classification and functional classes are out-lined. Adapted from (Humbert and McLaughlin, 2009; Galie et al., 2016).

In addition, PH remains a progressive and terminal disease. Current treatments have shown limited ability to reverse vascular and cardiac remodeling (Bubb et al., 2015). Thus, the search for novel and breakthrough treatments for PH continues in earnest.

In order to establish new PH treatments, it is important to determine how existing treatments can be improved. The first part of this review outlines the current treatments of PH. Subsequently, we review the effects of the sensory C-fiber-derived neuropeptides on the lung circulation. Several neuropeptides show promising effects and are being investigated for PH treatment. These include neuropeptides released from C-fibers: substance P and calcitonin gene-related peptide (CGRP). Both are important regulators of the pulmonary circulation. Modulation of either of the C-fiber-derived neuropeptides can reverse progression of experimental PH. Yet, their use has not progressed beyond pre-clinical research. We also discuss their potential as novel treatments for PH.

CURRENT TREATMENTS FOR PULMONARY HYPERTENSION

Current treatment options for all forms of PH include primary therapies directed at treating the underlying cause of the disease and broad therapies that alleviate the symptoms. General treatment prescribed at the discretion of the primary care physician includes the use of warfarin, diuretics and oxygen. The aims of these therapies are to alleviate volume and viscosity-induced pressure within the pulmonary circulation, reduce hypoxia, and treat right heart failure. Non-pharmaceutical approaches include lung transplant and double heart-lung transplant. Surgical approaches can be utilized in some cases, often when all other options have been exhausted. The primary pharmaceutical therapies that are approved for PH treatment are the main focus of this review and are detailed in subsequent sections.

Targeting the Pulmonary Vascular Endothelium for Treatment of Pulmonary Arterial Hypertension

For the purposes of this review we will focus mainly on Group 1 PAH. Vasoregulatory treatments are most successful in this type of PH (Austin and Loyd, 2015; Bubb et al., 2015; Prior et al., 2016). Thus, neuropeptides that we are investigating are most likely to impact PAH rather than other forms of PH. Disease-targeted treatment options for PAH are centered mainly around the control of pulmonary vascular tone. As outlined in **Figure 2**, major pathways that are currently targeted include endothelium-derived vasodilators and constrictors. The vasodilators of main interest are nitric oxide (NO) and prostacyclin. Both of these are susceptible to being decreased in bioavailability in PAH. These dilators are targeted at multiple levels. Treatments that either modulate their activity or inhibit their metabolism may be used. The endothelium-derived vasoconstrictor, endothelin (Galié et al., 2004) is the main culprit

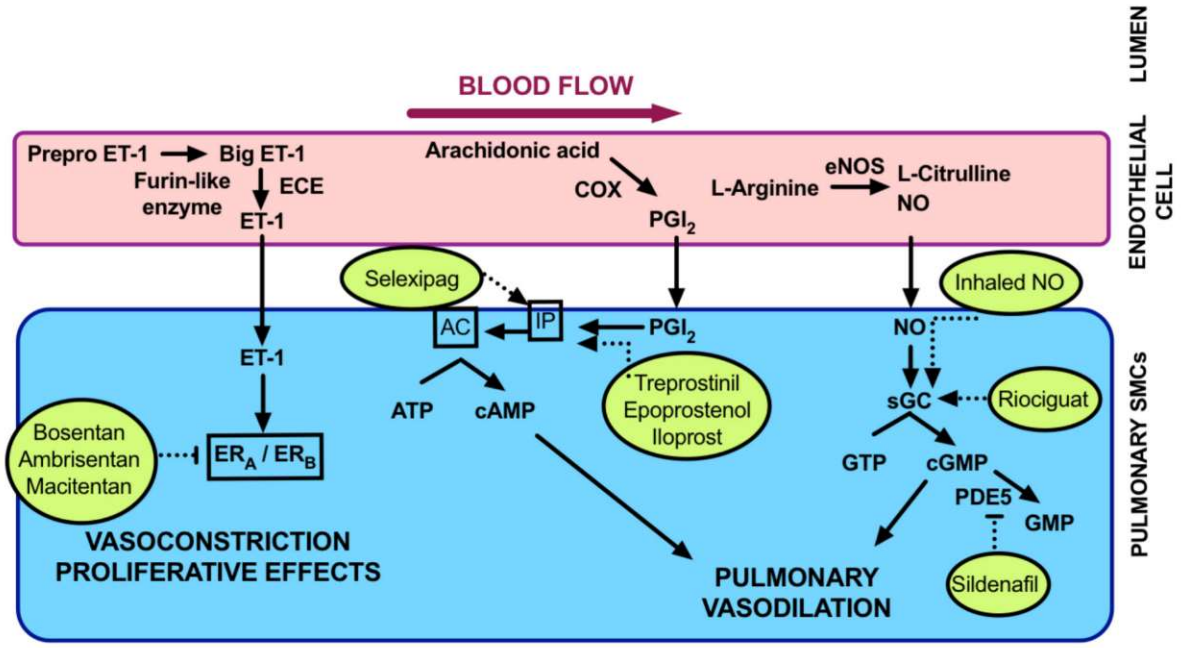


FIGURE 2 | Summary of current PAH treatments. Currently licensed treatments for PAH are shown inside the circles with broken lines indicating their mode of action within vascular signaling pathways. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; ECE, endothelin converting enzyme; eNOS, endothelial nitric oxide synthase; ET-1, endothelin; $ER_{A,B}$, endothelin receptor A,B; GTP, guanosine triphosphate; IP, inositol monophosphate; NO nitric oxide; PDE5, phosphodiesterase 5; PGI_2, prostacyclin; sGC soluble guanylate cyclase.

causing excessive vasoconstriction. Antagonists of the endothelin receptor/s are in clinical use for PAH. Calcium-channel blockers are also used to inhibit the calcium-dependent smooth muscle contraction.

Endothelium-Dependent Pulmonary Vasodilation

The reduction in pulmonary pressure by stimulating pulmonary endothelium-dependent vasodilation resulted in the first approved treatment for PAH. This continues to be the basis behind the development of the majority of PAH treatments.

Nitric oxide-cyclic guanosine monophosphate signaling

NO and the associated signaling pathways have a major role in promoting pulmonary vasodilation. The NO signaling pathway can be summarized as follows: NO activates soluble guanylate cyclase (sGC); this increases the second messenger, cyclic guanosine monophosphate (cGMP); cGMP then activates protein kinase G (PKG); PKG mediates vasodilation by decreasing intracellular calcium by a number of mechanisms. NO also mediates anti-inflammatory and anti-coagulatory effects. The low bioavailability of NO is a major occurrence in patients with PH. This is replicated in pre-clinical models of the disease (Michelakis, 2003). Upregulation of NO, either exogenously or endogenously, has been the primary aim of many decades of trials. Inhaled NO (Steiner et al., 2005) is useful in neonates suffering from persistent PH of the newborn. However, in adults it tends to result in methemoglobinemia, increased pulmonary oedema and a potentially life-threatening hypertensive rebound when therapy is ceased. It also suffers a short half-life and is prohibitively expensive and cumbersome for patients to administer (Michelakis, 2003; Steiner et al., 2005; Baliga et al., 2011). NO donors were developed in an attempt to improve the delivery of NO. Nebulised NONOates (Vanderford et al., 1994; Hampl et al., 1996) and inhaled glyceryl trinitrate (Goyal et al., 2006) effectively lower PVR. Yet they notoriously lack specificity for the pulmonary circulation (Baliga et al., 2011). Thus, targeting of the intermediates in the NO signaling pathway is a more attractive option for improving vasodilation.

In vascular endothelial cells, L-arginine is converted to NO (and the by-product L-citrulline) by endothelial NO synthase (eNOS) in a two-step oxidation reaction. This reaction utilizes nicotinamide adenine dinucleotide phosphate (NADPH), molecular oxygen and the essential cofactor, tetrahydrobiopterin (BH_4) (Palmer et al., 1988; Andrew and Mayer, 1999; Forstermann, 2010). Phosphorylation of eNOS is an important regulatory mechanism for NO generation. It can also occur in response to shear stress, estrogen, vascular endothelial growth factor, and insulin. In normally functioning arteries the major stimulus for eNOS phosphorylation is increased intracellular calcium. This proceeds by binding of calcium to calmodulin in the oxygenase domain of eNOS, increasing the rate of NADPH electron transfer within the domains. In PAH (like other cardiovascular diseases) eNOS can exist in an 'uncoupled state.' Usually the substrate L-arginine or co-factor BH_4 become rate-limiting. In this scenario, eNOS transfers electrons to molecular oxygen, rather than oxidizing L-arginine and superoxide is produced. Superoxide is a reactive oxygen species that is prevalent in vascular dysfunction (Verhaar et al., 2004).

Supplementation with BH_4 improves systemic hypertension (Landmesser et al., 2003). Evidence suggests that this strategy may also be effective in PAH (Baliga et al., 2011). Stimulating the coupling of eNOS and BH_4 to promote NO rather than superoxide production has proven beneficial in experimental (Jin et al., 1992) and clinical PH (Saadjian et al., 1998). Increasing the substrate for NO by L-arginine administration can likewise attenuate PH in the experimental setting (Mitani et al., 1997) and acutely decreases PVR in PH patients (Mehta et al., 1995). Despite the extensive research, no therapeutic targets have been licensed that target NO generation.

Drugs that target the receptor for NO, sGC have proven to be more successful. Activation of sGC by NO is dependent on binding of NO to a heme prosthetic group. This stimulates a conformational change which activates the enzyme. Changes to the redox state of sGC, which can occur in PAH, can result in modulation of sGC expression or activity (Priviero and Webb, 2010; Baliga et al., 2011). Mice lacking sGC are sensitive to experimentally induced PH (Vermeersch et al., 2007). This makes sGC an important target in PH. Pharmacological stimulation of sGC is complicated and drugs fall into two distinct classes. sGC activators, (e.g., cinaciguat) are effective at activating sGC when it is in an oxidized or heme-free state. sGC stimulators (e.g., riociguat) stabilize the enzyme in its active configuration and upregulate sGC activity. There is an advantageous synergistic relationship of sGC stimulators and NO when they are co-administered (Stasch et al., 2011). Experimental PH was ameliorated after treatment with either sGC activators or stimulators (Dumitrascu et al., 2006; Stasch et al., 2011; Lang et al., 2012). Both classes of drugs are promising, but, sGC stimulators have progressed to the clinic first. The PATENT trials demonstrated improved clinical outcome for patients with PAH treated with riociguat (Ghofrani et al., 2012). Riociguat was first FDA-approved for treatment of PAH in 2013 and is now licensed in many countries. It is also the only drug licensed to treat group 4 PH after success in the CHEST trial (Lian et al., 2017).

The other major class of drugs to successfully treat PAH by targeting the NO-cGMP signaling pathway are phosphodiesterase (PDE) 5 inhibitors. PDEs metabolize cyclic nucleotides to their inactive form and some, including PDE5, are specific to cGMP. PDE5 is substantially upregulated in most forms of experimental and clinical PH (Murray et al., 2002; Sebkhi et al., 2003; Wharton et al., 2005; Schermuly et al., 2007) and this led to extensive testing of the efficacy of PDE5 inhibition for PH. Pre-clinical studies have provided insight into the therapeutic mechanisms of PDE5 inhibitors. It has been well-established that sildenafil is effective in inhibiting PH in experimentally induced models (Zhao et al., 2001; Schermuly et al., 2004; Steiner et al., 2005), and this is cGMP-dependent. Both sildenafil (Galie et al., 2005) and tadalafil (Galie et al., 2009) are effective in improving symptoms and outcomes of PH in patients.

Prostacyclin

Prostacyclin was first identified in 1976 (Moncada et al., 1976) and is the main eicosanoid produced by vascular endothelial cells. It is synthesized by cyclooxygenase-dependent conversion from arachidonic acid. Like NO, prostacyclin is also a classic endothelium-dependent vasodilator and also possesses anti-inflammatory and anti-coagulatory properties. Prostacyclin acts predominantly by binding with cell-surface IP receptors which stimulates adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production with downstream effects mediated by protein kinase A (Mitchell et al., 2008). It is a particularly potent vasodilator in the lung circulation and PAH patients often exhibit decreased prostacyclin levels and/or reduced lung prostacyclin synthases (Mitchell et al., 2014). Prostacyclin analogs are a mainstay of PAH treatment but are generally restricted to functional class III and IV patients due mainly to limitations with their administration as outlined below. Prior to the use of epoprostenol (therapeutic prostacyclin) in 1995 (Barst et al., 1996), there was no therapy for PAH, and patients had a 1-year survival of 69% and a 5-year survival of 38% (D'Alonzo et al., 1991). When first introduced, epoprostenol was the only treatment available for PAH. It was widely used even though administration was challenging and side-effects were marked. It has been largely superseded by the next generation of therapeutic prostacyclins, iloprost and treprostinil. These are available in inhalers or by subcutaneous or intravenous routes. Recently the first non-prostacyclin that activates the cAMP signaling pathway has been developed. Selexipag is an IP receptor agonist, which has the benefit of being available in oral formulation (Mitchell et al., 2014). It has proven successful in Phase III trials (Sitbon et al., 2015) and has recently been licensed for PAH treatment.

Pulmonary Vasoconstriction by Endothelin

There are at least three isoforms of endothelin: endothelin (ET)-1, ET-2, and ET-3, of which ET-1 is the most potent and abundant in lungs (Matsumoto et al., 1989; Giaid et al., 1993a). First discovered in 1988 (Hickey et al., 1985; Yanagisawa et al., 1988), ET-1 is a peptide mainly produced by vascular endothelial cells. A number of physical factors affect ET-1 production, including shear stress, epinephrine, angiotensin II, growth factors, cytokines and free radicals (Marasciulo et al., 2006). ET-1 activity is mediated by ET receptors, ET_A and ET_B, both expressed on vascular smooth muscle cells (Seo et al., 1994). ET-1 is produced by ET converting enzyme (ECE) from a precursor molecule of ET (La and Reid, 1995), big ET-1. ET-1 induces increased intracellular calcium via ET_A and ET_B receptor-mediated activation of phospholipase C (PLC) (Pollock et al., 1995) and increases mitogenesis by ET_B-mediated activation of protein kinase C (Ohlstein et al., 1992), leading to both vasoconstriction and cell hyperplasia. ET_B, which is also found on endothelial cells, stimulates local vasodilators such as NO and prostaglandins. It also plays a role in the clearance of ET-1, a feature unique to the lungs (Fukuroda et al., 1994; Kelland et al., 2010). PAH is associated with increased circulating ET-1 levels (Miyauchi et al., 1993). Expression of ET-1 has been found in plexiform lesions of lungs, where higher levels of ET-1 correlate with increased PVR and structural abnormalities (Giaid et al., 1993b; Bressollette et al., 2001). PAH patients may also have reduced clearance of ET-1 in the lung (Stewart et al., 1991), contributing to vasoconstriction.

Modulation of ET-1 has proven a successful strategy for treating PAH (Channick et al., 2004) and several endothelin receptor antagonists (ERA) are licensed for use. One of the first ERAs approved for PAH was bosentan, which antagonizes both ET_A and ET_B receptors. The clinical trials BREATH I and II (Rubin, 2002; Humbert et al., 2004) showed that non-selective oral bosentan delayed clinical worsening of PAH by improved exercise capacity, hemodynamics, and WHO functional class. Studies assessing longer term outcome found reduced mortality rates after bosentan use for PAH (McLaughlin et al., 2005; Sitbon et al., 2005). The EARLY trial found that bosentan slowed the deterioration of patients with group 2 PH. Ambrisentan is another ERA currently used for PAH, and is a selective antagonist of ET_A receptors (ET_A:ET_B selectivity >4000:1), whereas bosentan (ET_A:ET_B selectivity ∼300:1) is a non-selective antagonist (Elshaboury and Anderson, 2013). Ambrisentan improved mortality and survival times when tested in ARIES 1 and 2 randomized, double-blind, placebo-controlled, multi-centre efficacy studies (Galie et al., 2008). Macitentan is a dual ET_A and ET_B antagonist developed by modifying the structure of bosentan to increase safety and efficacy (Bolli et al., 2012). The phase III clinical trial (SERAPHIN), showed reduced morbidity and mortality, with improved cardiac hemodynamics and 6-min walk distance with macitentan use (Pulido et al., 2013). Macitentan is characterized by sustained receptor binding and has better tissue penetration (Iglarz et al., 2008; Gatfield et al., 2012). Common side effects of ERAs include peripheral oedema and flushing, nasopharyngitis, headache, and anemia. Bosentan is associated with teratogenesis and elevated transaminases in 10–12% of patients due to liver toxicity. Liver toxicity forced another ERA, sitaxentan, to be withdrawn from the market in 2010 due to hepatic necrosis (Odili and Abdullahi, 2014; Tran Thao et al., 2018). Ambrisentan (Galie et al., 2008) and macitentan appear superior in this respect, with lower risk of aminotransferase abnormalities.

INVESTIGATION OF C-FIBER-DERIVED NEUROPEPTIDES AS NOVEL PULMONARY HYPERTENSION THERAPIES

Pulmonary vascular tone is controlled by many other factors, aside from those discussed above. There are a number of promising candidates for new PH treatment options, including the neuropeptides, substance P and CGRP. This pair of neuropeptides are of interest as both are released from sensory C-fibers. This complex has a well-characterized role in nociception and pain transduction, but this is not the focus of this review. Beyond their afferent role as nociceptors, sensory C-fibers have an efferent role. They can be stimulated in the periphery to release vasoactive neuropeptides. This leads to direct and immediate modulation of vascular tone (Holzer, 1991). The endogenous stimulation of C-fibers largely involves inflammatory signals. C-fibers are pharmacologically characterized by their sensitivity to noxious stimuli such as heat, acidic pH and capsaicin, the 'hot' component in chili peppers.

Use of Capsaicin in Isolating Neuropeptide Activity

Capsaicin has proven to be a useful pharmacological tool for investigating the actions of the C-fiber-derived neuropeptides (**Figure 3**). Acutely, capsaicin can activate C-fibers to release substance P and CGRP. Yet, with prolonged use it leads to depletion of nerve endings of their neuropeptide content via retrograde transport of nerve growth factor (NGF) to the cell bodies of sensory nerves (Burks et al., 1985; Juranek and Lembeck, 1997). As an example of this, in rats, capsaicin can permanently degenerate C-fiber afferents leading to reductions of neuropeptide levels. This is a promising strategy for alleviating chronic pain (Lynn, 1990). Capsaicin has been used extensively to characterize the contributions of neuropeptides to vascular regulation. The role of CGRP and substance P in the cardiovascular system, are discussed in detail in the following sections.

Calcitonin-Gene Related Peptide (CGRP)

First identified in 1982, CGRP is a polypeptide which has 37- amino acids (Amara et al., 1982; Rosenfeld et al., 1983; Wimalawansa, 1996) and belongs to a larger peptide family including calcitonin. CGRP is expressed in neuronal tissue and in central, peripheral and enteric nervous systems (Mulderry et al., 1985b). It can be found in both unmyelinated sensory C fibers and myelinated Aδ fiber. Immunocytochemistry studies showed cells within the central nervous system (CNS), peripheral nerves in the heart, airway mucosa and general vasculature all have high amounts of CGRP (Rosenfeld et al., 1983; Mulderry et al., 1985c; Tjen et al., 1998). It was originally reported that CGRP could mediate sympathetic efflux from brain (Fisher et al., 1983), and that intravenous delivery could lower blood pressure and increase heart rate (Fisher et al., 1983; Marshall et al., 1986). It was then crucially discovered that CGRP

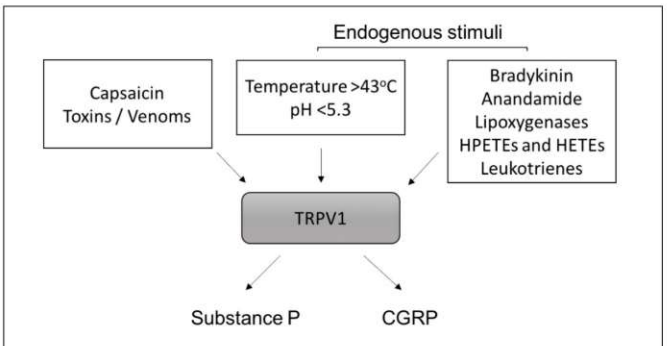


FIGURE 3 | Activation of TRPV1 and neuropeptide release. Exogenous and endogenous stimuli that activate TRPV1 are shown. HPETEs (hydroperoxyeicosatetraenoic) and HETEs (hydroxyeicosatetraenoic) refer to eicosanoid derivatives of arachidonic acid. Once activated, TRPV1 on sensory C-fibers releases substance P and calcitonin-gene related peptide (CGRP) from nerve endings.

has direct vasodilator activity (Brain et al., 1985). Perivascular nerves are the major source of plasma CGRP and deficiency of perivascular nerves has been shown in certain types of hypertension (Wimalawansa, 1996; Smillie and Brain, 2011). CGRP is widely believed to have anti-inflammatory effects (Gomes et al., 2005). Inflammation-mediated nerve damage can upregulate CGRP synthesis (Amara et al., 1982; Rosenfeld et al., 1984; Donnerer and Stein, 1992). In vasculature, terminal CGRPergic sensory fibers are located in all layers of the vascular smooth muscle (Rosenfeld et al., 1983) (Amara et al., 1985; Mulderry et al., 1985a). CGRP can be found in perivascular neurons common to all vascular beds, where expressions are higher in arterial than venous tissues in human coronary arteries (Amara et al., 1985). Receptors for CGRP have also been found in both the media and intima of resistance vessels and in endothelial cells (Mulderry et al., 1985a; Hagner et al., 2002). The wide distribution of CGRP and its receptor in the vasculature makes CGRP a promising future drug target.

CGRP Is a Potent Vasodilator

Calcitonin gene-related peptide is a potent vasodilator in the systemic circulation and other localized vascular beds. (Brain et al., 1985; Kubota et al., 1985; Greenberg et al., 1987; van Rossum et al., 1997). CGRP achieves vasodilation by receptor-mediated activation of adenylyl cyclase and cAMP (Aiyar et al., 1997), and may also have some cross-talk with NO (Chen and Guth, 1995). Patients with hypertension have increased plasma CGRP concentrations (Masuda et al., 1992; Li et al., 2009). Circulating CGRP levels correlate with systolic and diastolic pressures in severe hypertension (Edvinsson et al., 1992). Dose-dependent increases in plasma CGRP levels were found when blood pressure was artificially raised with angiotensin II in normotensive volunteers (Portaluppi et al., 1993). These studies suggest that raised plasma CGRP is a compensatory mechanism to overcome high blood pressure. While CGRP is the most potent neuropeptide vasodilator, it suffers from a short half-life that may limit its clinical use. To overcome this a long lasting acylated α-CGRP analog (half-life $\geq$ 7 h) was used in a pre-clinical study. This effectively alleviated hypertension (Aubdool et al., 2017), further supporting the notion of CGRP acting as an endogenous anti-hypertensive agent.

CGRP in Pulmonary Hypertension

Similar to systemic circulation, CGRP is also a major vasodilator in pulmonary arteries (McCormack et al., 1989a). Using experimental models, it appears that upregulating CGRP signaling protects against increased PVR in PAH (**Table 1**). Pulmonary vascular remodeling, right ventricular systolic pressure and right ventricular hypertrophy can all be ameliorated by the infusion of CGRP in experimental PH (Keith and Ekman, 1992; Tjen et al., 1992). Decreased CGRP activity, by CGRP receptor antagonism resulted in exacerbated PH in rats (Tjen et al., 1992). More interestingly, gene therapy with CGRP has also proven to be effective in preventing PH characteristics *in vivo* and *in vitro* in experimental PH models (Champion et al., 2000; Bivalacqua et al., 2002; Chattergoon et al., 2005; Zhao et al., 2007). Endothelial progenitor cells that were transfected with CGRP and directed to the pulmonary vasculature of rats led to decreased PVR (Zhao et al., 2007). This suggests that PH-associated endothelial dysfunction may be corrected by CGRP.

Interestingly, patients with higher CGRP levels had more severe PAH (Bartosik et al., 2002; Zhang et al., 2006). Likewise, chronic hypoxia-induced PH in rats was associated with higher levels of CGRP. The latter correlated with increased arterial medial hypertrophy and right ventricular systolic pressure and hypertrophy (Keith and Ekman, 1992). These studies hint at a role for CGRP in the pathophysiology of PAH. Taken in context with the therapeutic potential of CGRP for lowering pulmonary pressure, it is likely that increased CGRP levels reflect a compensatory upregulation to overcome the high pulmonary pressure. If this is the case, CGRP may be a useful clinical biomarker for PAH diagnosis.

Substance P

Substance P was first discovered in 1931 after tissue extract containing a previously unknown compound was demonstrated to stimulate rabbit intestinal contraction (V Euler and Gaddum, 1931). Several decades later the structure of substance P, as a polypeptide containing 11 amino acids, was resolved (Chang et al., 1971). It is now well established that substance P is involved in many physiological and pathological effects, mediating touch, pain and temperature (Lembeck and Holzer, 1979; Holzer, 1988). While substance P is mostly known for its role in pain and neurogenic inflammation and is found in the central nervous system (Mantyh, 2002), it also mediates effects via receptors in non-neuronal tissues. Substance P modulates cell proliferation and cytokine production, mediates interaction between immune cells and neurons (de Avila et al., 2014; Mashaghi, 2016) and is involved in immunoregulation (Payan and Goetzl, 1985; Stead et al., 1987, 1989; Haines et al., 1993; Ho et al., 1997; Schratzberger et al., 1997). The main receptor for substance P is neurokinin 1 receptor (NK1R), which belongs to the tachykinin receptor family of G protein-coupled receptors. When substance P binds to NK1R a scaffold complex can be formed, resulting in endocytosis and stimulation of intracellular signaling via mitogen activated protein kinases (Grady et al., 1995; DeFea et al., 2000).

Substance P Is an Indiscriminate Regulator of Vascular Tone

In the vasculature, substance P promotes proliferation of smooth muscle cells (Payan, 1985). It also influences vascular tone in a complex manner. It has a dual role of being a vasoconstrictor or vasodilator, depending on the circumstances and the type of vessel it stimulates (Worthen et al., 1985). The vascular response to substance P is largely mediated by NK1R activation. Substance P has a higher affinity for NK1R compared to NK2R and NK3R. There is little evidence to suggest a role for the other isoforms in vascular reactivity (Maggi et al., 1990; Constantine et al., 1991; Hall and Brain, 1994; Berthiaume et al., 1995; Corboz et al., 1998). Whether the effect of Substance P is to cause vasodilation or vasoconstriction is dependent on where NK1R is located. Activation of NK1R on smooth muscle cells may induce vasoconstriction. If

TABLE 1 | Role of neuropeptides in experimental models of PH.

Species	PH model	Treatment	Effect	Reference
TRPV1				
Rat	Monocrotaline	Capsaicin	↓ PH, ↓RVH	Zhou and Lai, 1993; Katzman and Lai, 2000
Rat	Pulmonary banding	Capsaicin	↓ PH, ↓RVH	Xu et al., 2017
Rat	Hypoxia	Capsaicin	↑ PH, ↑RVH	Tjen et al., 1998
Rat	Perinatal hypoxia/ monocrotaline	Capsaicin	↓ PAP	Chen et al., 2012
CGRP				
Rat	Post-natal hypoxia		↓ CGRP ↑ PH	Keith et al., 2000
Mouse	Hypoxia	CGRP gene transfer	↓ PH, ↓RVH	Champion et al., 2000; Bivalacqua et al., 2002
Rat	Hypoxia	CGRP	↓ PH, ↓RVH	Tjen et al., 1992
Rat	Hypoxia	CGRP receptor impaired	↑ PH	Qing and Keith, 2003
Rat	Hypoxia	CGRP infusion	↓ PH, ↓RVH	Qing and Keith, 2003
Substance P				
Rat	Perinatal hypoxia/ monocrotaline		↑ Substance P	Chen et al., 2012
Rat	Monocrotaline		↑ Substance P	Zhou and Lai, 1993
Rat	Hypoxia	NK1R antagonist	↓ PAP	Chen et al., 1999
Rat	Hypoxia	NK1R activation	↑ PAP	Chen et al., 1999

CGRP, calcitonin gene-related peptide; NK1R, neurokinin 1 receptor; PH, pulmonary hypertension; RVH, right ventricular hypertrophy.

NK1R on endothelial cells are stimulated, calcium-induced activation of the vascular endothelium can occur. This results in production of endothelium-derived vasodilators. Yet, endothelial NK1R activation can also result in thromboxane-mediated vasoconstriction (**Figure 4**). Interestingly, global knockout of the NK1R gene (*Tacr1*) resulted in no alteration to vascular function in isolated systemic arteries (Moyes et al., 2016). Given that both smooth muscle and endothelial cells were lacking NK1R in these mice, it may explain the phenotype. Vascular function would be best explored using tissue-specific knockout mice (i.e., smooth muscle and/or endothelial cell knockout) to truly determine functional relevance of NK1R.

Substance P as a vasodilator

Substance P can be a potent vasodilator of some vessel types and this has been shown in isolated vessels from both humans (Bodelsson and Stjernquist, 1992; Onoue et al., 1994;

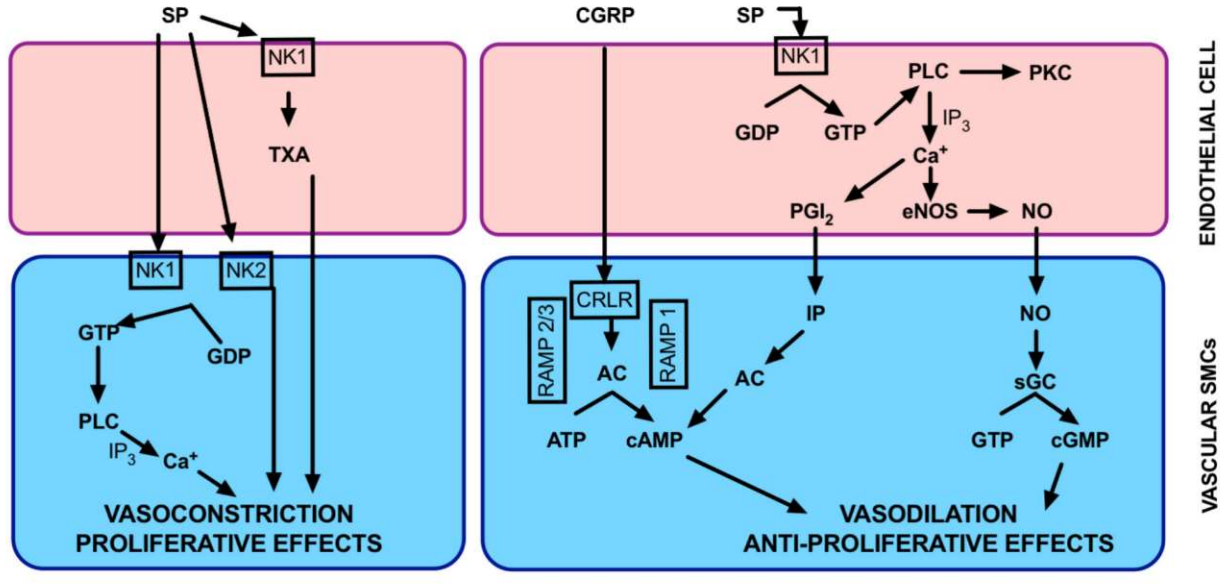


FIGURE 4 | Overview of neuropeptide function in the lung circulation. Proposed mechanisms by which CGRP and Substance P may be effective in regulating pulmonary vascular tone. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CRLR, calcitonin receptor-like receptor; eNOS, endothelial nitric oxide synthase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; IP_3, inositol triphosphate; MAPK, mitogen activated protein kinase; NK, neurokinin; NO nitric oxide; PGI_2, prostacyclin; PKC, protein kinase C, PLC, phospholipase C; RAMP-1, receptor activity-modifying protein-1; sGC, soluble guanylate cyclase; TXA, thromboxane.

Wallerstedt and Bodelsson, 1997) and animals (D'Orleans-Juste et al., 1985; Bolton and Clapp, 1986; McCormack et al., 1989b; Toda et al., 1991) and *in vivo* (McEwan et al., 1988; Beattie et al., 1993; Hall and Brain, 1994; Strobel et al., 1996). Vasodilation induced by substance P via NK1R can occur through both endothelium-dependent (D'Orleans-Juste et al., 1985; Bolton and Clapp, 1986; Onoue et al., 1994) and -independent mechanisms (Enokibori et al., 1994). Endothelium-dependent vasodilation (**Figure 4**) after substance P stimulation can be via any of the major endothelium-dependent pathways; NO (Rosenblum et al., 1993; Holton et al., 2010), prostacyclin (Bodelsson and Stjernquist, 1994) or endothelium-dependent hyperpolarization of the smooth muscle membranes (Petersson et al., 1995; Wallerstedt and Bodelsson, 1997). This can also occur through NK1R in isolated pulmonary vessels (Corboz et al., 1998; Pedersen et al., 2000). Substance P-induced pulmonary vasodilation tends to occur at low concentrations (McCormack et al., 1989b) and can be transient in nature (Maxwell, 1968).

Substance P as a vasoconstrictor

Whilst it is often considered a systemic vasodilator, substance P can also induce a myogenic 'response,' or pressure-induced constriction (Scotland et al., 2004). When substance P release is stimulated, it can induce coronary vasoconstriction and increased blood pressure (Bubb et al., 2013) and it may have a role in the pathogenesis of hypertension (Faulhaber et al., 1987; Kohlmann et al., 1997). Activation of the NK1R on smooth muscle cells results in activation of phospholipase C. This creates a transient increase of inositol 1,4,5 triphosphate (Takeda et al., 1991) and increased intracellular calcium (Krause et al., 1992), resulting in vasoconstriction. Substance P primarily causes vasoconstriction in pulmonary arteries (Selig et al., 1988; McCormack et al., 1989b; Shirahase et al., 1995), which can be mediated via NK1R via thromboxane or NK2R (**Figure 4**). This appears to be largely pulmonary-specific (Selig et al., 1988) and likely involves complex receptor interactions. At higher substance P concentrations, pulmonary vasoconstriction dominates over vasodilation (McCormack et al., 1989b).

Substance P in Pulmonary Hypertension

The complexities around the dual vasodilator/vasoconstrictor role of substance P in the pulmonary circulation (**Figure 4**) make it difficult to predict what role it has (if any) in PH. Infusion of substance P has minimal vasodilating effect on patients with PAH (Uren et al., 1992; Brett et al., 1996; Cailes et al., 1998). Thus, is has been postulated that substance P dysfunction is an underlying cause of PAH. On the other hand, the dominant role of substance P as a pulmonary–specific vasoconstrictor led to the hypothesis that substance P overactivity is causative in PAH. Pre-clinical models of PH are associated with increased lung substance P (Zhou and Lai, 1993; Chen et al., 2012). Pulmonary pressure can be decreased by depleting substance P (**Table 1**) or by using NK1R antagonists (Zhou and Lai, 1993; Chen et al., 1999, 2012). Likewise, activation of NK1R can induce increased pulmonary pressure (Chen et al., 1999). Substance P is involved in lung vascular remodeling, possibly due to increased oxidative stress (Springer and Fischer, 2003). Increased PVR has also been attributed to inflammatory stimulation of mitogen-activated protein kinase pathway by substance P. This is alleviated by capsaicin-induced depletion (Xu et al., 2017). In model systems using hypoxia to simulate PH, substance P release is increased (Lindefors et al., 1986). Based upon this body of literature it would appear that substance P is indeed a promising candidate for reducing PVR.

Further research should be conducted to pursue the intricate mechanisms of substance P in PAH. As NK1R antagonists can lower pulmonary pressure in rats, they may be the best pharmacological target to consider for humans. NK1R antagonists are in clinical use as anti-emetics. They are usually prescribed to take prior to chemotherapy or surgery. Aprepitant is available as an oral formulation and is well tolerated (Martinez and Philipp, 2016). It has been investigated for use as an anti-inflammatory agent. Interestingly, reported side-effects include low blood pressure. If subsequent pre-clinical studies are positive, a clinical trial in PH could conceivably proceed using this safety-approved formulation.

Transient Receptor Potential Vanilloid Type 1 (TRPV1) in Vascular Regulation

There are a number of signaling intermediates in C-fiber-derived neuropeptide release. One of the best characterized in the vasculature is TRPV1. TRPV1 activation results in substance P and/or CGRP release from peripheral nerve terminals (Holzer, 1991; Gibbons et al., 2010). TRPV1 receptors are non-selective cation channels located predominantly on sensory nerve endings (Tominaga and Julius, 2000) but they also reside on cells of many peripheral tissue types. They are present in the entire respiratory tract (Zholos, 2015) and located on both smooth muscle and endothelial cells of the vasculature. TRPV1 receptors are nociceptors that are highly responsive to pro-inflammatory stimuli and are activated by a range of endogenous and exogenous factors (**Figure 3**). These include temperature, pH, bradykinin, anandamide, arachidonic acid metabolites such as 20-hydroxyeicosatetraenoic acid (Wen et al., 2012), spider toxins, and most famously, capsaicin, (Caterina et al., 1997; Devesa et al., 2011). TRPV1 receptors have a binding site for capsaicin. The complicated mechanism for activation is still being unraveled and great progress has been made since 2013 when breakthrough structural information was first reported (Yang and Zheng, 2017). TRPV1 activation is involved in a variety of cardiovascular pathologies, including the modulation of atherosclerosis (Li et al., 2014; Xiong et al., 2016), myogenic tone (Scotland et al., 2004; Bubb et al., 2013), systemic arterial pressure (Bubb et al., 2013) and hypertension (Hao et al., 2011), congestive heart failure (Gao et al., 2014; Lang et al., 2015), vascular remodeling (Chen et al., 2010), haemorrhagic shock (Akabori et al., 2007) and sepsis (Chen et al., 2018).

Evidence of a Role for TRPV1 in Pulmonary Hypertension

Pulmonary hypertension is characterized by pulmonary smooth muscle cell hyperproliferation causing remodeling of the smooth muscle cell layer and impacting on PVR. Although it is likely that TRPV1 activation in sensory nerves is the key to modulation

of pulmonary circulation, it is possible that some direct effects in smooth muscle cells could contribute to the PH phenotype. Activation of TRPV1 on cultured pulmonary smooth muscle cells (SMC) results in enhanced proliferation (Randhawa and Jaggi, 2017), increased intracellular calcium and stimulates cell migration (Martin et al., 2012). Under laboratory conditions, PH can be simulated by exposing cells to chronic hypoxia. Using this model, TRPV1 expression can increase (Wang et al., 2008) with corresponding increased intracellular calcium and reorganization of cytoskeletal architecture. TRPV1 blockade with capsazepine can abolish these effects (Parpaite et al., 2016). There are a number of studies assessing the role of TRPV1 by using capsaicin to desensitize it (**Table 1**). These studies are further evidence of the importance of TRPV1 in PH. The contribution of individual neuropeptides downstream of TRPV1 activation is complex and has been discussed in the relevant sections of this review.

DIRECT CARDIAC EFFECTS OF SENSORY C-FIBER NEUROPEPTIDES

The increased PVR in all groups of PH generally leads to right ventricular cardiac remodeling due to the increased right ventricular afterload. This remodeling eventually leads to right heart failure and accounts for mortality in most patients. The right heart remodeling is the most difficult component to treat. Similar to left-sided heart failure, there are a paucity of drugs that are effective in reversing cardiac remodeling. Many PH patients remain undiagnosed until right heart remodeling is already established. Therefore, this is a significant clinical problem. It is important to investigate the direct cardiac effects of potential therapies for PH. Even if the effect on vascular resistance is modest, any direct effects on cardiac remodeling would increase the usefulness of the therapy. Current treatments tend to be disappointing in this respect (Michelakis, 2003; Steiner et al., 2005; Baliga et al., 2011). In PAH, where the treatments cause a significant decrease in right heart afterload, the impact on the right ventricular mortality is minimal (Westerhof et al., 2017). Promisingly, both CGRP and substance P have been reported to have direct cardiac effects (Ejaz et al., 2011) as outlined below (**Figure 5**).

CGRP in Heart Failure and Cardiac Remodeling

Calcitonin gene-related peptide receptors are located throughout the myocardium (Ieda et al., 2006). There is evidence of densely

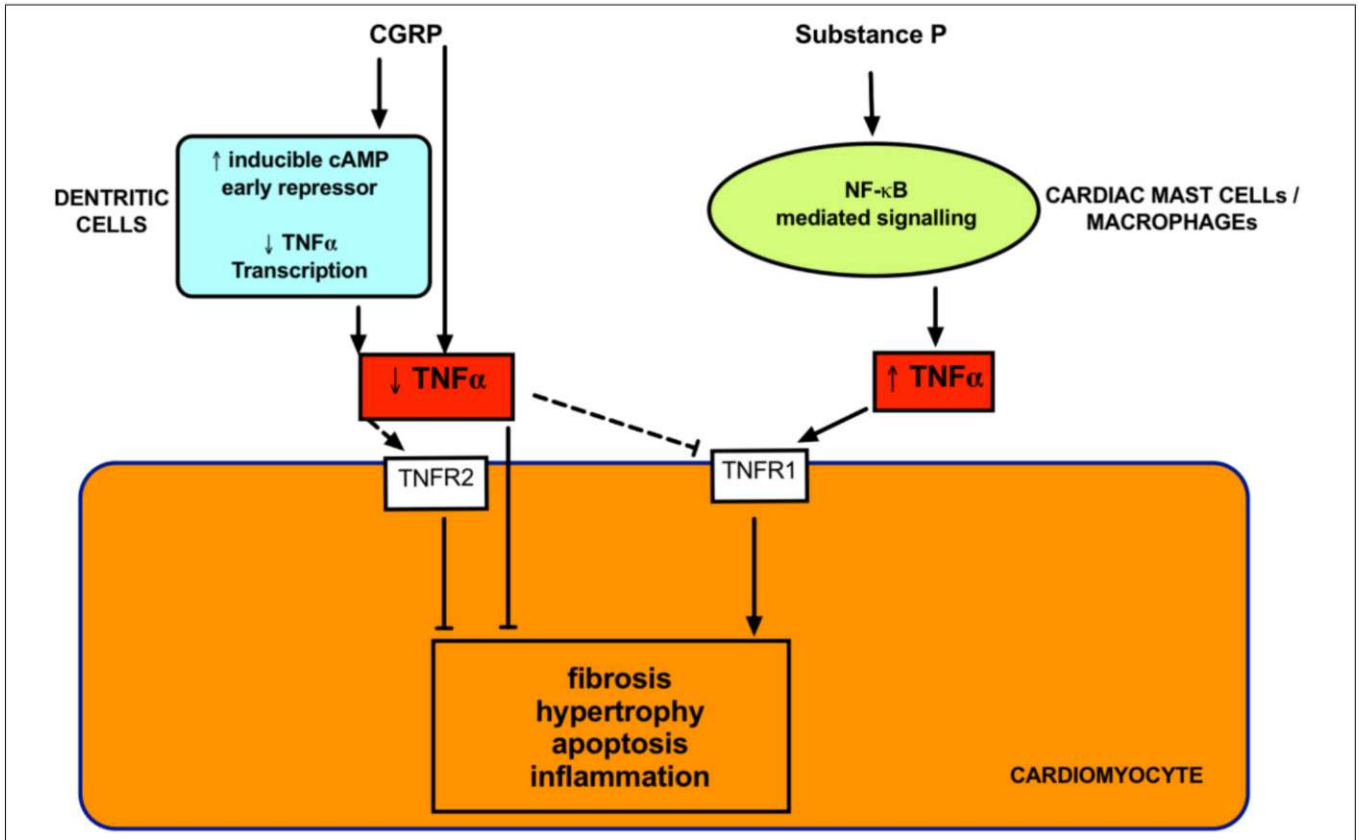


FIGURE 5 | Proposed direct cardiac effects of neuropeptides. Both Substance P and CGRP modulate tumor necrosis factor α (TNFα) signaling. TNF-α is crucial in mediating cardiac remodeling and these effects can occur via activation of receptors (TNFR1 and TNFR2) on cardiomyocytes. Substance P promotes cardiac remodeling via TNFα release from inflammatory cells. CGRP inhibits TNFα and decreases remodeling in diseased models. Dashed lines indicates unknown but postulated mechanisms.

located CGRP-containing nerve fibers around the coronary arteries, papillary muscles, sinoatrial and atrioventricular nodes (Mulderry et al., 1985b). CGRP protects against cardiac hypertrophy in a pre-clinical model of heart failure (Li et al., 2010). CGRP levels can be increased in heart failure (Hsu et al., 2005). Whether this is causative or compensatory is unknown. However, there is evidence to support the latter notion. Infusion of CGRP increases myocardial contractility (Gennari et al., 1990) and improves circulation in coronary disease (Gennari et al., 1990; Dubois-Rande et al., 1992; Stevenson et al., 1992). On a cellular level, CGRP inhibits generation of pro-inflammatory tumor necrosis factor (TNF)-α and prevents interstitial and perivascular fibrosis (Li et al., 2013). CGRP also ameliorates cardiomyocyte apoptosis via the Bcl-2/Bax pathway (Ma et al., 2013) and has direct anti-proliferative effects on cardiac fibroblasts (Li et al., 2016). CGRP has positive cardiac ionotropic effects that are mostly abolished by both a CGRP antagonist and a PI3 kinase inhibitor (Al-Rubaiee et al., 2013). A CGRP analog alleviated pre-clinical diabetes-induced heart failure and remodeling due to reduced α-smooth muscle actin and transforming growth factor (TGF)-β_1 (Aubdool et al., 2017). Therefore, CGRP appears to be cardioprotective. Even more promising for PH, is the recent finding that rutacarpine, which stimulates CGRP release, was able to reverse right heart remodeling in experimental PH and this was attributed to p27-dependent signaling (Li et al., 2016).

Substance P in Heart Failure and Cardiac Remodeling

In the human heart, substance P has been found in intrinsic cardiac ganglia (Wharton et al., 1990; Hoover et al., 2009), coronary vessels (Laine et al., 2000), and within myocardium (Weihe et al., 1981; Rechardt et al., 1986). Substance P appears to have a damaging role in heart disease (Dehlin and Levick, 2014), hinting at a direct effect of substance P on myocardial tissue. Pro-substance P (a stable surrogate for substance P) is an independent predictor of recurrent acute myocardial infarction, heart failure and cardiac mortality (Ng et al., 2014). Levels of substance P are increased in congestive heart failure (Valdemarsson et al., 1991). Substance P promotes hypertrophy in isolated cardiomyocytes, suggesting this effect is direct and independent of systemic factors (Church et al., 1996). Several pre-clinical models have demonstrated a role for substance P in mediating heart failure. Cardiac hypertrophy, apoptosis and dilated cardiomyopathy were absent from substance P knockout mice (D'Souza et al., 2007). Genetic deletion of *TAC1,* which encodes substance P was protective against increased cardiac mast cell density and TNFα upregulation and associated cardiac remodeling (Melendez et al., 2011). Inhibition of NK1R also improved cardiac function and ameliorated cardiac hypertrophy in models of heart failure (Melendez et al., 2011; Robinson et al., 2015). Substance P appears to be a mediator of cardiac toxicity induced by doxorubicin, which is a chemotherapy agent that causes cardiomyocyte death. Inhibition of substance P resulted in lower cardiomyocyte apoptosis after doxorubicin treatment to an isolated cell line (Robinson et al., 2016). Taken together, these findings are clinically exciting, as it suggests that substance P-antagonist based therapies may be able to directly improve right ventricular parameters above simply relieving right ventricular afterload for PAH treatment.

WHAT IS NOT KNOWN? FUTURE PROSPECTS FOR NEUROPEPTIDES AS A TREATMENT STRATEGY FOR PULMONARY ARTERIAL HYPERTENSION

From a relatively small pool of research we can conclude that the neuropeptides, substance P and CGRP, have the ability to regulate both systemic and pulmonary arterial pressure. We have also provided evidence that these neuropeptides regulate vascular smooth and cardiac muscle cell remodeling under diseased conditions. However, there is not yet a comprehensive answer on whether these neuropeptides are strong signaling targets for treating PAH. CGRP is a proven potent dilator of systemic and pulmonary vasculature and appears to have favorable effects on cardiac remodeling. Based on what we know, stimulation of endogenous CGRP signaling or exogenous administration of stable analogs should be good candidates for the treatment of PAH. Yet only a handful of studies have investigated this and it has not gone further than pre-clinical investigation. The next step would be to thoroughly characterize the potential for CGRP in reversing all aspects of PAH. It is important to show this in three or more different PH models, before embarking on a Phase II trial.

The clinical appeal of substance P is more distant as there are still inconsistencies in identifying the precise vascular regulatory function of this neuropeptide. From the pulmonary literature, it appears that the over-arching role of substance P is likely to be as a vasoconstrictor. This is contrary to its effect in the systemic circulation, where it seems to predominantly cause vasodilation. If this is proven to be the case, it is a very attractive property for PAH treatment. A major adverse effect for some PAH therapies has been the unacceptable lowering of systemic arterial pressure. This is an important factor to consider for PAH treatment, especially in regard to using combination therapies.

Combination therapies are emerging as a promising treatment regimen for PAH, while we wait for development of novel compounds. An effective PVR-lowering combination has involved upregulation of cGMP generation with concurrent inhibition of cGMP metabolism. However, this is not always possible. For example, the use of sildenafil and long-acting nitrates can cause life-threatening hypotension (Cheitlin et al., 1999). Using pre-clinical models, an alternative strategy has emerged. Stimulation of a different 'pool' of cGMP, derived from particulate guanylate cyclase activity rather than sGC (Baliga et al., 2008), and combining this with sildenafil, has been a success. This has resulted in a far more pulmonary-specific vasodilation, negating the hypotensive effects. A similar approach could apply for substance P. Antagonism of NK1R would presumably block the pulmonary vasoconstriction of

substance P, but also prevent excessive systemic dilation to some degree. A hypothetical treatment strategy could involve using NK1R antagonists alongside already approved PAH therapies such as PDE5 inhibition or sGC stimulation. This may result in a pulmonary-specific dilation, as substance P-induced systemic vasodilation would be prevented at the same time as pulmonary vasoconstriction is lowered. Pre-clinical studies should focus on investigating the substance P axis alongside current treatments, or in combination with other promising candidates. This could identify new strategies that are truly able to target multiple etiologies of PAH and are an essential starting point. A similar strategy should be utilized when investigating CGRP for PAH therapy. It is likely that CGRP-based pharmaceuticals may have significant and unacceptable blood pressure lowering effects, similar to the organic nitrates. Therefore dose-selection and combination approaches would be essential in establishing CGRP as a therapy.

As TRPV1 activation results in release of both neuropeptides, it may also make an interesting target. If antagonism of TRPV1 lowers substance P, this may be beneficial for PAH. It would also presumably lower CGRP, which may not be beneficial. It probably depends on the release pattern of CGRP/substance P in PH lungs and little is known about that. The majority of studies so far have found that depleting the C-fiber nerve endings by using capsaicin has generally resulted in improvements in PH models (**Table 1**). This suggests that inhibiting sensory C-fibers predominantly impairs substance P-induced pulmonary constriction without a major influence on CGRP-induced dilation. This may be reflective of the mechanisms at play in regards to the role of TRPV1 in PAH. Future research should focus on whether complete inhibition of TRPV1 could be effective as a PH therapy, or whether the balance could be shifted more toward CGRP-production with inhibition of substance P. Of course, with any strategy, consideration must be given to the consequences for other organ systems. Complete inhibition of either TRPV1 or substance P may elicit unexpected effects and this should be thoroughly investigated in PH models. Use of targeted therapy to the pulmonary vasculature and/or heart would help to elucidate the role of both neuropeptides. Also, receptor-specific therapy could be considered. One study has investigated all three neurokinin receptors, but it indicated no role of NK2R and NK3R (Corboz et al., 1998). The little that has been done suggests that NK1R is likely to be the primary mediator of pulmonary vasoconstriction, but the other neurokinins should not yet be ruled out.

Another interesting area to explore could be related to neutral endopeptidases (NEP), which are the most important enzymes in the degradation of tachykinins, including substance P (Skidgel et al., 1984). Interestingly, there is a decent body of evidence to suggest that NEP inhibitors could be a promising PAH therapy, and they are currently in clinical trial. In addition to tachykinins, NEPs also degrade a selection of other peptides, including pulmonary vasodilating natriuretic peptides and pulmonary vasoconstrictors such as ET-1 (Abassi et al., 1992). NEPs appear to have an underlying role in the pathogenesis of PH (Dempsey et al., 2009). NEP inhibitors have produced promising results in models of PH, both as monotherapy (Klinger et al., 1993; Thompson et al., 2012) or in combination with PDE5 inhibition (Baliga et al., 2008). This is largely due to their ability to increase circulating atrial natriuretic peptide. However, in addition to increasing pulmonary vasodilators, NEP inhibitors may result in increased circulating substance P (in addition to other vasoconstrictors such as ET-1 and angiotensin II) and this would likely counteract their beneficial effects on the pulmonary circulation. An alternative strategy could be to introduce NEP inhibition in combination with an NK1R antagonist, for example. A similar strategy has been trialed for hypertension, using combined NEP/ angiotensin II inhibition with promising results (Cuculi and Erne, 2011).

Another exciting avenue worth pursuing is the interaction of neuropeptides with ET-1. CGRP inhibits the interaction of ET-1 with ET_A on vascular smooth muscle cells. This can decrease smooth muscle contraction and is reversible with CGRP antagonists (De Mey and Vanhoutte, 2014). This suggests that intact CGRP may be important in preventing ET-1 activity. Upregulation of CGRP may act as an endogenous ERA inhibitor and attenuate ET-1 over-activity in PAH. There are also emerging findings that substance P may directly interact with ET-1. In non-cardiovascular (melanocyte) cells, substance P can stimulate ET-1 (Park et al., 2015). An NK1R antagonist was able to prevent an increase in ET-1 expression seen in spontaneously hypertensive rats, which was associated with reduced cardiac fibrosis (Dehlin et al., 2013). If the relationship between substance P and ET-1 is proven, this could also have important implications for PAH treatment, given that ET-1 is already a prime target for licensed therapies. Taken together, it would seem that selectivity in regulating neuropeptide release/ activity is of prime importance. Most of the *in vivo* studies in this area have investigated broad-spectrum C-fiber depletion. This is unlikely to give meaningful information on the mechanisms at play in PH. Many of these studies were conducted years ago, prior to the advancements in the efficiency of genome manipulation. It is worth revisiting these neuropeptides in PH. The use of elegant and well-designed studies, utilizing advanced technology, should fully interrogate the vascular interactions of substance P and CGRP in the pulmonary circulation.

SUMMARY

Pulmonary arterial hypertension is a fatal disease that afflicts people of any age and causes substantial reduction in quality of life. It tends to be more prevalent in young people, particularly women. While treatments developed in the past few decades improved the prognosis for PAH patients, mortality rates still remain high. Current treatment methods are primarily centered on enhancing pulmonary vasodilation. They are not effective at reducing mortality. There is great potential to develop new treatments that target both cardiopulmonary re-modeling and PVR. Altered release of neuropeptides such as substance P and CGRP have been implicated in the pathophysiology of PAH. Selective control of the balance on these neuropeptides in the pulmonary circulation is a promising approach to combating this fatal disease.

AUTHOR CONTRIBUTIONS

CL and KB created the figures. CL, SM, and KB researched the literature, prepared the table, and drafted the manuscript.

FUNDING

KB is funded by Rebecca L. Cooper Medical Research Foundation and University of Sydney Kickstart Grants related to this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.